MECHANISM OF INCREASED SUSCEPTIBILITY OF BLEACHED PEA SEEDS TO SEED AND SEEDLING ROT

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ABSTRACT

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By

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Maturation of pea (Pisum sativum L. 'Miragreen') seeds during moist, hot, sunny weather causes many green pea seeds to turn yellow. This process, known as bleaching, has been reported to increase seed and seedling rot of peas in growth chamber studies. Bleached and nonbleached seeds were placed in beds of glass beads in the laboratory, inoculated with 1×10^8 conidia of Fusarium solani f. sp. pisi per seed. Some seeds were leached with distilled water. Disease severity of leached seeds was reduced below that of nonleached controls, apparently due to the removal of nutrients from the host-pathogen interface. Green seeds were placed in beds of glass beads, inoculated as before, and exudates of bleached or nonbleached seeds were added. Seeds incubated in exudates from bleached seeds had a higher disease rating than those incubated in exudates from nonbleached seeds. When bleached and nonbleached seeds were allowed to inbibe water, and conidial suspensions (10, 100, or 1,000 conidia/seed) were injected beneath the seed coat, disease severity was similar.

Exudates from bleached and nonbleached seeds contained similar amounts of ninhydrin-positive substances. Fourteen amino acids were

present in exudates from both seed types; no qualitative or quantitative differences in the amino acid composition were observed. Bleached seed exudates contained approximately three times more soluble carbohydrate than did nonbleached seed exudate. Sucrose, glucose, and fructose were identified from both types of seed exudate, but all three sugars were present in larger quantities in bleached seed exudate. Bleached seed exudate was especially rich in sucrose. When the seed exudates were fractionated into anionic, cationic, and neutral components on ion exchange resins, only the carbohydrate-containing (neutral) fraction stimulated chlamydospores of F. solani f. sp. pisi to germinate in natural soil. The neutral fraction of bleached seed exudate caused 40% of the chlamydospores to germinate while the same fraction of nonbleached seed exudate promoted 10% germination. When a 0.1 M solution of sucrose was applied to inoculated nonbleached seeds, disease severity was increased over distilled water controls.

Pea seeds were planted in the field in ten footrows, in soil artificially infested with F. solani f. sp. pisi chlamydospores. Plots planted with nonbleached, partially-bleached, and bleached seeds had 69, 58, and 31% emergence, total fresh weights (grams of stems, leaves and pods) of 2398, 1965, and 1327, and shelled pea weights (grams) of 477, 445, and 237 per plot, respectively. Since the three types of seeds were equally viable when germinated in vitro, these data indicate that bleaching appears to increase the susceptibility of pea seeds to seed and seedling rot in the field. Differences in carbohydrate (especially sucrose) exudation from bleached and nonbleached seeds is probably the basis for differences in seed and seedling susceptibility to F. solani f. sp. pisi.

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Rosemary Loria

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To Lenny,

my best friend

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Pea seed viability and seedling vigor appear to depend on environmental conditions during seed maturation, and on a timely harvest (31). Maturation of pea seeds during moist, hot sunny weather causes many green seeds to turn yellow, a process known as bleaching (29). Bleached seeds are of questionable value as seed, since there is evidence that they may be more susceptible to seed and seedling-infecting fungi than nonbleached seeds of the same variety (55).

Germinating pea seeds exude nutrients such as sugars (17, 29, 54) and amino acids (51) which diffuse into the spermosphere soil (56). These nutrients stimulate spore germination and vegetative growth of the spermosphere microflora, including seed-infecting fungi. Exudates from germinating bleached seeds contained more carbohydrates than exudates from germinating nonbleached seeds (29, 54). Preemergence damping-off and carbohydrate exudation were higher among wrinkled-seeded pea cultivars than among smooth-seeded pea cultivars (17). Carbohydrate exudation was also a determining factor in the susceptibility of some soybean varieties to Pythium infection (25).

Amino acids are also components of pea seed exudates (51). Those from pea apparently have not been evaluated as stimulants of germination of pathogens of pea seeds, but those from other plants have. For example amino acids from bean and pine seeds have the ability to stimulate germination of chlamydospores of Fusarium solani

f. sp. phaseoli (52), and zoospores of Pythium afertile (3), respectively. Amino acids or other nitrogen-containing compounds in tomato root exudate may be responsible for stimulating germination of conidia and microsclerotia of Verticillium albo-atrum in soil (46). Conidia of F. solani f. sp. phaseoli were more virulent to bean stems when supplied with organic sources of nitrogen than when supplied with only glucose (61). Both organic and inorganic sources of nitrogen increased the incidence of the root disease of bean caused by F. solani f. sp. phaseoli under field conditions (69).

The evidence suggests that the increased nutrients in exudates from bleached, as compared with nonbleached pea seeds, may cause increased seedling susceptibility to F. solani f. sp. pisi. The objectives of this study were to determine if nonbleached pea seeds are more resistant to F. solani f. sp. pisi than bleached pea seeds in the field, to investigate the role of seed exudates in the differences in susceptibility expressed by nonbleached and bleached seeds, and to determine the effect of some components of pea seed exudate on chlamydospore germination and pathogenesis of F. solani f. sp. pisi.

LITERATURE REVIEW

Exudation of nutrients from germinating seeds and roots is known for a number of plant species, and is probably common to all higher plants. Although there have been extensive investigations of the composition of exudates from plant roots (5, 24, 62, 63, 64, 65, 66), few data are available on the composition of exudates from germinating seeds. There is ample evidence that carbohydrates, as a class, are present in the seed exudates of peas (26, 32, 33, 34, 51, 54, 57), beans (25, 47, 51), and cotton (51), and that amino acids or related compounds are found in bean (42, 47, 51), cotton (51), and pea (51) seed exudate. In addition, specific compounds have been identified from seed exudates: several sugars and amino acids from cotton (19), pine (3), and bean (28, 52), and aliphatic and aromatic acids from cotton, pea, and barley (27).

The relation of seed and root exudates to relatively high microbial activity in the soil around these structures, termed the spermosphere (67) or rhizosphere (21), is well documented. These exudates influence pathogenic fungi directly by inducing germination, enhancing nutritional status before penetration, and inhibiting growth; or indirectly by stimulating competition or antagonism from other microorganisms (49). Therefore, the kinds and amounts of substances exuded from seeds or roots have a strong influence on host-pathogen interactions in the spermosphere or rhizosphere.

