Time and Site of Infection of Resistant and Susceptible Germinating Pea Seeds by *Pythium ultimum*

T. E. Stasz, G. E. Harman, and G. A. Marx

Graduate research assistant, associate professor, and professor, respectively, Department of Seed and Vegetable Sciences, New York State Agricultural Experiment Station, Geneva 14456.
We thank G. Nash, G. Lowery, and E. P. Carney for laboratory and greenhouse assistance.
Portion of PhD thesis submitted by the senior author to the Department of Plant Pathology, Cornell University, Ithaca, NY 14853.
Approved by the Director of the New York State Agricultural Experiment Station as Journal Series Paper 3243.
Accepted for publication 21 January 1980.

ABSTRACT

STASZ, T. E., G. E. HARMAN, and G. A. MARX. 1980. Time and site of infection of resistant and susceptible germinating pea seeds by *Pythium ultimum*. Phytopathology 70:730-733.

Pea seeds with seed coats colored by anthocyanins were resistant to *Pythium ultimum* seed and seedling diseases, whereas seeds with uncolored seed coats were susceptible. Aqueous extracts of colored seed coats inhibited *P. ultimum* hyphal growth in vitro, but uncolored seed coats contained no fungistatic compounds. Nicking colored seed coats greatly reduced resistance and removing them reduced it still further. After planting, fewer hyphae developed on the surfaces of colored seeds than on uncolored seeds. Hyphae also were found on unimbibed seeds. Colored seed coats were not penetrated by hyphae 100 hr after seeds were planted, whereas uncolored seed coats were penetrated within 40 hr after which hyphal growth was profuse on inner seed coat tissues. Susceptibility of seeds without seed coats (ie, embryos) varied among lines independently of seed coat color and decreased dramatically during germination In some lines this decrease occurred within 16–27 hr after planting which was before the radicles had emerged through the seed coats and before the fungus could penetrate even uncolored seed coats. In other lines, embryos remained susceptible longer. Decreased susceptibility was due not only to fewer infections, but also to less severe disease symptoms developed following infection. Hyphal growth in and maceration of host tissue were confined to discrete lesions after susceptibility decreased.

Additional key words: seed rot, damping-off.

Germinating seeds of peas are subject to seed and seedling disease caused by *Pythium ultimum*. Generally seeds with seed coats colored by anthocyanins are considered more resistant than seeds with uncolored seed coats; several USDA Plant Introduction (PI) accessions with colored seed coats have proven to be highly resistant (2,5,11,12). The major gene *A* is required for anthocyanin production and for seed coat color and is therefore associated with resistance, but it also is associated with horticulturally undesirable seed characteristics (1,2,6,12).

Resistance to *P. ultimum* associated with colored seed coats may be due to preformed fungistatic compounds (2,5,6). Extracts of colored seed coats suppress activity of some pea pathogens (1,6,15,18) and compounds leaching from seed coats may suppress pathogen activity in soil (5–7). However, unheated, nonhydrolyzed extracts of colored seed coats have been reported to inhibit *P. ultimum* growth only slightly (2,6). Also, even a small nick in a colored seed coat has been reported to eliminate resistance to *P. ultimum* (2,12) even though nicking would not be expected to greatly reduce the leaching of fungistatic compounds.

Susceptibility of germinating seeds also is affected by exudates which stimulate pathogen activity in soil (3,6,9,10,13,17). The genes *R* (round seeds) and *I* (yellow cotyledons) may affect resistance (2,3), possibly by affecting exudation of compounds from cotyledons.

Mechanisms other than those associated with colored seed coats and exudates also may affect resistance of germinating seeds to pathogens. For example, seedlings of some pea lines limit the spread of *Fusarium solani* f. sp. *pisi* in infected epicotyls (5), which suggests that an inducible resistance mechanism exists.

The present study was undertaken to determine the levels of resistance of several pea lines with colored or uncolored seed coats; to determine the levels of resistance attributable to various host tissues in these lines; to determine if levels of susceptibility change during germination; and to compare the invasion by *P. ultimum* of resistant and susceptible seeds.

