Effects of Soybean Seed Coat Cracks on Seed Exudation and Seedling Quality in Soil Infested with Pythium ultimum

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ABSTRACT

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Nonblemished soybean seeds with intact seed coats exuded 5.3 μg glucose equivalents per hour per seed when buried in glass beads and leached by dripping distilled water at 3 ml/hr for 24 hr. Anthrone-positive leachates of 17.5 μg glucose equivalents per hour per seed were collected after soaking nonblemished seeds in 1 ml water for 3 hr. Exudation from soaking seeds was not influenced by cracks in the hypodermal seed coat layer, but scarifying through the remaining layers increased exudation to 217 μg/hr. Exudation of ninhydrin-positive substances from leached intact seeds was 1.7 μg glycine equivalents per hour per seed. This was increased two-fold by soaking and an additional five-fold by scarification. Germination of washed sporangia of *Pythium ultimum* was low in distilled water or in a sucrose-asparagine solution containing 1 μg of carbon/ml but increased to 48% or more with 5 μg or more carbon/ml. Spermosphere effects could be detected up to 5 mm from intact and 7.5 mm from scarified seeds after 5 hr in soil at 24 ± 1 C and −0.3 bar matric water potential. Lower quality seedlings resulted from scarified seeds than from seeds with intact or cracked seed coats when planted in saturated soil or soil infested with sporangia of *P. ultimum* (130 propagules/g dry soil) at 15 C, but addition of sucrose (12.7 mg) to each seed had no added effect. A high population of *P. ultimum* at 24 C reduced quality of seedlings produced from both intact and scarified seeds to the same level. Apparently adequate sugar is exuded from nondamaged soybean seed for optimum development of *P. ultimum* and scarification effects on seedling quality are not the result of additional stimulation of *P. ultimum* by exudates.

Pythium ultimum Trow. is the primary *Pythium* sp. associated with damping-off of soybean (*Glycine max*) (2, 9). Increased damage from *Pythium* sp. has been associated with several environmental factors that cause increased exudation such as low oxygen levels (3), high or low temperature (11), and high soil moisture (10). Damage caused by *P. ultimum* to plants of specific soybean cultivars has been associated with high levels of seed exudation; however, low soybean seed vigor also is correlated with high exudation (5).

Seed exudation also is increased by seed coat blemishes (wrinkled or cracked seed coats) in several plants (6, 19). A common seed coat condition found in soybean is an irregular break in the hypodermal layer of the seed coat. This will be referred to as a crack and is distinct from mechanical damage in which all the seed coat layers are split by fine, hair-line fractures. Hypodermal cracks appear as areas of separation of the hour-glass cells thereby exposing the underlying spongy parenchyma layer of the seed coat (22). The exact cause of the cracking is not understood; however, cracks have been associated with adverse weather conditions at the time of maturity and may be genetically linked (12).

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Most investigators (6, 9, 10, 19) have concluded that seed exudation is a primary factor in preemergence damping-off caused by *Pythium* sp. under environmental conditions favorable for pathogenesis. In many studies of exudation seed of specific cultivars or stress conditions are used to achieve varying exudation levels. However, the relation of the initial quality of seed and seedling to damping-off often has been overlooked.

The purpose of this study was to determine the rate of exudation from soybean seed with intact and damaged seed coats and their role in preemergence damping-off caused by *P. ultimum*.

MATERIALS AND METHODS

Hand-harvested soybean seeds of the cultivar Amsoy 71 were used. Seed weight varied from 0.13 to 0.24 g and moisture content was about 10.5%. Seeds were stored at 24 ± 2 C in the laboratory for 6-10 mo before use.

The seeds were sorted into two groups: (i) seeds with no visible seed coat cracks (intact seeds) and (ii) seeds with hypodermal cracks (cracked seeds). Some seeds were scarified by scraping with a scalpel to remove parenchyma exposed by the hypodermal cracks. Percentage scarified area was determined using black and white photographs of both sides of the seed magnified ×2.3. Seeds in the pictures were cut out and weighed.

Cracks with a surface area greater than 0.1 mm^2 in actual size were cut out and the photographs then were reweighed.