Effect of Exudates
on Spore Germination

Propagules of seed and root-infecting fungi remain dormant in soil until stimulated to germinate by a nutrient source (10, 11, 18, 56). In most cases this stimulatory effect is nonspecific, and there are many reports of dormant fungal propagules germinating in the rhizosphere of nonsusceptible plants (46, 48, 58). The ability of various components of seed or root exudates to stimulate germination of dormant fungal propagules has been investigated. Conidia and microsclerotia of Verticillium albo-atrum in natural soil were stimulated to germinate by the basic fraction of tomato-root exudate, which contain amino acids, but not by the neutral-acidic fraction, which contains sugars and organic acids (46). Since sugars have been shown to stimulate germination of microsclerotia in soil (16), the lack of germination in response to the neutral-acidic fraction may have been due to a level of carbohydrate in the exudate lower than that necessary to stimulate germination, or to an inhibition of germination by organic acids in the exudate. Organic acids have been reported to inhibit zoospore germination of Pythium afertile (3).

All sugars and most amino acids identified from seed exudates of Pinus resinosa stimulated zoospore germination of Pythium afertile when supplied singly; glucose and asparagine were particularly effective in accelerating germ tube development (3). Exudates were collected from germinating bean seeds, and amino acids and sugars were identified and assayed individually for their ability to stimulate germination of chlamydospores of F. solani f. sp. phaseoli in soil. While all sugars and most amino acids stimulated germination to some

degree, glucose and asparigine stimulated chlamydospore germination to a greater extent than any of the other components tested (52).

Effect of Exudates on Competition and Antagonism

Seed and root exudates stimulate microorganisms which are antagonistic to pathogenic fungi in the spermosphere or rhizosphere. Slykhuis (59) found that the number of bacteria in soil apart from grass seeds was not correlated with the amount of suppression of Fusarium blight, while the numbers in soil around germinating seeds was. The primary mechanism by which soil bacteria inhibited F. oxysporum was by immobilization of nitrogen when the carbon supply was high (30). This inhibition was removed when the ratio of carbon to nitrogen was less than five. The ability of bacterial isolates to compete with F. oxysporum was not related to their growth rates, but was correlated with their ability to develop in the absence of growth factors.

Soil moisture influences the survival of germinated spores by affecting bacterial activity. In soil amended with carbon and nitrogen, chlamydospores of F. roseum f. sp. cerealis 'Culmorum' germinated and grew well at soil water potentials below 10 bars (13). At higher water potentials, germ tubes lysed due to high bacterial activity. When antibiotics were added to the soil with the nutrients the germ tubes did not lyse, even at the highest water potential tested (-1.0 bars). In soil near pea seeds, high soil water potentials increased germination of chlamydospores of F. solani f. sp. pisi due to increased seed exudation. Soil water potential also influenced germling survival by modifying bacterial activity (12). In soil containing more than 8.7% moisture, germination at 20 hours after planting was

high; but by 48 hours up to half of the germ tubes were lysed. In drier soils, germination was lower but no lysis was detected. The number of germ tubes that successfully penetrated the foot region of the plant was greater at 8% soil moisture than at all other moisture levels tested.

Effect of Exudates on Disease Incidence

There is ample evidence for a relationship between seed and seedling exudation and susceptibility of seedlings to damping-off diseases. Resistance of soybean varieties to preemergence mortality caused by Pythium was negatively correlated with the amount of carbohydrate exuded from the germinating seed (25). When the seeds of a resistant variety were coated with powdered sucrose and planted in infested soil, preemergence mortality was increased. The quantity of electrolytes leaching from seeds of 11 cultivars of pea or 16 cultivars of french bean that were soaked in distilled water was directly related to the incidence of damping-off in the field (33). In the same study, the amount of soluble carbohydrate exuded from pea seeds was also correlated with preemergence mortality in the field. There was a direct relationship between preemergence damping-off of three bean varieties and the quantity of fluorescent, ninhydrin-positive, and silver nitrate-positive substances in the seed exudates (47). Flentje and Sakena (17) found that susceptibility of wrinkled-seeded peas to Pythium was due to high quantities of carbohydrates in the seed exudate. Smooth-seeded peas which exuded small quantities of carbohydrates were resistant to the damping-off disease caused by this fungus. The incidence of foot-rot disease of rice caused by F. moniliforme was

reduced when germinated, rather than ungerminated, rice seeds were sown in infested soil (44). Most of the nutrients had been exuded from the seeds before they were planted, and therefore were not available to increase inoculum potential. Kerr (26) found that the effect of soil moisture on infection of peas by Pythium ultimum was due to its influence on seed exudation. More sugar was exuded from germinating peas at higher soil moistures, and the amount of sugar in the seed exudate was directly related to disease incidence.

Temperature has been shown to influence exudation from seeds and roots. An increase in amino acid exudation from strawberry roots at low temperatures increased the severity of Rhizoctonia root decay (22). Seven and three times as much total amino acids and sugars were exuded at 18 and 24°C, respectively, than at 30°C by germinating cotton seeds (19). Evidence suggested that this increase in seed exudation at low temperatures was associated with the preemergence mortality of cotton seedlings caused by R. solani under these conditions. Vancura (63) observed that 'cold shock' stimulated higher exudation from the roots of maize and cucumber. An increase in the number of fungi in the spermosphere of maize was also observed after exposure to low temperatures (53).

Bleaching of Seeds

Extremes of temperature, moisture, and light following seed maturation result in a loss of chlorophyll from the cotyledons of peas (29), lima beans (43), and other species (40), a process known as bleaching. There were no differences in the constituents of bleached and nonbleached pea seeds when mineral composition (6), amino acids, sugars, and carboxylic acids (29) were examined. However, exudates

from germinating bleached seeds have been shown to contain more carbohydrates than exudates from germinating nonbleached seeds (29, 54). Other components of the exudates from bleached and nonbleached seeds apparently have not been compared.

MATERIALS AND METHODS

Maintenance of the Pathogen and Preparation of Inoculum

A culture of Fusarium solani (Mart.) Sacc. f. sp. pisi (Jones) Snyder & Hansen isolated from Pisum sativum L. was obtained from J. L. Lockwood, Michigan State University, and maintained on potato-dextrose agar petri plates (PDA) at 24°C under continuous fluorescent light. Conidia were harvested by flooding colonies with 10 ml of sterile distilled water, then washing the conidia by centrifugation in three changes of sterile, distilled water. The pelleted conidia were resuspended in sterile distilled water and the concentration was adjusted as necessary using a hemacytometer. Chlamydospores were produced in shaken culture as follows: Conidia, prepared as described above, were incubated in potato-dextrose broth (PDB) for 24 hours, centrifuged, washed, and placed in agitated, sterile aqueous soil extract for 10-14 days (prepared by mixing 1 liter Conover loam soil with one liter of water, allowing to stand for 48 hours, and filtering the supernatant through a 2.2-μm Gelman membrane filter). Chlamydospores formed by macroconidia were then washed by centrifugation and agitated at a low speed in a Sorvall Omni-Mixer for 1 hour to break up chlamydospore aggregates. The concentration of chlamydospores was adjusted as necessary using a hemacytometer.