0031-949X/80/08073004/$03.00/0

MATERIALS AND METHODS

Pea lines reported to be resistant or susceptible to *P. ultimum*-caused seed and seedling diseases, P. ultimum root rot, or other fungal root rots were chosen for study. The *A, R,* and *I* genes also were considered in the selection (Table 1). PI accessions were obtained from the Northeast Plant Introduction Station, Geneva, NY 14456. PI 257593 is highly resistant to Fusarium and Pythium root rots (5). PI 253968, PI 165965, and PI 138945 are considered susceptible to root rot (5). Minnesota 108 (obtained from F. L. Pfleger, University of Minnesota, St. Paul, MN 55108), and G213 (a selection from PI 175227 [8]) are resistant to root rot caused by *Aphanomyces euteiches* (8,16). Seedlings of cultivars Dark Skin Perfection (DSP) and Alaska (both obtained from Asgrow Seed Co., Twin Falls, ID 83301) and a Perfection-type line (PTL) developed by the third author, were susceptible to Pythium and other root rots in preliminary experiments.

Seeds of PI 257593 from four plants selected from a greenhouse planting were increased for two generations at Geneva. Seeds of PI 257593, DSP, and Alaska for experimentation were grown at the Asgrow Seed Co. Experimental Farm, Twin Falls, ID 83301. Seeds of all other lines used were grown in the greenhouse at Geneva. All seeds were stored at 10 C and 20% relative humidity.

Autoclaved soil was infested with *P. ultimum* oospores produced in vitro and the density of germinable oospores per gram of moist soil (GO/g) was determined by a plate count method and adjusted as previously described (19). Seeds were planted with seed coats intact, nicked by removing a 1–3 mm^2 section distal to the embryonic axes, or completely removed. Seedlings were grown for 10 days at 16 C and the disease severity of each seedling was rated as previously described (19). Each data point represents the mean disease severity rating of 10 seedlings expressed as a percentage of the maximum possible rating. Disease severity-inoculum density data were statistically analyzed by regression of log-log transformed data as previously described (19) to determine slopes of regression lines and inoculum densities required to cause 50 % of the maximum possible disease severity rating (ED_{50}). Tests of 110 F_3 progeny lines from the cross PTL × PI 257593 and of

parental lines were made at an inoculum density of 20 GO/g and could not be replicated due to lack of sufficient seed.

The time required for hyphae to reach germinating seeds was determined by transplanting seeds from infested soil to noninfested soil at various times after planting. Soil particles adhering to seeds being transplanted were removed by gently rinsing them with water.

The length of time germinating seeds remained susceptible was determined by transplanting seeds from noninfested soil to soil infested with *P. ultimum* at the ED_{70} inoculum density of each pea line as previously determined. Seeds transplanted from infested to infested soil served as controls.

The outer seed coat surface was examined by bright-field microscopy following staining for 1–5 min with acid fuchsin (ICN Pharmaceuticals, Inc., Plainview, NY 11803) in dilute lactic acid (4). Inner seed coat tissues, the outer surface of cotyledons, and the surfaces of embryonic axes were examined by fluorescence microscopy following staining with coriophosphine (distributed by Atomergic Chemicals, Plainview, NY 11803), congo red (Fisher Scientific Co., Rochester, NY 14624), and acridine orange (Fisher Scientific Co., Rochester, NY 14624) by the method of van Vuurde and Elenbaas (20). Embryonic tissues were sectioned by hand and restained for 10 min in acridine orange to detect hyphae within them. A Bausch and Lomb fluorescence microscope and vertical illuminator were used with a 436-nm (maximum transmission) exciter filter and a 500-nm dichroic reflector coupled with a 515-nm barrier filter. The number of hyphal colonies, including solitary hyphae and hyphal aggregates, observed on seed surfaces after staining are reported as the mean number per seed $\pm t_{p=0.05}$ with nine degrees of freedom.

