The Role of Delphinidin and Sugars in the Resistance of Pea Seedlings to Fusarium Root Rot

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ABSTRACT

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Pea seed and seedling exudates and extracts from testae of all Plant Introduction accessions with the *A* gene for anthocyanin production, whether resistant or susceptible to Fusarium root rot, contained the anthocyanidin (anthocyanin-aglycone) pigment delphinidin. Delphinidin, located primarily in the testae of all P. I. accessions tested, was fungistatic to conidial germination of *Fusarium solani* f. sp. *pisi*. In a bioassay, the pathogen was able to germinate in the presence of delphinidin when glucose was present in sufficient amounts. These results suggest that P. I. accessions, with the *A* gene, can be susceptible in the seedling stage, despite the presence of delphinidin if they exude sufficient sugar.

Additional key words: *Pisum sativum*, fungistatic effect of delphinidin.

Delphinidin, the anthocyanin (anthocyanin-aglycone) pigment present in the testae of pea cultivars bearing the *A* gene, was reported to be responsible for resistance of seed and seedlings to Pythium and Ascochyta (Mycosphaerella) seedling rot (1, 3). Seedling exudates from resistant Plant Introduction (P. I.) accessions with the *A* gene for anthocyanin-pigmented testae, were reported to inhibit sporulation of *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (Jones) Snyd. & Hans. in vitro and conidial germination in soil (6, 9). In contrast, seedling exudates from susceptible accessions, with pigmented or green testae, did not inhibit sporulation or germination. Seedling exudates from resistant and susceptible P. I. accessions with pigmented testae contained similar amounts of total phenols (6), which indicated that perhaps factors other than total phenols in seedling exudates play a role in the resistance of pea seedlings to *F. solani* f. sp. *pisi*.

This study was designed to determine: (i) if both susceptible and resistant P. I. accessions with pigmented seed, contained delphinidin in the testae; (ii) whether delphinidin was responsible for the inhibition of germination of conidia of *F. solani* f. sp. *pisi*, and (iii) whether other compounds modified any fungistatic effect of delphinidin.

MATERIALS AND METHODS

Preparation of inoculum.—Macroconidia of an isolate of *F. solani* f. sp. *pisi* recovered from diseased peas in eastern Washington, were washed with sterile, glass-distilled water from 7-day-old cultures on dehydrated potato-dextrose agar. The spore suspension was centrifuged at about 3,000 *g* for 10 min and the supernatant liquid was discarded. The spores were resuspended in sterile water and the process was repeated. The resultant spore suspension was stored at 6 C until needed.

Seed storage.—All seeds used in this study were stored at 10 C at about 50% relative humidity to maintain seed vigor. All seed lots had a germination of 80-90% and only healthy appearing seed with no cracked or split testae were used.

Collection of seedling exudates.—Seed and seedling exudates were collected aseptically according to the described methods (8). However, for the purposes of this study, 10 g of surface-disinfected seed (8) were placed in 50 ml of sterile glass-distilled water in 125-ml flasks. Three seed lots of each source were incubated on a rotary shaker operated at one cycle per sec at room temperature in the dark. Seedling exudates were collected when about 90% of the seeds in a flask had germinated and radicle length was 1 cm or less. For bioassay studies, the exudates were concentrated in vacuo to about one-sixtieth of their original volume and diluted in absolute methanol to a final volume of 3 ml. The concentrated methanolic exudates were stored at 6 C in the dark until analyzed.

Seedcoat and seed analysis.—Testae and seed were analyzed and bioassayed separately for delphinidin to confirm or refute earlier reports that delphinidin is localized in the testae of seed from lines with the *A* gene (1, 3).

Delphinidin concentration in testae and remaining seed parts was determined separately from two resistant (P.I. 140165 and 257593) and one susceptible (P.I. 253968) pea accession. Seed surface area, as determined by direct measurement and application of the formula $4\pi r^2$, was used to calculate the number of seeds to be analyzed for each accession. The surface area of ten seeds of pea cultivar Dark Skin Perfection (1.76 cm^2) was used as a standard. Number of seeds of the three test accessions then were adjusted so that total surface area of each accession approximated 1.76 cm^2.

Seeds were cracked with a pliers and the testae were removed with a forceps. The testae from each line were ground separately in an Omni-Mixer in 0.1% HCl-methanol (2 ml) for 5 min at high speed. After grinding, an additional 1 ml of HCl-methanol was added to the extract. The remaining seed parts (cotyledons and embryos) of each line were ground in 5 ml of HCl-methanol. Then the ground testae and seed extracts were stored in the refrigerator for 3 days for extraction (5). Extracts were centrifuged at 3,000 *g*, the precipitate was discarded, and the supernatant liquid was stored in the refrigerator until assayed.