Source and Preparation of Seeds

A wrinkled-seeded pea (Pisum sativum L. cv. Miragreen) obtained from Ferry-Morse Seed Company (1974 seed lot) or grown at the Michigan State University Botany Farm (1975 seed lot) was used. Individual seeds were selected on the basis of uniformity in size and freedom from spots or cracks on the seed coat, then sorted according to the degree of bleaching into lots of bleached and nonbleached. Sodium hypochlorite (NaOCl) is widely used for surface sterilizing seeds in exudate collection studies (3, 19, 27, 54). However, there is evidence that NaOCl may effect seed metabolism, and that it cannot be removed with distilled water (1, 2). Exudation of soluble carbohydrate from bleached pea seeds (1974) that had been surface sterilized with 1% NaOCl for 30 minutes was compared with that from seeds sterilized with 30% hydrogen peroxide (H_2O_2) for 5 minutes. These treatments provide equivalent degrees of surface sterilization. The seeds were treated as described, rinsed with three changes of sterile distilled water, and incubated in leaching dishes (a modified deep petri dish, with an outlet in the lower half of the dish, containing 300 g of moist 1 mm-diameter glass beads) for 24 hours at 22°C. The exudate was collected by flushing the beads with two 50 ml aliquots of sterile distilled water. The exudate was passed through a 2.2 µm Millipore filter, taken to dryness in a flash evaporator at 60°C, and redissolved in 10 ml of distilled water. Total soluble carbohydrate was determined with a modified anthrone analysis (39) using a standard curve derived from glucose. Seeds sterilized with NaOCl exuded more soluble carbohydrate than those sterilized with H_2O_2 (Table 1). It was concluded that H_2O_2 was a more desirable surface sterilant than NaOCl for seed exudation studies. For experiments

Table 1. Comparison of two surface-sterilizing agents on carbohydrate exudation from bleached Miragreen pea seeds in moist glass beads.

Surface Sterilant[a]	µg Carbohydrate exuded per seed[b]				
	Trial 1	Trial 2	Trial 3	Trial 4	Mean
1% NaOCl	689	595	121	131	384
30% H_2O_2	488	413	72	86	265

[a]Ten seeds were soaked in 1% NaOCl for 30 minutes or 30% H_2O_2 for 5 minutes followed by three rinses in sterile distilled water.

[b]Seeds were incubated in beds of glass beads for 24 hours at 22°C. Exudates were collected by flushing the glass beads with sterile distilled water. Carbohydrate was measured by the anthrone method and is expressed as glucose equivalents.

requiring aseptic conditions, seeds were treated for 5 minutes in 30% H_2O_2, followed by 3 rinses in sterile distilled water.

Source, Preparation, and Infestation of Soil

Conover loam soil from the Michigan State University Botany Farm, from an area free from recent pesticide application, was used for field as well as laboratory experiments. Prior to use in laboratory studies, the soil was air-dried and passed through a 30-mesh sieve, then stored in covered metal containers lined with plastic bags. Soil infested with chlamydospores of F. solani f. sp. pisi was obtained for laboratory experiments by placing air-dried, sieved soil in a small rotary mixer with baffles, and applying an aqueous spore suspension with an atomizer until the desired spore concentration and soil moisture were attained. Field soil was infested by spraying a suspension of chlamydospores into the seed furrow after planting but prior to covering seeds.

The Leaching Apparatus

A leaching system was employed which consisted of a modified deep petri dish, with an inlet in the lid and an outlet in the lower half of the dish, containing 300 g of 1 mm-diameter glass beads (Figure 1). Sterile distilled water was pumped from a reservoir to the leaching dish by a peristaltic pump. The water passed through the dish and into a drainage flask. Ten yellow or green seeds were placed in each leaching system. The seeds were then individually inoculated with 1 ml of a conidial suspension (1×10^8/ml) of F. solani f. sp. pisi and covered with glass beads. The peristaltic pump supplied 75 ml of water over a period of fifteen minutes during each hour. Noninoculated seeds served as controls.

Figure 1. Apparatus used for leaching pea seeds. Ten seeds were placed in the bed of glass beads, and the peristaltic pump supplied 75 ml of water during a period of fifteen minutes every hour.

Inoculation of Seeds by Injection

Surface-disinfested bleached and nonbleached seeds were allowed to imbibe water for 24 hours on moist filter paper in petri dishes. Seeds were then injected in one of the cotyledons with 1 µl of a conidial suspension containing 10, 100, or 1,000 conidia respectively, using a 10 µl Hamilton syringe which had been sterilized in 95% ethyl alcohol and rinsed in sterile distilled water. Noninjected seeds and seeds injected with sterile distilled water served as controls.

Evaluation of Disease

Seeds were incubated at 22°C for five days unless otherwise noted, then evaluated for disease severity. A rating system of 0 to 5 was used: 0 indicated no lesions; 1 = 1% to 10% of the seed was necrotic; 2 = 11% to 25% of the seed was necrotic; 3 = 26% to 50% of the seed was necrotic; 4 = 51% to 75% of the seed was necrotic; and 5 = > 75% of the seed was necrotic.

Collection of Exudates

Twenty-five mm-diameter test tubes were filled with 25 g of acid-washed silica sand. Four ml of glass-distilled water was added to each tube, and the tubes were capped and autoclaved. Miragreen pea seeds (1975) were prepared as described. One yellow or green seed was put into each tube and covered with 10 g of sterile sand. Three ml of sterile distilled water was added to each tube, and the tubes were sealed with Parafilm (American Can Company) and incubated at 22°C for 5 days. The seeds were then removed from the tubes and placed on PDA plates for 5 days. Tubes which contained contaminated seeds were discarded. The sand from the remaining tubes was washed with glass-distilled water, the leachate was filtered through a Gelman membrane filter (2.2 µm pore

size), evaporated to dryness in a flash evaporator at 60°C, and redissolved in 10 ml of glass-distilled water. This exudate solution was stored at -20°C until used.

