Mechanism of Increased Susceptibility of Bleached Pea Seeds to Seed and Seedling Rot

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ABSTRACT

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Seeds of some pea *(Pisum sativum)* cultivars may lose normal green color during maturation. The process, which is known as "bleaching," is associated with increased susceptibility to seed and seedling rot. When surface-sterilized unbleached seeds were inoculated with *Fusarium solani* f. sp. *pisi*, those incubated in exudate from bleached seeds had a higher disease rating than those incubated in exudate from unbleached seeds. Disease severity was similar, however, if exudate effects were minimized by injecting conidial suspensions directly into the cotyledons of bleached and unbleached seeds. Exudates from bleached and unbleached seeds contained similar quantities of fourteen amino acids, but bleached seed exudate contained approximately three times more soluble carbohydrate (glucose, fructose, and largely sucrose) than unbleached seed exudate. The carbohydrate-containing fraction of seed exudates stimulated chlamydospores of *F. solani* f. sp. *pisi* to germinate in field soil. The neutral fraction of bleached seed exudate caused 40% of the chlamydospores to germinate but the same fraction of unbleached seed exudate promoted only 10% germination. Differential carbohydrate, but not amino acid, exudation apparently causes differences in susceptibility of bleached and unbleached seed to *F. solani* f. sp. *pisi*.

Germinating pea seeds exude sugars (4,10,14,16) and amino acids (14) that diffuse into the spermosphere soil (15) and stimulate microbial growth which can result in seed or seedling disease when soilborne pathogens are present. Pea seeds that lose normal green color during maturation, a process known as bleaching (10), are more susceptible to seed- and seedling-infecting fungi than are unbleached seeds of the same cultivar (17,18). As in other host-pathogen interactions (4,7,8,11), increased susceptibility of bleached (yellow) seeds was correlated with increased exudation of carbohydrates (10,16). Kraft (9) showed that the fungistatic effect of delphinidin, an anthocyanidin (anthocyanin-aglycone) pigment in the testae of pea cultivars responsible for resistance of pea seeds and seedlings to *Pythium* and *Aschochyta (Mycosphaerella)* seedling rots, was overcome by addition of glucose.

Amino acids in seed and root exudates also stimulate spores of soilborne plant pathogens to germinate (1,12,13), but those from pea apparently have not been evaluated for that effect.

The objective of this study was to investigate the role of exudates from bleached and unbleached seeds in the differential susceptibility of pea seeds to *Fusarium solani* f. sp. *pisi*.

MATERIALS AND METHODS

Source and preparation of seeds. Seeds of a wrinkled-seed pea *(Pisum sativum* L. 'Miragreen') were obtained from Ferry-Morse Seed Company (Mountain View, CA 94040), or grown at Michigan State University. Individual seeds of uniform size and free from spots or cracks were selected and sorted into bleached and unbleached lots. All seeds were surface sterilized by soaking in 30% H_2O_2 for 5 min and rinsing three times in sterile distilled water.

Preparation of inoculum. A culture of *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (Jones) Snyd. & Hans. isolated from pea root was obtained from J. L. Lockwood, Michigan State University, and maintained on potato-dextrose agar (PDA) at 24 C under continuous fluorescent light. Conidia were harvested, washed by centrifugation in three changes of sterile distilled water, resuspended in sterile distilled water, and their concentration was adjusted with a hemacytometer. Chlamydospores were produced in shaken culture according to the method of Short and Lacy (15), washed in sterile distilled water by centrifugation, agitated at low speed in a Sorvall Omni-Mixer (Ivan Sorvall Co., Norwalk, CT 06856) for 1 hr to break up chlamydospore aggregates, and adjusted to final inoculum concentrations with a hemacytometer.

Evaluation of disease. Inoculated seeds were incubated at 22 C for 5 days and rated for disease: 0 = no necrotic lesions; 1 = 1–10%; 2 = 11–25%; 3 = 26–50%; 4 = 51–75%; and 5 = > 75% necrosis of the seed surface.

Application of seed exudates to inoculated seeds. To determine whether bleached or unbleached seed exudates at the host-pathogen interface would differentially affect disease severity, unbleached pea seeds were placed in 25 mm-diameter test tubes containing 25 g of sterile 1 mm-diameter glass beads, inoculated with 1 ml of a suspension of *F. solani* f. sp. *pisi* conidia (1×10^5/ml), and covered with 10 g of sterile glass beads. Then 6 ml of exudate from bleached or unbleached seeds or sterile distilled water was added to each tube. The tubes were capped, sealed with Parafilm (American Can Co., Greenwich, CT 06830), and incubated for 5 days at 22 C.

Inoculation of seeds by injection. Seeds were allowed to imbibe water for 24 hr on sterile, moist filter paper in petri dishes, then 1 μl of a conidial suspension which contained 10, 100, or 1,000 spores was injected aseptically into one of the cotyledons with a 10-μl Hamilton syringe. Uninoculated seeds and seeds injected with sterile distilled water served as controls.