Exudates.—Seed exudates were collected by either leaching or soaking. For the leaching method, three seeds weighing 0.195 ± 0.005 g were buried halfway in 5 ml of 1-mm-diameter glass beads and covered with 9 ml of 3-mm-diameter glass beads in a 40-mm-diameter Pyrex Büchner funnel with the neck removed. Nylon screening material was used to support the glass beads. The funnel mouth was covered with an aluminum cap. The cap contained four holes and a glass tube sealed with Tygon tubing to deliver 3 ml of water/hr for leaching. Components were autoclaved before seed placement. Leachates were collected in test tubes containing 1 ml of a solution composed of 500 μg/ml each of streptomycin sulfate and chloramphenicol. The exudates were stored frozen.

Exudates were collected from both propylene oxide treated (16) and nontreated seeds. Only exudates from noncontaminated seeds were used to obtain data for sterile seeds.

The seed-soaking method consisted of placing individually weighed seeds in vials (I.D. 12 mm) containing 1 ml of distilled water at 24 ± 1 C for 3 hr. Then 0.5 ml of the solution was removed from each vial and added to 4 ml of antibiotic solution containing 100 μg/ml streptomycin sulfate and chloramphenicol. This solution was filtered through Whatman No. 1 paper and stored in a freezer.

Total carbohydrates was determined using the anthrone test modified from Morris (13). Absorbance was determined at 620 nm in a Spectronic 20 colorimeter with 40 μg glucose plus antibiotics as a standard.

Clark's (4) modified ninhydrin test (23) was used to determine amino acids. Absorbance at 570 nm was determined with a Spectronic 20 colorimeter, with 5 μg glycine plus antibiotics as a standard.

Sporangium production and germination.—The isolate of *P. ultimum* was obtained from field soil in Wooster, Ohio, in 1975. Tests conducted in a greenhouse indicated that the isolate was pathogenic on soybeans. It was maintained on Difco cornmeal agar (CMA) slants at 8 C. Sporangia were used because they occur naturally in soil (20), will form in liquid culture, and germinate rapidly.

Sporangia were produced in hemp seed broth (HSB) which was made by placing 5 g hemp seed in 1 liter of distilled water and autoclaving for 15 min. The extract was filtered, dispensed to 250-ml flasks (50 ml/flask), capped, and autoclaved. Each HSB flask was seeded with a CMA plug from a 3-day-old culture of *P. ultimum*. Flasks were incubated for 10 days in the light (2,400 lux) at 24 C and then stored in the dark at 24 C from 1 to 90 days before use. At harvest, the agar plug was removed from a HSB culture and the remaining mycelium was minced in a Sorvall Omni-Mixer (Du Pont, Sorvall Operation, Newtown, CT 06470) at half speed for 1 min. The resulting suspension was filtered through a nylon screen (46-μm-mesh), then refiltered through a 10-μm screen. The screen containing the sporangia was covered with an additional 10-μm screen and was washed with distilled water at the rate of 8 liter/hr which decreased to 25 ml/hr after 16 hr as the spores settled into the pores. The total amount of wash water was approximately 15 liters.

The in vitro germination requirements of sporangia were determined by mixing a drop of different sucrose-asparagine solutions (C:N, 20:1) with an equal volume of washed sporangia (200 per 0.07-ml drop) in plastic petri dishes. The dishes were covered and placed in the light (1,000 lux) at 24 ± 1 C. After 0 and 4 hr spores were stained with cotton blue in lactophenol, and 100 spores in each of the drops were examined. Sporangia were considered germinated if the germ tube length was half the width of the spore. The experiment was repeated four times each with three replications.

A modification of Adams' technique (1) was used to determine the extent of the spermosphere. Sporangia washed for 1 hr were collected on membrane filters (Gelman Metricel GA-1, 47 mm diameter, 5 μm pore size) that were cut into pieces 0.5 × 1 cm. Three pieces were placed in a row with 1 cm sides touching on a piece of nylon cloth (240 μm pore size) which was lying on saturated soil (Wooster silt loam plus sand v/v). A seed was placed with the hilum facing the 1 cm side of the filter piece at the end of the row. Filter pieces then were covered with nylon cloth. The nylon cloth and seed were covered with a 1-cm layer of moist soil at approximately −1.2 bars matric water potential, thereby resulting in an overall soil moisture of −0.3 bar matric water potential. After 5 hr, the filters were removed and cut into two pieces (0.25 × 1 cm), placed on a slide, stained and treated with glycerine. After the filters were sufficiently translucent, spore germination was determined by observing 25 spores on each 1-cm side. Twenty scarified and 20 intact seeds were tested.