Fractionation of Exudates

Concentrated solutions of pea seed exudate were fractionated into cationic, anionic, and neutral components. Thirty ml of cation exchange resin (Bio Rad AG 50-X8, H+ form) was prepared by passing 5N NaOH through the resin in a 1.5 cm-diameter column until the pH was 14, rinsing with glass-distilled water until the pH was 5, passing 5N HCL through until the pH was 1, then rinsing again until the pH was 5. Thirty ml of anion exchange resin (Bio Rad AG 1-X8, formate form) was prepared similarly, in a column of 1.5 cm-diameter, by rinsing sequentially with 2N NaOH until the pH was 14, glass-distilled water until the pH was 5, 1N acetic acid until the pH was 1, then glass-distilled water until the pH was 5. Five ml of a concentrated pea seed exudate solution was applied to the cation exchange column and washed through with 300 ml of .01 N HCL. The wash from the cation resin was taken to dryness twice, redissolved in glass-distilled water, then applied to the anion exchange resin. The anion resin was rinsed with 300 ml of glass-distilled water, and this solution was designated the neutral fraction. The cation exchange resin was eluted with 300 ml of 4 N HCL (cationic fraction), and the anion exchange resin was eluted with 1 N acetic acid (anionic fraction). All three fractions were taken to dryness twice and redissolved in 5 ml of glass-distilled water for chlamydospore germination studies.

Chemical Analysis of Exudates

Total water-soluble carbohydrate was determined by a modified anthrone analysis (39) in which 1 ml of exudate was mixed with 9 ml of anthrone reagent, placed in a boiling water bath for 10 minutes, and immediately cooled in an ice bath. The solution was then allowed to warm to room temperature, and optical density at 600 nm was measured with a Bausch and Lomb Spectronic 20 spectrophotometer. Carbohydrate concentrations were determined using a standard curve derived from glucose. Total amino acids and related compounds were determined by the ninhydrin method of Moore and Stein (38), using glycine as a standard.

Carbohydrates were separated on thin layer chromatography plates coated with silica gel (layer thickness 0.25 mm) obtained from E. Merck Laboratories Inc., Rahway, N.J. The solvent system used was n-butanol: acetone: water (4:5:1, v/v/v) and the carbohydrates were detected by spraying the chromatogram with ADOP indicator (Aniline, 4 ml; diphenylamine, 4 g; orthophosphoric acid 20 ml in 200 ml acetone mixed with 100 ml acetic acid), then heating for 10 minutes at 100°C (23).

Carbohydrates were also separated and quantified by gas-liquid chromatography of trimethylsilyl (TMS) derivatives according to the method of Sweeley et al (60). TMS derivatives of the carbohydrates were formed in pyridine containing hexamethyldisilazane and trimethylchlorosilane at 22°C in Kimax vials (100 x 13 mm) covered with a teflon-lined cap. The Hewlett Packard (Model 402) gas chromatograph, with hydrogen flame ionization detector, employed a U-shaped glass column (2 mm diameter) packed with 80-100 mesh Gas Chrom Q (Applied Science Laboratories Inc.). Separation of the carbohydrates was done using linear temperature-programmed analysis from 140-240°C at 2°C min^{-1}. The flow rate of the nitrogen carrier gas was 25 ml min^{-1}. Inositol was used as

an internal standard and the detector response factor was calculated using standards of glucose, fructose, sucrose, and inositol that were silyated by the same procedure used for the exudates.

Individual amino acids were identified and quantified. Exudate samples containing 2,000 - 3,000 nm of glycine equivalents per ml were taken to dryness at 60°C in a flash evaporator, and redissolved in 2.5 ml of 70% ethanol. The samples were then incubated for 12 hours 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 .01 N HCL. Samples (250 µl) were then run on an amino acid analyzer (modified Technicon system) which used Chromo Beads C2 (Technicon Corporation, Tarrytown, N.Y.)

Chlamydospore Germination

The ability of seed exudates or their components to stimulate germination of chlamydospores of F. solani f. sp. pisi was determined using the method of Schroth et al (52). Air-dried Conover loam soil (.5 g) that had been infested with chlamydospores of F. solani f. sp. pisi (1×10^6 spores/g) was placed in a small petri dish (5 cm diameter), moistened with 0.2 ml of a test solution, and incubated in a humid container for 15 hours at 22°C. The percentage germination was determined by making a soil smear on a slide, staining it with 0.1% aniline blue in lactic acid, and counting spores at a magnification of X 430. One hundred chlamydospores were counted per slide.

All experiments were repeated at least once with similar results, except those conducted in the field.

RESULTS

Emergence, Fresh Weight, and Seed Viability

Bleached, partially bleached, and nonbleached pea seeds were planted at the Michigan State University Botany Farm on 9 May 1975, in soil artificially infested with chlamydospores of F. solani f. sp. pisi. Seeds were planted 25-30 mm below the soil surface in rows 3.8 meters long, spaced 60 cm apart. Each treatment was replicated four times, with 100 seeds per replicate. Seeds coated with 0.8 g thiram (tetramethylthiuram disulfide) (active ingredient) per kg seed served as controls. All seeds which extended a pumule above the soil surface were counted as emerged. Emergence of untreated bleached seeds was less than that of untreated nonbleached seeds, while emergence of partially bleached seeds was intermediate (Table 2). No significant differences in emergence were observed between thiram-treated seeds in the field regardless of bleaching. All seedlings were a normal green color following emergence and appeared normal in growth habit and equal in vigor.

Plants were harvested at maturity by severing vines from the roots at the soil line, and the fresh weight of the above-ground portion (leaves, vines, and pods) of the total plant population was determined. The pods were then shelled mechanically to obtain the fresh weights of the peas. There were no significant differences in total fresh weights or shelled pea yields between plants grown from fungicide-treated bleached or nonbleached seeds. However, plants from untreated

Table 2. Effects of seed bleaching and seed treatment on emergence, fresh weight, and yield of Miragreen peas.

Seed Treatment[a]	Degree of Bleaching	Emergence	Green Fresh Weight (g)	
			Leaves, Vines, Pods & Peas	Shelled Peas
Fungicide	Nonbleached	82 A[b]	2,092 AB	464 A
	Partially Bleached	73 AB	1,539 AB	343 AB
	Bleached	78 A	1,936 AB	432 A
No Fungicide	Nonbleached	69 AB	2,398 A	477 A
	Partially Bleached	58 B	1,965 AB	444 A
	Bleached	30 C	1,326 B	238 B

[a]Fungicide = 0.8 g thiram (active ingredient) per kg of seed. Seeds were sprayed with a chlamydospore suspension of Fusarium solani f sp. pisi after planting but before covering the rows.

[b]Means of 4 replicates, 100 seeds per replication. Means in each column followed by the same letter do not differ significantly by Duncan's multiple range test.

nonbleached seeds had higher total fresh weights and shelled pea yields than plants grown from untreated, bleached seeds. When total fresh weights and shelled pea weights were calculated on a per-plant basis (Table 3), individual plants from untreated bleached seeds did not differ significantly in fresh weights or pea yield from untreated nonbleached seeds, indicating that the plants were similar in vigor once they were established.