Chlamydospore germination. Pea seed exudates were concentrated and fractionated into cationic, anionic, and neutral components with exchange resins (Bio Rad AG 50-X8, H^+ form and Bio Rad AG 1-X8, formate form, Bio-Rad Laboratories, Richmond, CA 94800). The three fractions each were taken to dryness and redissolved in 5 ml of glass-distilled water twice. The ability of these seed exudate fractions, or complete seed exudates, to stimulate germination of chlamydospores of *F. solani* f. sp. *pisi* (1×10^6 spores/g) was determined by the method of Schroth et al (13). Air-dried Conover loam soil (0.5 g) that had been infested with chlamydospores of *F. solani* f. sp. *pisi* was placed in a 5 cm-diameter petri dish, moistened with 0.2 ml of a test solution, and

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incubated in a saturated atmosphere for 15 hr at 22 C. Percentage germination was determined in a soil smear stained with 0.1% aniline blue in lactic acid. A minimum of 200 spores was counted per treatment at × 430 magnification.

Collection of seed exudates. Acid-washed silica sand (25 g) and glass-distilled water (4 ml) were placed in 25 mm-diameter test tubes which then were capped and autoclaved. Individual surface-sterilized pea seeds were placed in the tubes, covered with 10 g of sterile sand, and moistened with 3 ml of sterile glass-distilled water. The tubes were sealed with Parafilm, incubated for 5 days at 22 C, and the peas were removed from the tubes and placed on PDA for 5 days. Tubes that contained visibly contaminated seeds were discarded. Sand from tubes with sterile seeds was washed with glass-distilled water to obtain a leachate which was filtered through a Gelman membrane filter (2.2 μm pore size), evaporated to dryness in a flash evaporator at 60 C, redissolved in 10 ml of glass-distilled water, and stored at -20 C until used.

Analysis of carbohydrates and amino acids. Carbohydrates were separated on silica gel thin-layer chromatography plates (layer thickness 0.25 mm, E. Merck Laboratories Inc., Elmsford, NY 10523) with *n*-butanol:acetone:water (4:5:1, v/v). Separated carbohydrates were detected by spraying the chromatogram with ADOP indicator (aniline, 4 ml: diphenylamine, 4 g; orthophosphoric acid, 20 ml in a mixture of 200 ml acetone and 100 ml acetic acid), followed by heating for 10 min at 100 C (6).

Carbohydrates also were separated and quantified by gas-liquid chromatography of trimethylsilyl (TMS) derivatives according to the method of Sweeley et al (19) with a Hewlett-Packard Model 402 gas chromatograph with hydrogen flame ionization detector, and a U-shaped glass column (2 mm diameter) packed with 177 to 149-μm (80 to 100-mesh) Gas Chrom Q (Applied Science Laboratories Inc., State College, PA 16801). Column temperature was programmed linearly from 140–240 C at 2 C/min. The flow rate of the nitrogen carrier gas was 25 ml/min. Inositol was used as an internal standard and the detector response factor was calculated with glucose, fructose, sucrose, and inositol standards that were silylated by the same procedure used for the exudates.

To assay for individual amino acids, exudate samples containing 2,000–3,000 ng glycine equivalents per milliliter were dried at 60 C in a flash evaporator, redissolved in 2.5 ml of 70% ethanol, incubated for 12 hr at 10 C, and the precipitate was removed by centrifugation. The supernatant was taken to dryness at 60 C in a flash evaporator and redissolved in 1 ml of 0.01 N HCl. Samples (250 μl) were analyzed with an amino acid analyzer (modified Technicon system) with a column of Chromo Beads C2 (Technicon Corporation, Tarrytown, NY 10591).

RESULTS

Influence of seed exudates on infection. Necrosis was more severe in seeds treated with bleached seed exudate than on those treated with unbleached seed exudate (Table 1). Disease severity for seeds treated with unbleached seed exudate did not differ significantly ($P = 0.05$) from that of seeds treated with distilled water, although disease severity always was greater when exudates were supplied.

When inoculum was injected directly into the cotyledon of the pea seed, which eliminated the external influence of seed exudates on disease development, disease ratings of bleached and unbleached seeds did not differ at three inoculum levels (Table 2), but disease severity increased as inoculum concentration increased. Therefore, the greater susceptibility of bleached seeds to *F. solani* f. sp. *pisi* apparently was due to differences in seed exudation.

Carbohydrate and amino acid analysis. Sucrose, glucose, and fructose were identified and quantified from bleached and unbleached seed exudates by gas-liquid chromatography (Table 3) and thin layer chromatography. All three sugars, especially sucrose, were present in larger quantities in bleached seed exudate than in unbleached seed exudate.