Pythium ultimum populations in soil.—A modification of Schmitthenner's medium (SA-PBNC) (18) was used to estimate the population of *P. ultimum* in soil. The medium consisted of 1,000 ml distilled water, 2.5 g sucrose, 0.27 g asparagine, 150 mg KH_2PO_4, 150 mg K_2HPO_4, 100 mg $MgSO_4 \cdot 7H_2O$, 2 mg thiamine hydrochloride, 10 mg cholesterol (in 2 ml N,N-dimethylformamide), 20 mg Benlate (50% benomyl), 27 mg Terraclor (75% pentachloronitrobenzene), 0.1 g neomycin sulfate, 10 mg chloramphenicol, and 20 g Bacto agar.

Soil agar plugs were prepared by placing a known weight of soil in 15 ml of water agar (3%) held at 50 C and containing 0.01 g neomycin sulfate and 0.001 g chloramphenicol per liter. The agar and soil were mixed in a Sorvall Omni-Mixer at medium speed (10 sec) then poured into a 9-cm diameter petri dish to solidify. Fifty plugs (4 mm in diameter) from the soil-agar suspension were plated on 10 petri dishes of the isolation medium. After 48 to 72 hr, the outside edges of colonies characteristic of *P. ultimum* were transferred to the identification medium. The medium used for identification was the same used for isolation but with antibiotics and fungicides replaced by three micronutrients: 4.4 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $FeSO_4 \cdot 7H_2O$, 0.07 mg $MnCl_2 \cdot 4H_2O$.

Wooster silt loam soil was seeded with an equal volume of infested sand. One 50 ml HSB culture of *P. ultimum* was minced in a Sorvall Omni-Mixer for 1 min and mixed with 120 ml of autoclaved sand. Noninfested soil consisted of a mixture of equal volumes of sand and

Wooster silt loam. This soil mixture was incubated for 24 hr at 5 C before use in seed germination studies.

Soybean seeds were germinated in sand furrows containing soil. Three furrows (length, 35 cm; width, 3 cm; depth, 5 cm) were made in coarse sand in plastic containers (39 × 19 × 12 cm, tomato baskets) containing drainage holes. The furrows were lined with a 40 cm length of paper toweling and filled with soil to a depth of 3 cm. The soil was saturated and after draining, 20 seeds were placed on the surface and covered with 2 cm of soil (−1.5 bars matric water potential). The overall equilibrated soil moisture was −0.4 bar matric water potential. A high moisture condition was established in some containers by flooding with deionized water after planting. Trays were placed in three growth chambers at 24 C and two at 15 C with a 12-hr photoperiod and illumination of 30,000 lux. The study was terminated after 15 days in 24 C chambers and 21 days in the 15 C chambers.

Seedling quality rating.—The rating consisted of the number of emerged plants minus the emerged plants with severe stunting of the apical meristem (baldhead) or which had damped-off, minus the number of cotyledons on emerged plants with at least half their tissue missing or deeply lesioned. If any 20 plants in a furrow had a negative seedling quality rating, this was considered as zero for statistical analysis. A seedling was considered emerged if part of its hypocotyl was above ground. To determine dry weights of the plants, cotyledons were removed and roots were washed before drying at 85 C for 24 hr.

RESULTS

Seed exudation.—Total carbohydrates leached per hour from three bacteria-free seeds (propylene oxide-treated) and nontreated seeds were not significantly different (16.01 and 15.85 μg glucose equivalents, respectively). Carbohydrate leachate concentrations from either treated or nontreated seed did not change significantly over a 24-hr period even though seed germination usually had occurred by the end of the period. Chromatographic analysis (7) of leachates indicated sucrose to be the most predominant mono- and/or disaccharide present. The concentration of amino acids present in nonsterilized seed leachates was significantly higher than that from propylene oxide-treated seeds (5.10 and 2.34 μg glycine equivalents, respectively, $P = 0.05$). Ninhydrin-positive material from nontreated and treated seeds was significantly reduced after 3 hr ($P = 0.05$) from 8.05 and 6.59 to 4.68 and 1.73 μg glycine equivalents per hour per three seed samples, respectively.