Qualitative determinations of sugars and anthocyanidins.—Sugars were determined by spotting 10 μliters of the concentrated seedling exudates on silica gel (60F-254, E. Merck and Company, Rahway, NJ 07065) thin-layer (TLC) glass plates, developing the plates in *n*-butanol:acetic acid:ether:water (9:6:3:1, v/v), and comparing R_f values to known standards. Sugar standards were made according to described procedures (5). The solvent was allowed to ascend 12 cm, the plates were air-dried, sprayed with aniline hydrogen phthalate (5), and oven-dried at 100 C for 10 min. After heating, R_f values were determined for sugar standards and for sugars present in the seedling exudates.

Anthocyanidin detection was as follows: Nonconcentrated seedling exudates, testae, and naked seed extracts (cotyledons and embryos) were diluted 1:1 (v/v) with 2N HCl and heated in a boiling water bath for 30 min. The solutions were cooled, washed twice with ethyl acetate to remove flavones (5), the ethyl acetate was discarded, and the washed solutions were heated for 3 min at 80 C to remove the remaining ethyl acetate. The solution was reextracted with iso-amyl alcohol, concentrated to dryness, and redissolved in 1 ml of 0.1% HCl-methanol. This preparation then was chromatographed both on paper (Whatman No. 1 paper in a descending tank) using either concentrated HCl:acetic acid:water (3:30:10, v/v) or *n*-butanol:acetic acid:water (4:1:5, v/v) as a solvent system, and on cellulose TLC plates (Cellulose F, E. Merck and Company, Rahway, NJ 07065) using a *n*-butanol:acetic acid:ether:water (9:6:3:1, v/v) solvent system.

TABLE 1. Chromatographic properties of the acid hydrolysate of seed exudates (A) and testae (B) of *Fusarium solani* f. sp. *pisi*-susceptible and -resistant peas with anthocyanin-pigmented testae R_f (× 100)

		Paper chromatographs					
		Forestal[b]		BAW[c]		Thin-layer chromatographs[d]	
Pea accession	Root rot reaction[a]	A	B	A	B	A	B
138945	S	36[e]	36	38	38	24	24
140165	R	36	36	38	38	23	23
253968	S	37	37	38	38	23	23
257593	R	37	37	38	38	23	23
Delphinidin-HCl		34	34	39	39	24	24

[a]Symbols: S = as susceptible as the commercial pea cultivar Dark Skin Perfection; and R = more resistant than Dark Skin Perfection (6, 7). Disease reactions were based on plant reactions at seedling stage.
[b]Solvent system for Forestal = conc. HCl:acetic acid:H_2O (3:30:10, v/v).
[c]Solvent system BAW = *n*-BuOH:acetic acid:H_2O (4:1:5, v/v).
[d]Thin-layer chromatography was run on silica gel plates and the solvent was *n*-BuOH:acetic acid:ether:H_2O (9:6:3:1, v/v).
[e]All R_f values are an average of three replications per accession.

TABLE 2. Capacity of concentrated seed exudates and extracts of testae, and of naked seed of three Plant Introduction (P.I.) pea accessions to suppress the germination and growth of macroconidia of *Fusarium solani* f. sp. *pisi*

		Biological assay		
P.I. accession	Root rot reaction[a]	Conc. seed exudate	Testa extract[b]	Naked seed extract[c]
140165	R	–[d]	–	+
253968	S	+	–	+
257593	R	–	–	+

[a]Disease reaction based on seedling stage: S = as susceptible as commercial pea cultivar Dark Skin Perfection; and R = more resistant than Dark Skin Perfection (6, 7).
[b]Number of seeds of each accession used to make a testa extract was determined by using formula for area of sphere ($4\pi r^2$) whereby gm weights were: 140165, 0.2527; 253968, 0.2789; 257593, 0.3466.
[c]Gram weight of seed used to make each extract was: 140165, 1.6725; 253968, 1.8005; 257593, 2.8657.
[d]Germination and growth was determined by microscopic observation of macroconidia on agar disks infiltrated with each test solution: + = all or majority of conidia germinated and producing a viable, nonlysed germtube; and – = no germination or growth.

Delphinidin chloride was dissolved in 0.1% HCl-methanol (mg/ml) and used as a standard.