Similar amounts of 14 amino acids were present in exudates of

TABLE 1. Effect of exudates from bleached or unbleached pea seeds on infection of unbleached pea seeds by *Fusarium solani* f. sp. *pisi*

Treatment[a]	Disease rating	
Exudate from unbleached seeds-inoculated	2.1[b]	A[c]
Exudate from bleached seeds-inoculated	3.7	B
Glass distilled water-inoculated	1.9	A
Glass distilled water-uninoculated	0.6	C

[a] Seeds were incubated in exudate solution or glass distilled water for 5 days at 22 C. Inoculum was adjusted to 1×10^5 conidia/seed.
[b] Disease rating scale: 0 = no lesions; 1 = 1–10%; 2 = 11–25%; 3 = 26–50%; 4 = 51–75%, and 5 = >75% of the seed was necrotic. Mean of two replicates of 10 seeds each.
[c] Means followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

TABLE 2. Disease development following injection of bleached or unbleached Miragreen pea seeds with conidia of *Fusarium solani* f. sp. *pisi*

	Disease rating[b]	
Treatment[a]	Unbleached seeds	Bleached seeds
10 conidia per seed	1.0 A[c]	0.9 A
100 conidia per seed	1.6 B	1.6 B
1,000 conidia per seed	1.9 B	1.9 B
Distilled water	0.4 C	0.4 C
Uninjected	0.1 C	0.1 C

[a] Conidial suspensions were injected under the seed coat and seeds were incubated under sterile, moist conditions for 5 days at 22 C.
[b] Disease rating scale: 0 = no lesions; 1 = 1–10%; 2 = 11–25%; 3 = 26–50%; 4 = 51–75%; and 5 = >75% of the seed was necrotic. Mean of two replicates of 10 seeds each.
[c] Means followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

TABLE 3. Carbohydrates exuded from bleached and unbleached pea seeds

	Concentration (nmoles per seed)[a]	
Carbohydrate	Bleached seeds	Unbleached seeds
Fructose	102	30
Glucose	156	47
Sucrose	184	18

[a] Means of two replicates of 43 seeds each. Leachates were collected aseptically from seeds incubated in moist sand and assayed by gas-liquid chromatography.

TABLE 4. Amino acids in exudates from bleached and unbleached pea seeds

	Concentration (nmoles per seed)[a]	
Amino acid	Bleached seeds	Unbleached seeds
Aspartic acid	6	9
Threonine	10	8
Serine	8	13
Glutamic acid	8	11
Glycine	9	11
Alanine	12	16
Valine	5	7
Isoleucine	3	5
Leucine	7	6
Phenylalanine	4	4
Lysine	4	3
Histidine	4	4
Arginine	2	3
Tyrosine	3	2

[a] Means of two replicates of 43 seeds each. Leachates were collected aseptically from seeds incubated in moist sand and assayed with an amino acid analyzer.

TABLE 5. Stimulation of *Fusarium solani* f. sp. *pisi* chlamydospore germination in field soil by exudates or exudate components from bleached or unbleached pea seeds

Exudate source	Exudate fraction	Chlamydospore germination (%)[a]	
Unbleached seed	Unfractionated	16	B[b]
	Anionic	0	A
	Cationic	0	A
	Neutral	10	B
Bleached seed	Unfractionated	48	C
	Anionic	0	A
	Cationic	0	A
	Neutral	40	C
Distilled water control	...	0	A

[a] Means of two replicates of 100 chlamydospores each.
[b] Means followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

both bleached and unbleached seeds (Table 4). All amino acids identified were those commonly found in proteins.

Chlamydospore germination. Unfractionated exudate from bleached seed and the neutral (carbohydrate) fraction stimulated more chlamydospore germination than unfractionated exudate from unbleached seed (Table 5). Neither the cationic (amino acid) nor anionic (non-amino acid) fractions of either unbleached or bleached seed exudate stimulated chlamydospore germination. The unfractionated seed exudates promoted more chlamydospore germination than did the neutral fractions of exudates from both bleached and unbleached seeds.

DISCUSSION

Differences in seed exudates were associated with differences in susceptibility of bleached and unbleached pea seeds to *F. solani* f. sp. *pisi*. Bleached pea seed exudate added to unbleached seeds significantly increased their susceptibility compared to that of seeds treated with exudate from unbleached seed. Further, bleached and unbleached pea seeds did not differ in susceptibility when inoculum was introduced into the cotyledons of the seed to minimize the influence of seed exudates. Similar techniques have been used in other systems to determine the effect of seed or root exudates on the occurrence and severity of diseases caused by soilborne pathogens (2,4).

Amounts of sucrose, glucose, and fructose exuded from pea seeds were correlated with susceptibility of pea to seed- and seedling-infecting fungi, which confirmed the conclusions of Short and Lacy (16) and Maguire et al (10). In our study there were quantitative but no qualitative differences in sugars, and no qualitative or quantitative differences in amino acids exuded from bleached and unbleached pea seeds. Similar arrays of sugars and amino acids have been detected in exudates from pine (1), cotton (5), and bean (14) seeds. Although other workers (3) concluded that available carbon was the principal factor limiting germination of chlamydospores of *Fusarium* spp. in soil, germination was higher when both amino acids and sugars were supplied. Chlamydospores of *F. solani* f. sp. *pisi* germinated only in response to the fractions of seed exudates that contained carbohydrate. Since unfractionated pea seed exudates were more stimulatory to germination than their carbohydrate fractions, other unidentified components of the exudate, such as amino acids, probably enhanced germination.

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