Seed coat material for extraction was separated from embryo material by fragmenting dry seeds ~10 sec in a Waring Blendor, removing fine debris with a sieve with 1.3-mm-diameter round holes, and collecting seed coat pieces in a seed blower. Seed coat material was extracted overnight in 30 ml of sterile distilled water per gram. Each extract was filtered through a Millipore filter (Millipore Filter Corp., Bedford, MA 01730) with 0.45-μm pores, lyophilized, redissolved in water, and serially diluted. Aliquots were combined with Difco Corn Meal Agar (Difco Laboratories, Detroit, MI 48232) tempered to 50 C and poured into sectored plastic petri plates. Quadrants were seeded with 5-mm-diameter plugs cut from the advancing edges of two-day-old *P. ultimum* colonies on corn meal agar.

RESULTS

Transformed disease severity-inoculum density curves differed significantly ($P = 0.05$) in position, as reflected by ED_{50} inoculum density values (Table 1), but did not differ in slope among pea lines and seed coat conditions. ED_{50} inoculum density values of all lines with colored seed coats were greater than 2,500 GO/g whereas those of uncolored lines ranged from 6.3 to 480 GO/g (Table 1). Removal of seed coats reduced the ED_{50} values of colored lines by more than 100-fold but reduced those of uncolored lines by only 4.3-fold or less. ED_{50} values of embryos planted without seed coats varied among lines independently of seed coat color.

None of the seeds of a composite sample from the 83 F_3 progeny lines with colored seed coats from the cross PTL × PI 257593 was diseased in soil with 20 GO/g, whereas mean disease severity ratings of the 27 uncolored lines ranged from 34 to 93% of the maximum rating (Fig. 1). Nicking the seed coats increased the mean disease severity ratings of colored progeny lines to as high as 23%, whereas nicking did not increase those of uncolored lines (Fig. 1). Colored progeny lines with nicked seed coats, retested at 300 GO/g, had mean disease severity ratings ranging from 0 to 62%. Most uncolored progeny lines were more resistant than the uncolored parent and two lines were as resistant as nicked seeds of the colored parent (Fig. 1).

Extracts of colored seed coat material inhibited *P. ultimum* radial growth but the level of inhibition by similarly prepared extracts varied among pea lines. Extracts of uncolored seed coats were not inhibitory (Table 1). Slopes of dosage-response curves also varied among colored lines (Fig. 2). Slopes of log-probit

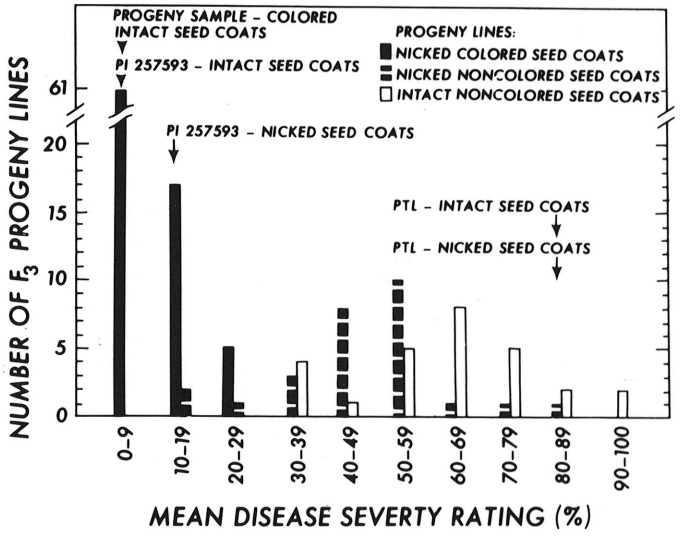

Fig. 1. Distribution of mean disease severity ratings of 110 F_3 progeny lines from the cross Perfection-type line (PTL) × PI 257593 in soil with 20 germinable *Pythium ultimum* oospores per gram. Mean disease severity ratings of parental lines and of a composite sample from progeny lines with colored seed coats are indicated by arrows. Seed coats were either intact or nicked distal to the embryonic axes at planting.