TABLE 1. Influence of seed coat conditions on exudation of nutrients from soybean seeds soaked for 3 hr in 1 ml of distilled water at 24 ± 1 C

Seed coat condition	Carbohydrates (μg glucose equivalents per seed)	Amino acids (μg glycine equivalents per seed)
Intact	52.5 ± 15.9[a]	12.1 ± 4.8
Cracked	60.4 ± 24.2	13.2 ± 5.7
Scarified[b]	650 ± 450	35.9 ± 21.0

[a]Mean of two tests of 20 individual seeds.

[b]Seeds were scarified with a scalpel by removing the spongy parenchyma in the area of hypodermal cracks.

Scarification increased the amount of material exuded (Table 1). Percentage area scarified (0.3 to 18.5%) was more highly correlated with exudation of carbohydrates ($r = 0.37$) than with the presence of ninhydrin-positive material ($r = 0.29$). Scarified seed absorbed more water in 3 hr (0.21 g) than did intact seeds (0.11 g), but increasing amounts of water absorbed was not correlated with either increasing carbohydrate or amino acid exudation. Water absorption and carbohydrate exudation were significantly correlated in intact seeds ($r = 0.50$). There was no correlation between seed weight (0.187 ± 0.031 g) and the amount of carbohydrates or amino acids exuded.

Production and germination of sporangia.—Hemp seed broth was effective in producing large numbers of sporangia (2,000/ml) with little mycelial growth; less than 1% of the propagules were oospores. Spores that did not lyse during washing with 15 liters of water over a 16-hr period germinated 2.8% after 4 hr in distilled water. Addition of 1 μg carbon/ml mainly as sucrose did not increase germination significantly. However, after 4 hr germination was 48, 62.1, and 67.1% in solutions containing 5, 10, and 100 μg carbon/ml, respectively.

Sporangia washed for 1 hr did not germinate in soil in the absence of seeds but germinated up to a distance of 5 mm from a seed (Fig. 1). The radius of the spermosphere was 2.5 mm larger around scarified seed than intact seed. The percentage of the seed surface which was scarified (0.5% to 10.2%) correlated with the size of the resulting spermosphere ($r = 0.51$). The germ tube length decreased as the distance from the seed increased, and extensive mycelium development was only observed within 2.5 mm of the seed.

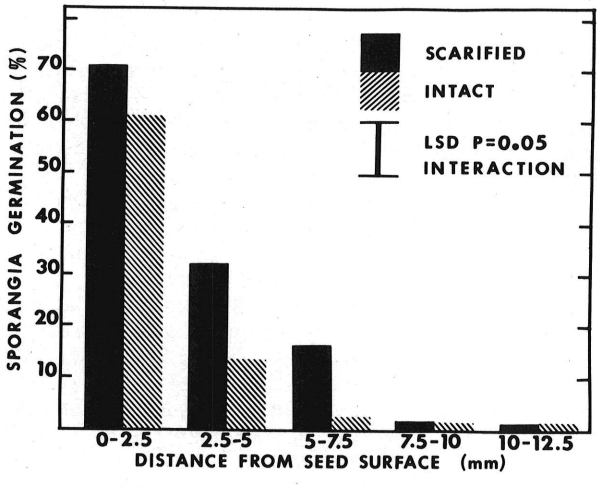

Fig. 1. Influence of soybean seed coat condition on germination of adjacent *Pythium ultimum* sporangia in soil after 5 hr at 24 ± 1 C and −0.3 bar matric water potential. Percentage germination was determined by counting 50 spores at each distance from 20 seeds. Seeds were scarified with a scalpel by removing the spongy parenchyma in the area of hypodermal cracks.

Seed germination in Pythium-infested soil.—In one experiment, the effects of autoclaved soil, soil moisture, temperature, and seed coat condition were studied in all possible combinations and replicated four times. The nonautoclaved soil contained 15 *P. ultimum* propagules per gram of dry soil. Autoclaved soil contained only airborne microorganisms present as contaminants.

Most damage occurred in nonautoclaved saturated soil (Table 2). At 15 C scarification resulted in a significantly lower ($P = 0.01$) seedling quality rating than did intact or cracked seeds (Table 3). The combination of saturation and scarification resulted in the most severe damage. At 24 C no significant difference in seedling quality occurred as a result of variation in seed coat conditions. The interactions which were significant ($P = 0.05$) with the seedling quality rating also were significant using plant dry weight data with the exception of the soil moisture × soil sterility interaction at 15 C.