Seeds were germinated in vitro to determine if the differences in emergence in the field were due to differences in the viability of bleached, partially bleached, and nonbleached pea seeds. Surface disinfested seeds were placed on sterile, moist paper towels for 6 days, at 22°C. The germination rates for all the seeds, regardless of amount of bleaching, were between 95% and 98%. It was concluded that differences in emergence between bleached and nonbleached seeds in the field were not due to differences in seed viability.

Influence of Exudates on Infection

Bleached and nonbleached pea seeds that had been inoculated with 1×10^8 conidia per seed of F. solani f. sp. pisi were leached with sterile distilled water to remove seed exudates from the host-pathogen interface. Disease severity was lower for leached seeds of both colors than for nonleached seeds (Table 4), apparently due to removal of nutrients exuded. Leached bleached seeds had a higher disease incidence than leached nonbleached seeds.

It appeared that disease severity could be reduced by continually removing nutrients from the infection court by leaching the seeds with distilled water. An experiment was then conducted to determine whether addition of exudates from bleached or nonbleached seeds to the

Table 3. Effects of seed bleaching and seed treatment on the fresh weights and yields of individual pea plants grown from nonbleached, partially bleached, or bleached Miragreen pea seeds.

Seed Treatment[a]	Seed Color	Percent of Original Stand	Fresh Weight per plant (g)	
			Leaves, Vines Pods & Peas	Shelled Peas
Fungicide	Nonbleached	82	25.88 AB[b]	5.73 AB
	Partially Bleached	73	20.79 A	4.61 A
	Bleached	78	25.14 AB	5.65 AB
No Fungicide	Nonbleached	69	34.64 BC	6.96 ABC
	Partially Bleached	58	32.78 BC	7.35 BC
	Bleached	30	43.96 C	8.16 C

[a]Fungicide = 0.8 g thiram (active ingredient) per kg of seed. Seeds were sprayed with a chlamydospore suspension of Fusarium solani f. sp. pisi after planting but before covering the rows.

[b]Means of 4 replications, 100 seeds per replication. Means in each column followed by the same letter do not differ significantly (P = 0.05) by Duncan's multiple range test.

Table 4. Effect of leaching with distilled water on infection of bleached or nonbleached seeds by F. solani f. sp. pisi.

Degree of Bleaching	Inoculated[a]	Disease Rating[b]	
		Leached[c]	Nonleached
Nonbleached	yes	0.2 AB	2.1 D
Bleached	yes	2.3 D	3.0 E
Nonbleached	no	0.1 A	0.3 B
Bleached	no	0.0 A	0.7 C

[a] 1×10^8 conidia/seed.

[b] 0 = no lesions, 1 = 1-10%, 2 = 11-25%, 3 = 26-50%, 4 = 51-75%, 5 = > 75% of the seed was necrotic. Each value is the mean for two replicates of ten seeds each.

[c] Leaching dishes (10 seeds/dish) were flushed with 75 ml of sterile distilled water every hour. Seeds were incubated for five days at 22°C.

[d] Means followed by the same letter do not differ significantly (P = 0.05) by Duncan's multiple range test.

host-pathogen interface would enhance disease expression. Surface disinfested nonbleached pea seeds (1975) were placed in 25 mm-diameter test tubes containing 25 g of sterilized 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 nonbleached seeds was added to each tube. Sterile distilled water served as a control. The tubes were sealed with Parafilm and incubated for 5 days at 22°C. Inoculated seeds treated with exudate from bleached seeds had a higher disease incidence than those treated with exudate from nonbleached seed exudate (Table 5). Disease in inoculated seeds treated with nonbleached seed exudate did not differ statistically at P=.05 from that in seedling treated with distilled water, although disease severity was always higher where exudates were supplied.

An experiment was conducted to determine whether bleached seeds were more susceptible to F. solani f. sp. pisi than nonbleached seeds when the inoculum was injected into the cotyledon, thereby eliminating the influence of seed exudates on disease development. Disease ratings between bleached and nonbleached seeds did not differ at three inoculum levels (Table 6). Disease severity increased as inoculum concentration increased. Therefore, it appears that the greater susceptibility of bleached seeds to F. solani f. sp. pisi was due to differences in seed exudation.

Chemical Analysis of Seed Exudates

Exudates collected from nonbleached or bleached pea seeds were analyzed for total water-soluble carbohydrate and ninhydrin-positive substances to determine if quantitative differences in these components were associated with the differences in susceptibility expressed by

Table 5. Effect of exudates from bleached or nonbleached pea seeds on infection by F. solani f. sp. pisi of nonbleached pea seeds in sterile, moist glass beads.

Treatment[a]	Disease Rating	
Nonbleached Seed Exudate-Inoculated[b]	2.1[c]	A[d]
Bleached Seed Exudate-Inoculated	3.7	B
Glass Distilled Water-Inoculated	1.9	A
Glass Distilled Water-Noninoculated	0.6	C

[a]Seeds were incubated in exudate solution or glass distilled water for 5 days at 22°C.

[b]1×10^5 conidia/seed.

[c]0 = no lesions, 1 = 1-10%, 2 = 11-25%, 3 = 26-50%, 4 = 51-75%, 5 = > 75% of the seed was necrotic. Each value is the mean for two replicates of ten seeds each.

[d]Means followed by the same letter do not differ significantly (P = 0.05) by Duncan's multiple range test.

Table 6. Effect of injection of bleached or nonbleached Miragreen pea seeds with conidia of F. solani f. sp. pisi.

Treatment[a]	Disease Rating[b]	
	Nonbleached	Bleached
10 conidia/seed	1.0 A[c]	0.9 A
100 conidia/seed	1.6 B	1.6 B
1,000 conidia/seed	1.9 B	1.9 B
Distilled Water	0.4 C	0.4 C
Noninjected	0.1 C	0.1 C

[a]Conidial suspension was injected under the seed coat and the seed was incubated under sterile, moist conditions for five days, at 22°C.

[b]0 = no lesions, 1 = 1-10%, 2 = 11-25%, 3 = 26-50%, 4 = 51-75%, 5 = > 75% of the seed was necrotic. Each value is a mean for two replicates of 10 seeds each.

[c]Means followed by the same letter do not differ significantly (P = 0.05) by Duncan's multiple range test.

bleached and nonbleached seeds. Individual sugars and amino acids were identified from nonbleached and bleached seed exudates to determine if there were qualitative differences in these components.