TABLE 1. The soil population density of germinable *Pythium ultimum* oospores required for 50% disease severity rating for seeds planted with seed coats intact, nicked, or removed as affected by pea line and genotype, and inhibition of *P. ultimum* hyphal growth caused by pea seed coat extracts

Pea line	Genotype[b]	ED_{50} oospore density (no./g soil)[a]			Inhibition of *P. ultimum* by seed coat extract (%)[c]
		Seed Coat intact	Seed Coat nicked	Seed Coat removed	
G 213	*AA II RR*	>2,500	530 A	55 F	46 B
PI 138945	*AA II RR*	>2,500	400 AB	130 E	55 B
PI 257593	*AA II RR*	>2,500	350 AB	46 FG	72 A
PI 253968	*AA II RR*	>2,500	220 CD	15 H	32 C
PI 165965	*AA II RR*	>2,500	160 CD	70 F	22 C
Minnesota 108	*aa II RR*	480 A	250 BC	140 DE	− 4 D
Alaska	*aa ii RR*	130 DE	31 G	30 G	− 3 D
Dark Skin Perfection	*aa ii rr*	11 H	6.0 I	5.3 I	2 D
Perfection-type line	*aa ii rr*	6.3 I	4.5 I	4.5 I	1 D

[a] Values followed by the same letter do not differ significantly ($P = 0.05$) as determined by comparison by a least significant difference criterion of values and confidence intervals from regressions of disease severity-inoculum density curves.

[b] Seed coats colored (*A*) or uncolored (*a*); cotyledons yellow (*I*) or green (*i*); seeds round (*R*) or wrinkled (*r*).

[c] Inhibition on corn meal agar amended with extract of 0.1 g seed coat per milliliter. Values followed by the same letter do not differ significantly according to Waller and Duncan's Bayesian significant difference test (K = 100).

transformed curves (inhibition ranging from 15 to 85%) ranged from 1.3 for PI 257593 to 5.2 for PI 165965.

Hyphae reached DSP and nicked PI 257593 seeds within 10 hr of planting in soil with ~400 GO/g. Exposing DSP and nicked PI 257593 seeds to their ED_{70} inoculum densities for 30 and 40 hr, respectively, resulted in 70% disease severity ratings. Longer exposures did not result in more severe disease ratings.

Twenty hours after intact PI 257593 seeds were planted in soil with ~400 GO/g, there were 17 ± 6 fungal colonies (hyphae and hyphal aggregates) per seed on the outside seed coat surface; after 40 hr there were 62 ± 18 colonies per seed by which time radicles had emerged through the seed coats and were about 0.5 cm long. Hyphae also were observed on seed coats of hard, unimbibed seeds 20 hr after planting. Hyphae were not observed in the inner seed coat tissues or on the cotyledons of imbibed PI 257593 seeds even after 100 hr, by which time plumules were emerging above the soil surface. Solitary hyphae were occasionally observed on elongating radicles but the development of root hairs made observations difficult after 60 hr.

The number of fungal colonies on seed coats of PI 257593 seeds was not affected by nicking seed coats, but cotyledon tissue exposed by nicking seed coats had 0.5 ± 0.3 and 3 ± 2 colonies per seed 20 and 40 hr after planting, respectively. After 100 hr, cotyledon tissue was discolored and soft near the nick and hyphae were observed in seed coat parenchyma and on cotyledon surfaces not exposed by the nick. Radicles of infected seeds emerged later than those of noninfected seeds and usually were killed as elongation was beginning.

Twenty hours after PI 257593 embryos were planted without seed coats in soil with ~400 GO/g, there were 40 ± 10 colonies per seed on the outer surface of the cotyledons and more than 75 colonies after 40 hr. There were 4 ± 2 colonies per embryonic axis after 20 hr. After 40–60 hr, radicles ceased elongating and hyphae ramified profusely within cotyledons, which were soft and discolored. When PI 257593 embryos were grown for 20 hr in noninfested soil and subsequently grown for 40 hr in soil with ~400 GO/g, there were only 15 ± 5 colonies per seed on the cotyledons and hyphae only occasionally were seen on embryonic axes. Hyphae of some of the colonies on the cotyledons appeared to be robust and were surrounded by macerated host tissue 100 hr after transplanting, but lesions were restricted in size and the cotyledons remained firm.