Bioassay and alteration of resistance.—A bioassay, consisting of germinating macroconidia of *F. solani* f. sp. *pisi*, was used to detect and demonstrate the fungistatic effect of delphinidin. Test extracts were pipetted onto Whatman 3 MM chromatographic paper at 0.2 ml per spot. The diameter of each spot was restricted to 1 cm. Each spot was cut out and placed in a plastic petri dish with a moistened 9-cm diameter filter paper disk placed inside the top half to serve as source of humidity. A No. 4 cork borer (0.8-cm diameter) was used to punch out purified agar (Difco) disks from poured petri plates (10 ml/plate). These disks then were removed and placed on the extract-paper disk, and incubated overnight to allow imbibition of test compounds into the agar disks. A suspension of conidia of *F. solani* f. sp. *pisi* (0.01 ml) at a concentration of 1×10^6/ml was dispersed evenly on the surface of each agar disk and incubated an additional 16 hr. At that time, each agar disk was examined under low power (× 160) with a compound microscope. Germination and/or germtube or chlamydospore formation were recorded.

The hypothesis that sugar exudation could alter the resistance of a seedling to *F. solani* f. sp. *pisi* was further tested. Fifteen seeds each of two resistant P. I. accessions (P.I. 140165 and 257593) were surface disinfested (5) and placed either in 15 ml of a 5% glucose solution or in glass distilled water. The seed then were placed in a vacuum desiccator jar for 1 hr under 16 mm Hg of vacuum. The seeds then were removed and planted in a Warden fine sandy loam, artificially infested with *F. solani* f. sp. *pisi* at 1×10^5 chlamydospores/g air dry soil. Soil moisture was adjusted to 17% at time of planting. Five seeds of each test line per treatment were planted in each of three 5.1-cm-diameter plastic drinking cups, filled with infested soil. The plastic cups were placed in a growth chamber set at 23.9 ± 1 C day and an 18.4 ± 1 C night temperature with a 16-hr day and maximum illumination of 11,840 lux. Resultant seedlings were harvested 7 days after emergence. Disease severity (0-5 scale where 0 = healthy and 5 = completely rotted root), and fresh weight of tops and roots were recorded.

TABLE 3. Relative concentrations of glucose and fructose in seed and seedling exudates of peas as determined by thin-layer chromatography

Pea accession or cultivar	Root rot reaction[a]	Sugar concentration[b]	
		Glucose	Fructose
P.I. 138945	S	+[c]	+
P.I. 140165	R	Tr	Tr
P.I. 253968	S	+	+
P.I. 257593	R	Tr	Tr
Dark Skin Perfection	S	+	+

[a]Symbols: S = as susceptible as pea cultivar Dark Skin Perfection; and R = more resistant than Dark Skin Perfection (6, 7).

[b]Concentrated exudates were spotted (10 μliters) on silica gel plates and run in a *n*-butanol:acetic acid:ether:water solvent (9:6:3:1, v/v). Sugar determinations were made by comparing R_f values with known standards after spraying the plates with aniline hydrogen phthlate and drying at 100 C for 10 min for maximum color development of the spots.

[c]Symbols: + = spot readily detected; and Tr = trace amount detected.

RESULTS

All acid-hydrolyzed exudates and extracts of testae originating from pea accessions with the *A* gene contained delphinidin as the major pigment component (Table 1). The R_f values of two other common plant pigments, cyanidin and pelargonidin, are quite different from delphinidin (4) and were not detected.

Extracts from testae of seed of both resistant (P.I. 140165 and 257593) and susceptible (P.I. 253968) accessions were fungistatic in a bioassay (Table 2). The concentrated seed and seedling exudates from the susceptible P. I. accession were not fungistatic but exudates from the two resistant accessions were. Extracts from naked seed (cotyledons and embryos) of resistant and susceptible accessions were not fungistatic and did not contain delphinidin.