Exudates from bleached seeds contained approximately three times as much total soluble carbohydrate as exudates from nonbleached seeds (Table 7). Sucrose, glucose, and fructose were identified from both bleached and nonbleached seed exudates using thin layer chromatography (Figure 2). These sugars were also identified and quantified using gas-liquid chromatography (Figure 3, Table 8). All three sugars were present in larger quantities in bleached seed exudate than in nonbleached seed exudate. Sucrose was relatively higher in bleached than in nonbleached seed exudate.

Nonhydrin-positive substances were present at much lower concentrations in the seed exudates than were carbohydrates; however, no significant differences were found in amounts of ninhydrin-positive substances exuded from bleached or nonbleached pea seeds (Table 7). Fourteen amino acids were present in the exudates from both bleached and nonbleached seeds (Table 9). All amino acids identified from the seed exudates were among those known to be commonly found in proteins. No significant qualitative or quantitative differences in the amino acid composition of exudates from nonbleached and bleached pea seeds were observed.

Chlamydospore Germination

Chlamydospores of F. solani f. sp. pisi in natural soil were used to measure the ability of exudates from bleached and nonbleached seeds, and some components of these exudates, to stimulate germination in soil. Cationic, anionic, and neutral fractions of seed exudates, as

Table 7. Exudation of carbohydrates and ninhydrin-positive substances from pea seeds incubated for 5 days in a moist, sterile, sand environment at 22°C.

Degree of Bleaching	µg Carbohydrate per Seed[a]	µg Ninhydrin-Positive substances per seed[b]
Bleached	188.37[c] A[d]	1.68 C
Nonbleached	68.70 B	1.48 C

[a]Carbohydrate was measured by the anthrone method and is expressed as glucose equivalents.

[b]Ninhydrin-positive substances is expressed as glycine equivalents.

[c]Each value is a mean of two replicates of 43 seeds each.

[d]Means followed by the same letter do not differ significantly (P = 0.05) by Duncan's multiple range test.

Table 8. Carbohydrates exuded from bleached and nonbleached pea seeds.

Carbohydrate	Concentration (nanomoles/seed)[a]	
	Bleached	Nonbleached
Fructose	102.27	29.94
Glucose	155.72	46.94
Sucrose	184.09	18.02

[a]Values represent means of two replicates of 43 seeds each. Identification and quantification was accomplished using gas-liquid chromatography.

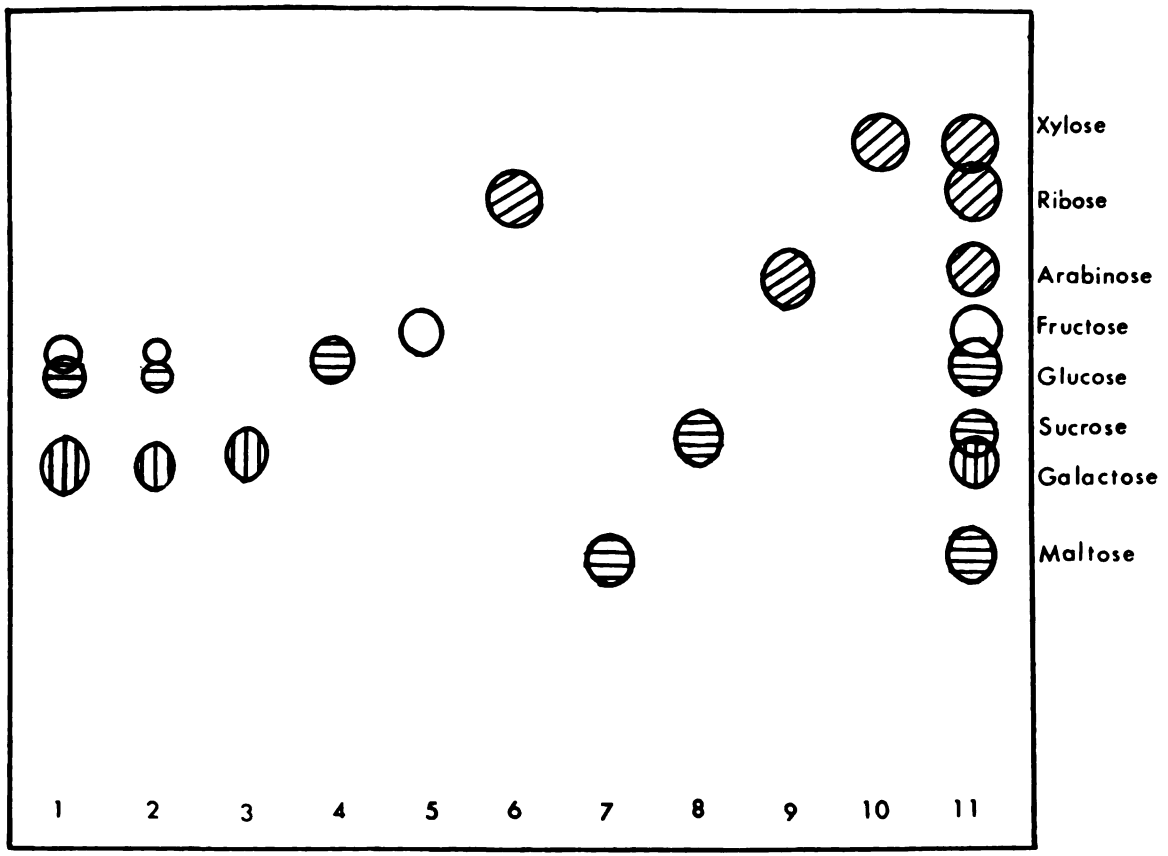

Figure 2. Representation of a thin layer chromatogram: 1, bleached seed exudate (20µl); 2, unbleached seed exudate (40 µl); 3-10, standard sugars (10 µg); and 11, mixture of standard sugars (10 µg each). The colors produced by the detector are depicted in the following manner: 0 = red-brown; 0 = green; 0 = brown; 0 = gray-brown.