Twenty hours after DSP seeds were planted there were more than 75 colonies per seed on the outer seed coat surface but only 1.5 ± 2.0 colonies per seed in soil with 15 GO/g. At the greater inoculum density, hyphae reached the inner seed coat tissues and the cotyledons 40 hr after planting, by which time radicles were beginning to emerge. After 60 hr hyphal growth was profuse in seed coat parenchyma and on the cotyledons. After 80 hr, hyphae were ramifying within the radicles, which had ceased developing, and within the cotyledons, which were beginning to decay.

Susceptibility of pea seeds decreased during germination. DSP seeds exposed to their ED_{70} inoculum density more than 40 hr after planting subsequently developed less severe disease symptoms than did seeds exposed immediately and seeds exposed after 72 hr developed no disease symptoms. Similarly, nicked seeds of PI 257593 exposed to their ED_{70} inoculum density after only 27 hr, by which time radicles had not yet emerged through the seed coats, did not subsequently develop disease symptoms. Susceptibility of PI 257593 embryos without seed coats to their ED_{70} inoculum density decreased greatly within 16 hr of planting, by which time radicles were just beginning to elongate (Fig. 3). Embryos of PI 253968, Minnesota 108, and the most resistant uncolored progeny lines from the PTL × PI 257593 cross were no longer susceptible to 400 GO/g 27 hr after planting, but embryos of PTL remained susceptible for at least 27 hr.

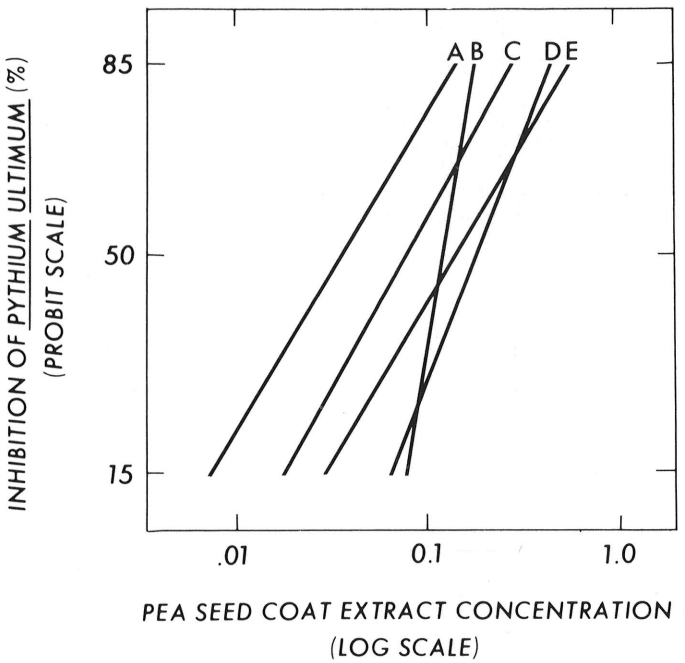

Fig. 2. Inhibition of *Pythium ultimum* radial growth by aqueous extracts of colored seed coats of various pea lines. Extract concentration is expressed as the grams of seed coat material extracted per milliliter of culture medium. **A**, PI 257593, slope = 1.3 ± 0.2 ($t_{.05}$); **B**, PI 165965, slope = 5.2 ± 1.0; **C**, PI 138945, slope = 1.4 ± 0.2; **D**, PI 253968, slope = 2.0 ± 0.6; and **E**, G 213, slope = 1.4 ± 0.3.

DISCUSSION

These results show that several types of resistance to *P. ultimum* seed and seedling disease exist in peas. Different types of resistance are associated with various seed parts and differ in the levels of resistance conferred.