The effects of low (15 propagules/g) compared with high (130 propagules/g) amounts of *P. ultimum* and high amounts of *P. ultimum* plus sucrose on germination of seeds with intact, cracked, or scarified seed coats were investigated. A population of 130 propagules/g is typical for cultivated soils in Ohio (17). The sucrose was added as a drop (12.7 mg in 0.05 ml water) on the seeds just before they were covered with soil.

At 15 C there was a significant interaction ($P = 0.057$) between the amount of *P. ultimum* and seed coat condition (Table 4). The seedling quality rating for scarified seeds was significantly higher in soil with the low population of *P. ultimum* than in soil with the high population or high population plus sucrose. Soil population of *Pythium* sp. did not significantly influence the seedling quality of intact or cracked seeds. In soil with a low population of *Pythium* sp. at 24 C the seedling quality rating (16.4) was significantly ($P = 0.001$) higher than for high *Pythium* (10.5) or high *Pythium* plus sucrose (9.1).

DISCUSSION

Depending on the method used, several levels of exudation were found in our tests. Using the glass bead leaching method our results for 24 hr (5.3 μg glucose equivalents and 1.7 μg glycine equivalents per hour per seed) were comparable to the findings of Hobbs and Obendorf (8) who found about 9.7 μg glucose equivalents and 1.2 μg glycine equivalents exuded per hour per seed in wet sand after 17 hr. Soaking intact seeds resulted in 17.5 μg glucose equivalents per hour per seed which is similar to that found by Keeling (9) who reported 205 to 500 μg after 16 hr for 13 to 31 μg glucose equivalents per hour per seed.

Exudation levels were correlated with water absorption by intact seeds, confirming the results of Kerr (10) who worked with peas under different soil bulk densities.

TABLE 2. Influence of soil moisture and soil sterility on seedling quality rating of soybean at 15 and 24 C

Soil moisture[b]	Matric water potential	Seedling quality rating[a] per temperature and soil treatment			
		15 C		24 C	
		Autoclaved	Nonautoclaved	Autoclaved	Nonautoclaved
Saturated	0 bar	10.7[c]	4.0	13.2	3.7
Nonsaturated	−0.4 bar	19.4	17.1	18.8	15.9
LSD (P=0.05)		2.3		2.8	

[a]Number of emerged plants minus the abnormal seedlings and number of lesioned cotyledons.

[b]Saturated condition was established by flooding the soil after planting and nonsaturated by covering seeds with moist soil resulting in an equilibrated soil of −0.4 bar. Soil was a mixture of sand and Wooster silt loam soil (1:1, v/v).

[c]Mean of four replications of 20 seeds each.

TABLE 3. Influence of soil moisture and seed coat condition on seedling quality rating of soybean at 15 C

Soil moisture[b]	Matric water potential	Seedling quality rating[a] per condition of seed coat		
		Intact	Cracked	Scarified[c]
Saturated	0 bar	10.5[d]	9.9	1.6
Nonsaturated	−0.4 bar	18.9	18.1	17.8
LSD (P=0.05)			2.9	

[a]Number of emerged plants minus the abnormal seedlings and number of lesioned cotyledons.

[b]Saturated condition was established by flooding the soil after planting and nonsaturated by covering seeds with moist soil resulting in an equilibrated soil of −0.4 bar. Soil was a mixture of sand and Wooster silt loam (1:1, v/v).

[c]Seed which have had their parenchyma layer scraped away in the area of hypodermal cracks.

[d]Mean of four replications of 20 seeds each.

TABLE 4. Seedling quality rating of soybean plants from seed with three types of seed coat conditions in soil with two population densities of *Pythium ultimum* at 15 C

P. ultimum population and treatment	Seedling quality rating[a] per condition of seed coat[b]		
	Intact	Cracked	Scarified
15 propagules/g soil	15.9[c]	15.4	15.1
130 propagules/g soil	12.8	13.4	9.8
130 propagules/g soil plus 12.7 mg sucrose/seed	14.1	14.3	8.4
LSD (P=0.05)		3.3	

[a]Number of emerged plants minus the abnormal seedlings and number of lesioned cotyledons.

[b]Intact seeds had no seed coat cracks, cracked seeds had hypodermal cracks exposing the spongy parenchyma layer, and scarified seeds had their parenchyma layer scraped away in the area of hypodermal cracks.