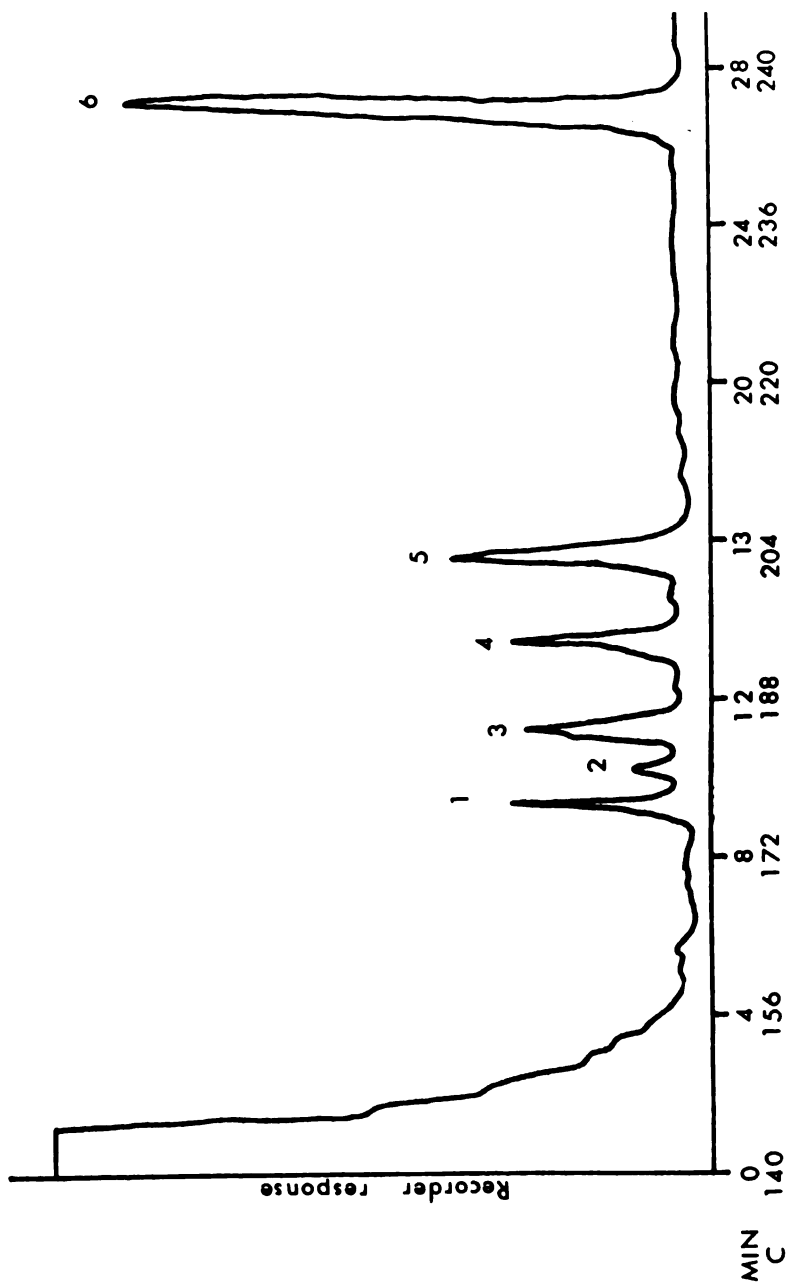

Figure 3. Gas chromatogram of TMS derivatives of carbohydrates from bleached pea seed exudate. Peaks are: 1, fructose; 2, unknown; 3 and 4, glucose; 5, inositol (internal standard); and 6, sucrose. Chromatographs of TMS derivatives of carbohydrates from nonbleached pea seed exudate did not differ qualitatively from this one.

Table 9. Amino acids exuded from bleached and nonbleached pea seeds.

Amino Acid	Concentration (nanomoles/seed)[a]	
	Bleached	Nonbleached
Aspartic acid	6.22	9.40
Threonine	9.94	8.23
Serine	7.91	13.16
Glutamic acid	8.50	10.52
Glycine	9.28	11.05
Alanine	11.77	15.74
Valine	5.43	7.04
Isoleucine	3.23	4.61
Leucine	6.74	5.74
Phenylalanine	3.92	4.39
Lysine	4.50	3.41
Histidine	4.19	4.35
Arginine	2.33	3.17
Tyrosine	2.75	2.26

[a]Values represent means of two replicates of 43 seeds each. Identification and quantification was accomplished with an amino acid analyzer.

well as unfractionated exudates, were assayed by applying .2 ml of the test solution to .5 g of air-dried soil, infested with chlamydospores of F. solani f. sp. pisi (1 x 10^6 spores/g). Glass-distilled water added to infested soil served as a control. Spore germination was determined with soil smears after 15 hours of incubation at 22°C in a humid environment.

Unfractionated exudate from bleached seeds stimulated more chlamydospore germination than unfractionated exudate from nonbleached seeds (Table 10). The neutral (carbohydrate) fraction of bleached seed exudate also stimulated more chlamydospore germination than did the neutral fraction of nonbleached seed exudate. Neither the cationic (amino acid) nor anionic (organic acid) fractions stimulated chlamydospore germination. The unfractionated seed exudates promoted more chlamydospore germination than did the neutral fractions for both bleached and nonbleached seeds.

Influence of Sucrose on Disease Incidence

Since other experiments had indicated that the carbohydrate fraction of the seed exudate was influential in the infection process, the effect of an exogenously applied carbohydrate source on infection was investigated. Sucrose was chosen as the carbohydrate source to be tested because it was the predominant sugar component of bleached pea seed exudate. Surface-sterilized nonbleached pea seeds (1975) were placed in 25 mm-diameter test tubes containing 25 g of washed silica sand (sterile), inoculated with 1 ml of a chlamydospore suspension of F. solani f. sp. pisi (1 x 10^2 chlamydospores/ml), and covered with 10 g of sterile, washed silica sand. Then 6 ml of either a 0.1 M sucrose

Table 10. Stimulation of F. solani f. sp. pisi chlamydospore germination in natural soil by bleached or nonbleached pea seed exudates, or components of these exudates.

Degree of Bleaching	Exudate Fraction	Chlamydospore Germination (%)[a]
Nonbleached	Unfractionated	16 A[b]
	Anionic	0 B
	Cationic	0 B
	Neutral	10 A
Bleached	Unfractionated	48 C
	Anionic	0 B
	Cationic	0 B
	Neutral	40 C
Distilled Water	-	0 B

[a]Values represent means of two replicates of 100 chlamydospores each.

[b]Means followed by the same letter do not differ significantly (P = 0.05) by Duncan's multiple range test.

solution or distilled water was added aseptically to each tube. The tubes were sealed and incubated at 22°C for 5 days; the seedlings were then rated for disease severity. Sucrose-treated seeds had a higher disease severity than seeds treated with distilled water (Table 11).

Table 11. Effect of exogenously applied sucrose on infection of nonbleached pea seeds by F. solani f. sp. pisi.

Treatment[a]	Disease Rating
Sucrose	3.2[b] A[c]
Distilled Water	2.1 B

[a]Seeds were incubated in a 0.1 molar sucrose solution for 5 days at 22°C.

[b]0 = no lesions, 1 = 1-10%, 2 = 11-25%, 3 = 26-50%, 4 = 51-75%, 5 = > 75% of the seed was necrotic. Each value is the mean for two replicates of ten seeds each.

[c]Means followed by the same letter do not differ significantly (P = 0.05) by Duncan's multiple range test.