That a high level of resistance was due to colored seed coats was shown by three lines of evidence: intact seeds of all lines with colored seed coats, whether PI accessions or progeny lines from a PTL × PI 257593 cross, were much more resistant than were seeds of uncolored lines; removing seed coats from colored seeds greatly reduced resistance whereas removing seed coats from uncolored seeds had little or no effect on resistance; and colored seed coats were not penetrated by the pathogen even after 100 hr but uncolored seed coats were penetrated within 40 hr and hyphal growth in the inner seed coat tissues was profuse within 60 hr. Nicking colored seed coats reduced resistance by exposing cotyledonary tissue to fungal attack, but undamaged portions of nicked seed coats were not penetrated by the pathogen and afforded a degree of protection to underlying embryonic tissues. The conclusion that colored seed coats may play a major role in

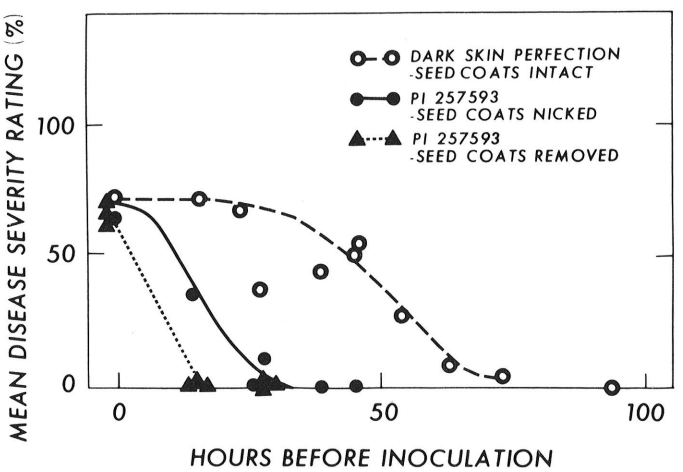

Fig. 3. Mean disease severity rating of peas inoculated with the ED_{70} density of *Pythium ultimum* oospores for each line and seed coat condition at various times after planting.

resistance of pea seeds to seed rots is consistent with the results of others (2,5,6,11). Field soils usually contain fewer than 1,500 *P. ultimum* propagules per gram (14), whereas colored lines suffered little or no disease in the present study in autoclaved soil infested with as many as 2,500 GO/g. Also, a higher *P. ultimum* inoculum density is required to cause diseases in field soil than in autoclaved soil (19).

Resistance to *P. ultimum* conferred by colored seed coats may be at least partially due to fungistatic compounds, as has been postulated (2,5,6). The presence of fewer fungal colonies on the surface of colored than on uncolored seeds may be partly due to fungistatic compounds leaching into the spermosphere of colored seeds since aqueous extracts of colored, but not of uncolored, seed coats inhibited hyphal growth in vitro. Further, the failure of the pathogen to penetrate colored seed coats in spite of colonizing the seed surface may be due to a higher concentration of fungistatic compounds in seed coat tissues than in the spermosphere. The nature as well as the concentration of fungistatic compounds in colored seed coats appears to vary among lines, since the curves relating concentration of seed coat extract and inhibition of hyphal growth varied in slope as well as in position.

Embryos also contribute to resistance. Resistant embryos were infected fewer times during germination than were susceptible embryos since resistant embryos planted without seed coats had fewer fungal colonies than similarly treated susceptible embryos. Also, resistant embryos became less vulnerable sooner after planting and at an earlier physiological age than did susceptible embryos as determined by delaying inoculation. For one line (PI 257593), embryo susceptibility decreased during germination partly because embryos became less infectible, since fewer colonies developed when inoculation was delayed. Also, less severe disease developed as a result of infection. Thus, whereas only three colonies per seed on cotyledons resulted in a mean disease severity rating greater than 50%, 15 colonies per seed resulted in a rating less than 10% when exposure to the pathogen was delayed.

Thus, seeds may be highly resistant not only because colored seed coats are not penetrated by the pathogen, but also because embryos become resistant before being exposed to the pathogen by rupture of seed coats during germination. Similarly, in moderately resistant seeds, seed coats may delay infection of cotyledons sufficiently long for embryo susceptibility to decrease unless inoculum potential is high. In contrast, embryos of highly susceptible seeds do not become less susceptible before seed coats are penetrated.