The presence of delphinidin in hydrolyzed extracts of testae and in hydrolyzed seedling exudates of both resistant and susceptible pea accessions seemed to lessen the importance of this pigment in the resistance mechanism. Because other studies had associated susceptibility with sugar exudation from germinating seed (4, 8, 9, 10, 11), the possibility existed that the fungistatic effect of delphinidin could be overcome with a corresponding sugar exudation. In addition, more glucose and fructose exuded from seed and seedlings of the susceptible P.I. accessions and Dark Skin Perfection

TABLE 4. Effect of glucose on the fungistatic effect of delphinidin to inhibit spore germination and growth of *Fusarium solani* f. sp. *pisi*[a]

Concentration in paper disks		
Delphinidin (mg)	Glucose (mg)	Growth[b]
1.0	0.0	−
0.5	0.0	−
0.4	0.0	−
0.3	0.0	−/+
0.2	0.0	+
0.1	0.0	+
1.0	1.0	−
0.5	1.0	+
0.4	1.0	+
1.0	0.4	−
0.5	0.5	−/+
0.5	0.4	−/+
0.0[c]	0.0[c]	+

[a]Agar disks were infiltrated with delphinidin at various concentrations (in methanol), as indicated, with and without glucose present. A 0.01 ml drop of macroconidia of *Fusarium solani* f. sp. *pisi* was placed on each disk and incubated in a moist chamber for 24 hr.

[b]Microscopic examination of the agar disk was used to determine conidial activity such that − = no growth, −/+ = slight growth or chlamydospore formation, and + = normal germ tube development. This test was repeated three times.

[c]Control consisted of germinating macroconidia on the agar disk alone.

than from the resistant P.I. accessions (Table 3).

Seed of two resistant P. I. accessions were immersed in either a 5% glucose solution or water for 30 min, under a vacuum, and planted in soil artificially infested with *F. solani* f. sp. *pisi*. Both accessions were more susceptible (10 days after emergence) when immersed in glucose than in water (2.5 vs. 1.5 disease index for P.I. 140165 and 3.7 vs. 1.9 disease index for P.I. 257593).

In a bioassay, delphinidin suppressed macroconidial germination of *F. solani* f. sp. *pisi* at decreasing concentrations from 1.0 - 0.3 mg/paper disk (Table 4). Only when the concentration of delphinidin decreased to 0.2 mg did germination and normal germtube growth ensue. In comparison, when the concentration of delphinidin was 0.5 and glucose concentration was 1.0 mg/paper disk, germination and normal germtube growth occurred. In this system, 1.0 mg of glucose was needed to overcome the effect of delphinidin. A reduction in glucose concentration to 0.5 mg/disk, where delphinidin concentration was 0.5 mg, resulted in macroconidial germination but chlamydospores rapidly developed (Fig. 1). The fungistatic effect of delphinidin on conidial germination was overcome when an adequate supply of sugar was present.

DISCUSSION

Pea root rot caused by *F. solani* f. sp. *pisi* begins as a black decay of the lower portion of the epicotyl, the upper portion of the tap root, and the hypocotyl. These plant parts are in very close proximity and even in direct contact with the cotyledons. Cook and Flentje (2) showed a direct relationship between the number of chlamydospores stimulated to germinate near pea seeds (24 hr after planting) with the number of thalli of *F. solani* f. sp. *pisi* on the nearby epicotyl, hypocotyl and root tissues 3 and 6 days after planting. Thus, exudates from a germinating pea seed exerts a significant influence not only on infection of the seed itself (3) but also on infection of root and stem tissue contiguous to the seed.

My results confirm earlier work (1, 6, 9) which indicated that pea seeds with pigmented testae had less disease caused by *Fusarium*. It should be emphasized, however, that this kind of resistance may be of little value against root and stem infection later in the life of the plant (7). Nevertheless, protection even if only in the seedling stage is significant in determining total damage caused by *F. solani* f. sp. *pisi*.

Sugars (and probably other organic nutrients) exuded from peas apparently nullify, at least partially, the fungistatic effect of delphinidin. This is indicated by: (i) conidial germination occurred in the presence of 0.5 mg or 0.4 mg of delphinidin only when 0.4 to 1.0 mg glucose also was added (germination without delphinidin occurred with or without glucose) and (ii) root rot was increased on the two resistant accessions with pigmented testae, when the seeds were impregnated under vacuum with a glucose solution before planting in infested soil. These findings perhaps can help to explain why certain accessions with pigmented testae may still be susceptible,