DISCUSSION

Bleached pea seeds were found to be more susceptible to preemergence damping-off than were nonbleached pea seeds under field conditions. This is in agreement with Short and Lacy (55) who showed that pea seed and seedling rot was higher among bleached pea seeds than among nonbleached pea seeds in growth chambers at 20% and 37% soil moisture. The differences in field emergence could not be explained by differences in viability of bleached and nonbleached seeds. Macquire et al (29) also reported that bleached and nonbleached seeds of the wrinkled-seeded cultivar Perfection were equally viable. However, bleached seeds of the round-seeded cultivar Alaska were considerably lower in viability than green seeds.

Total fresh and shelled pea weights of plants grown from non-bleached seeds were higher than those grown from bleached seeds because of higher plant populations. However, yields and fresh weights from individual plants grown from bleached or nonbleached seeds were not significantly different, suggesting that plants grown from bleached seeds were similar in vigor to those grown from nonbleached seeds. This is in contrast to reports that seedlings grown from bleached lima bean seeds were lower in vigor than those grown from nonbleached seeds (43, 68).

Qualitative or quantitative differences in pea seed exudates seemed to be at least partially responsible for the greater

susceptibility to seed and seedling-infecting fungi of bleached as compared with nonbleached pea seeds. Applications of exudate from bleached pea seeds to nonbleached seeds increased the susceptibility of these seeds to infection by F. solani f. sp. pisi as compared with seeds treated with nonbleached seed exudate. Flentje (17) showed that wrinkled-seeded peas were more susceptible to Pythium than smooth-seeded peas because of differences in seed exudates: smooth-seeded peas planted below wrinkled-seeded peas, and therefore influenced by the exudate from the wrinkled-seeded peas, had a higher disease rating than did those planted below the smooth-seeded peas. In these studies, removing seed exudates from the host-pathogen interface by leaching with distilled water did not eliminate the differences in susceptibility between bleached and nonbleached pea seeds, presumably because the larger amounts of nutrients exuded by bleached seeds were more difficult to remove. However, leaching with distilled water did serve to reduce disease incidence over nonleached controls, suggesting that seed exudates influenced disease incidence or that inoculum potential was being decreased by leaching. Bleached and nonbleached pea seeds did not differ in susceptibility when inoculum was introduced into the cotyledons of the seed, thereby eliminating the influence of seed exudates. Beute (7) showed that the increase in Fusarium root rot of virus-infected pea plants was due to an increase in root exudation by injecting spores of F. solani f. pisi into the cortical tissues of the stems of pea plants: lesions produced on healthy and virus-infected plants were equal in severity.

The quantity of carbohydrate exuded from pea seeds was correlated with susceptibility of pea seeds to preemergence mortality. Approximately three times more carbohydrate was present in the exudate from bleached seeds than in exudates from nonbleached seeds. This confirmed

work by Short and Lacy (54) and Maquire et al (29) showing carbohydrate exudation to be 3-5 times higher from bleached than from nonbleached pea seeds. There were no quantitative differences in ninhydrin-positive substances and no qualitative differences in sugars or amino acids in exudates from nonbleached or bleached pea seeds. Since optimal germination of chlamydospores of Fusarium in soil can be achieved with several sugars or nitrogen sources (14), stimulation of disease by specific sugars or amino acids was not expected. Similar arrays of sugars and amino acids have been reported to occur in exudates from other seeds: all three sugars and 10 of the amino acids were found in pine seed exudates (3), while the three sugars and amino acids were identified from exudates of cotton (19) and bean (52) seeds.

Chlamydospores of F. solani f. sp. pisi germinated only in response to the carbohydrate fractions of both bleached and nonbleached seed exudates, showing that the carbohydrate component of the seed exudate was primarily responsible for stimulating chlamydospores of F. solani f. sp. pisi to germinate in the spermosphere of pea. Higher chlamydospore germination in response to the carbohydrate fraction of exudates from bleached pea seeds than from nonbleached pea seeds reflected higher amounts of carbohydrate in exudates from bleached seeds. Cook and Schroth (14) have shown that a reduced amount of available carbon was the principle factor limiting germination of chlamydospores of Fusarium in soil. Unfractionated pea seed exudate was more stimulatory to germination than was the carbohydrate fraction, suggesting that other components of the exudate serve to enhance germination. It is possible that nitrogen was somewhat limiting to germination when only the carbohydrate fraction was supplied. Germination of chlamydospores of Fusarium in soil was higher when both amino acids and sugars were

supplied than when only simple sugars were added (14). Amino acids are sources of both carbon and nitrogen, and thus have the ability to stimulate germination of chlamydospores (3, 15, 50, 52). The fact that the amino acid fraction of the seed exudates did not stimulate chlamydospore germination may have been because the quantity of amino acids present was not high enough to fulfill the energy requirements for chlamydospore germination in soil.

Increased disease severity in response to the addition of sucrose to the host-pathogen interface suggested that the high carbohydrate exudation from bleached seeds could be responsible for increased pre-emergence damping-off in the field. Keeling (25) found that there was a direct relationship between the amount of preemergence seed and seedling rot of soybean caused by Pythium, and the amount of carbohydrates exuded by germinating seeds: more than twice the quantity of carbohydrate was exuded from seeds of a susceptible cultivar than from a resistant cultivar. Similar correlations of carbohydrate exudation and seed and seedling susceptibility to soil-borne pathogens have been made in other studies (17, 26, 33).

Carbohydrate exuded from seeds or roots serve to stimulate spore germination and increase the inoculum potential of the pathogen in the spermosphere or rhizosphere, resulting in increased disease incidence (49). Exudation of carbohydrate would be expected to increase the rate of spore germination and inoculum potential until other factors, such as nitrogen, become limiting to the fungus. However, the stimulation of the pathogen in the spermosphere or rhizosphere by host exudates is affected by the activities of other microorganisms which occupy the same microhabitat as the pathogen, and therefore influence

both its saprophytic and pathogenic activities (49). In certain circumstances, exudation may favor antagonistic microorganisms more than the pathogen, the net effect being a reduction in disease severity: a number of reports have indicated the importance of antagonists in suppressing the growth of Fusarium (9, 35, 36, 37, 41).

The effect of seed and seedling exudation on disease incidence depends to a large extent on the nature of the exudates. Exudation of nutrients from seeds and roots is dramatically influenced by environmental parameters, such as soil moisture (24, 26), high (20) and low (22) temperatures, and anaerobic conditions (8), as well as by the collection method used (5). Present methods of exudate collection and analysis do not permit quantification of the concentration of nutrients at the host-pathogen interface under natural conditions. The development of techniques to measure nutrient concentrations in the soil would greatly increase the significance of plant exudate analysis.

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