LITERATURE CITED

1. CLAUSS, E. 1967. The phenolic constituents of the testae of peas and their importance for resistance to the agents of root rot. Naturwissenschaften 61:106.
2. EWING, E. E. 1959. Factors for resistance to pre-emergence damping-off in pea (*Pisum sativum* L.) incited by *Pythium ultimum* Trow. PhD Thesis, Cornell University, Ithaca, NY. 118 pp.
3. FLENTJE, N. T. and H. K. SASKENA. 1964. Pre-emergence rotting of peas in South Australia. III. Host-pathogen interaction. Austr. J. Biol. Sci. 17:665-675.
4. HAMMERSCHLAG, F., and R. G. LINDERMAN. 1975. Effects of five acids that occur in pine needles on Fusarium chlamydospore germination in nonsterile soil. Phytopathology 65:1120-1124.
5. KRAFT, J. M. 1974. The influence of seedling exudates on the resistance of peas to Fusarium and Pythium root rot. Phytopathology 64:190-193.
6. KRAFT, J. M. 1977. The role of delphinidin and sugars in the resistance of pea seedlings to Fusarium root rot. Phytopathology 67:1057-1061.
7. KRAFT, J. M., and D. D. ROBERTS. 1970. Resistance in peas to Fusarium and Pythium root rot. Phytopathology 60:1814-1817.
8. MARX, G. A., W. T. SCHROEDER, R. PROVVIDENTI, and W. MISHANEC. 1972. A genetic study of tolerance in pea (*Pisum sativum* L.) to Aphanomyces root rot. J. Am. Soc. Hortic. Sci. 97:619-621.
9. MATTHEWS, S. 1971. A study of seed lots of peas (*Pisum sativum* L.) differing in predispositon to pre-emergence mortality in soil. Ann. Appl. Biol. 68:177-183.
10. MATTHEWS, S., and R. WHITBREAD. 1968. Factors influencing preemergence mortality in peas. I. An association between seed exudates and the incidence of pre-emergence mortality in wrinkle-seeded peas. Plant Pathol. 17:11-17.
11. McDONALD, W. C., and H. H. MARSHALL. 1961. Resistance to preemergence damping-off in garden peas. Can. Plant Dis. Surv. 41:275:279.
12. OHH, S. H., T. H. KING, and T. KOMMEDAHL. 1978. Evaluating peas for resistance to damping-off and root rot caused by *Pythium ultimum*. Phytopathology 68:1644-1649.
13. PERRY, D. A. 1973. Infection of seeds of *Pisum sativum* by *Pythium ultimum*. Trans Br. Mycol. Soc. 61:135-144.
14. PIECZARKA, D. J., and G. S. ABAWI. 1978. Populations and biology of *Pythium* species associated with snap bean roots and soils in New York. Phytopathology 68:409-416.
15. SCHNEIDER, A. 1952. Über das Vorkommen gerbstoffartiger Kondensationsprodukte von anthocyanidinen in der Samenschalen von *Pisum arvense*. Naturwissenschaften 39:452-453.
16. SHEHATA, M., C. R. GRAN, D. W. DAVIS, and F. L. PFLEGER. 1976. A technique for concurrently evaluating resistance in peas to *Fusarium oxysporum* f. sp. *pisi* and *Aphanomyces euteiches*. Plant Dis. Rep. 60:1024-1026.
17. SHORT, G. E. 1975. Determinants of pea seed and seedling rot. PhD Thesis, Michigan State University, East Lansing. 60 pp.
18. SORGEL, G. 1952. Über die Ursachen der unterschiedlichen Resistenz verschiedener Erbsensorten gegenumber den Fusskrankheitserregern *Ascochyta pisi* Lib., *Ascochyta pinodella* Jones und *Mycosphaerella pinodes* (Berk et Blox.) Stone. I. Vergleichende Untersuchungen zum Verhalten der Pilze auf einer stark und einer schwach anfalligen Sorte. Zuchter 22:4-26.
19. STASZ, T. E. and G. E. HARMAN. 1980. Interactions of *Pythium ultimum* with germinating resistant or susceptible pea seeds. Phytopathology 70:27-31.
20. VanVUURDE, J. W. L., and P. F. M. ELENBAAS. 1978. Use of fluorochromes for direct observation of microorganisms associated with wheat roots. Can. J. Microbiol. 24:1272-1275.