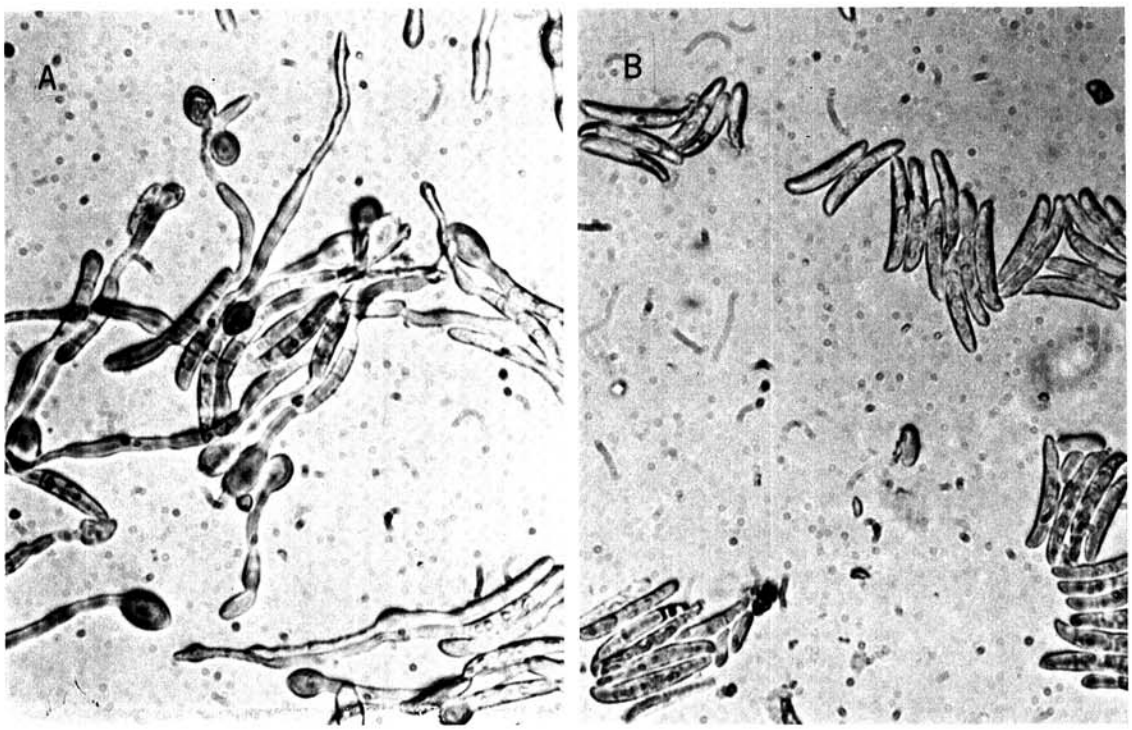

Fig. 1-(A,B). Bioassay of conidia of *Fusarium solani* f. sp. *pisi* on agar disks impregnated with delphinidin with and without the presence of glucose. **A)** Macroconidia of *F. solani* f. sp. *pisi* on an agar disk with an initial concentration of 0.5 mg/0.2 ml of delphinidin and 0.5 mg/0.2 ml of glucose. **B)** Macroconidia on a similar agar disk with 0.5 mg of delphinidin alone.

in spite of the presence of delphinidin; sugar exudation may be greater from seeds of such lines. Schroth and Cook (10) observed considerably less chlamydospore germination of *F. solani* f. sp. *phaseoli* in soil near seeds of the resistant dry bean (*Phaseolus vulgaris* L. 'N203', a cultivar with pigmented testae) than in soil near seeds of Pinto, a Fusarium-susceptible cultivar. However, when the seed coats of N203 were cracked to permit greater exudation of sugars and amino acids, chlamydospore germination increased to the percentage measured near seeds of the susceptible Pinto. Possibly with beans as well as peas, the suppressive effect of seed coat pigments on chlamydospore germination can be overcome by increased seed nutrients for the pathogen.

Ewing (3) stated that extracts from testae of pea lines with the *A* gene were not fungistatic to *Pythium ultimum* unless the extract was first heated. Heating was not necessary, however, for extracts from testae of all P. I. accessions and seedling exudates of resistant P. I. accessions were fungistatic to *F. solani* f. sp. *pisi* in an unheated and nonhydrolyzed state.

The fact that acid hydrolysis of seedling exudates and extracts from testae yielded a preponderance of delphinidin indicates that pea accessions with the *A* gene for anthocyanin formation, used in this study, contain the pro-anthocyanidin derivative of delphinidin (5). This confirms the earlier work of Ewing (3) and Clauss (1). However, in what form delphinidin is present in the nonhydrolyzed exudate was not determined and most probably was present as a glycoside (5). In addition, we were not able to determine the fungistatic properties of a glycosidic form of delphinidin as the compound was not available.

Previously, peas have been screened for resistance to *F. solani* f. sp. *pisi* by pipetting conidial inoculum on the seed at planting time. I believe this procedure will select for seedling resistance which is at least partially dependent upon delphinidin and a comparative lack of fungal nutrients being exuded during germination and early growth. However, seedling resistance is not always correlated with root resistance (7).

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