[c]Mean of eight replications of 20 seeds each.

Seeds which were scarified absorbed twice the amount of water than did intact seeds; however, total water absorption did not correlate with exudation. Scarified seeds absorb water at such a rapid rate that after 3 hr, total water absorption may not be a reflection of the initial rate which may be the correlative factor. Rapid absorption of water by the seed might result in damage due to an aerobiosis by filling the cavity between cotyledons with an excess of water and preventing free access of oxygen (14). Damage may be caused by physical stress imposed by differential hydration within the cotyledons (15). Such damage could disorganize membranes which could explain the observed high rates of exudation from scarified seeds.

The lowest level of carbohydrates exuded was 5.3 μg glucose equivalents per hour from seeds with intact seed coats. Our interest was to determine if this concentration would be sufficient to support germination and growth of *P. ultimum*. Stanghellini and Hancock (20) reported that more than 15 μg glucose equivalents/g soil were required for sporangia germination and for unlimited germ tube growth of *P. ultimum*. We found that 5 μg carbon/ml water (12.5 μg glucose equivalents/ml) resulted in 48% sporangium germination. Apparently even nondamaged soybean seed, under normal germination conditions, would supply enough exudates in a few hours for germination and growth of *P. ultimum*. It is not surprising, therefore, that within 5 hr sporangia germinated within 5 mm of a soybean seed. Increasing exudation by scarification resulted in a 2.5 mm increase in the spermosphere effect. We found extensive development of *Pythium* sp. mycelium only within 2.5 mm of the seed surface in both intact and scarified seed. Stanghellini and Hancock (21) found that the spermosphere of bean with regard to *P. ultimum* continued to enlarge over a 12-hr period to 8-10 mm at 50 cm water suction.

Many workers have associated increased exudation under various unfavorable germination conditions with increased activity of *Pythium* sp. and have assumed that increased stimulation of *Pythium* sp. is responsible for poor seed germination. However, Hobbs and Obendorf (8), using two soybean seed lots which differed in their sugar exudation rate by a factor of four, found no difference in their germination when the seeds were planted in autoclaved or nonautoclaved soil at 24 C. Flentje and Saksena (6) found that with peas, cracks in the testa increased sugar loss and preemergence rotting. However, cracking the testa near the micropyle did not increase percentage rotting by *P. ultimum* even though sugar exudation was increased.

We found that at 24 C increasing exudation by scarification did not result in more damage caused by *P. ultimum*. At 15 C, saturating either autoclaved or nonautoclaved soil significantly decreased the seedling quality rating of scarified seed compared to that of intact seeds, thereby indicating that scarified seeds may be of lower vigor and germinate less under stress regardless of the presence of soilborne microorganisms. Our results agree with the fact that a host under environmental stress is more susceptible to *P. ultimum* damage.

At 15 C fewer scarified than cracked seeds emerged in soil with a high level of *P. ultimum*. This response could have been caused by increased activity of *P. ultimum* induced by increased amounts of exudation. To test this hypothesis, 12.7 mg sucrose per seed, which is about 2×10^3 higher than the normal hourly exudation rate, was applied at planting time. The high level of sucrose did not increase the number of diseased seedlings. Seeds having high levels of exudation probably are damaged, and therefore germinate slower under stress conditions which provides more time for *P. ultimum* to colonize the seed before the natural seedling resistance has developed. Scarification also may provide wounds through which rapid invasion by *P. ultimum* could occur; however, hypodermal cracks did not provide such wounds. Naturally occurring hypodermal seed coat cracks did not increase exudation or decrease seedling quality.

We conclude that sufficient sugars are exuded from nondamaged soybean seeds for optimum development of *P. ultimum*. This conclusion is based on four facts: (i) extensive development of *P. ultimum* occurred adjacent to intact seeds; (ii) intact seeds exude adequate amounts of nutrients for growth of *P. ultimum*; (iii) addition of sucrose failed to reduce seedling quality; and (iv) *P. ultimum* at 24 C decreased seedling quality of scarified and intact seeds to the same level.

Seed scarification results in increased exudation and may result in decreased vigor. Poor seed germination probably would occur under stress conditions imposed by high numbers of pathogen propagules or unfavorable environmental conditions regardless of excessive exudation levels which may be present.

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