# Resistance and Susceptibility to Phytophthora megasperma Expressed in Alfalfa Cotyledons

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ABSTRACT

Cotyledons of 10-day-old alfalfa seedlings exhibited susceptible and resistant disease reactions within 3-7 days after inoculation with zoospores of *Phytophthora megasperma*. Susceptible reactions, characterized by rapid collapse of cotyledon tissue leading to shriveling and complete necrosis, contrasted with resistant reactions, in which only small, red-brown local lesions developed on upper surfaces of infected cotyledons. Symptoms on some plants were intermediate between resistant and susceptible types. Differential disease symptoms were also observed in detached leaflets inoculated with zoospores. Similar numbers of Vernal alfalfa seedlings were severely diseased at inoculum levels of from 9 to 151 zoospores per cotyledon; fewer plants were severely diseased when less than five zoospores were applied to each cotyledon.

Cotyledons of Vernal and Saranac were more susceptible to infection by zoospores than were cotyledons of six other lines and cultivars which had been selected for resistance to Phytophthora root rot. Vernal and Saranac plants also developed more severe root rot in artificially infested soil than did plants of the other six lines. The significant correlations observed between resistance and susceptibility in cotyledons and tap- and lateral roots of alfalfa ($r = .80$ and $r = .78$, 6 df) suggest that the cotyledon inoculation technique may provide a simple means for screening alfalfa lines for resistance to *P. megasperma*.

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Root rot of alfalfa, caused by *Phytophthora megasperma* Drechsler, is a serious disease in many parts of North America, and is likely to be widely distributed in alfalfa-growing areas (6, 16). The development of cultivars resistant to *P. megasperma* offers the only practical means of control. While cultivars not selected for resistance to *P. megasperma* differ in tolerance to root rot, none has high levels of resistance (3, 4, 9). Intercrosses and outcrosses of plants selected for resistance in the field (6) and greenhouse (4, 6) have recently led to the development of several *Phytophthora*-tolerant or resistant germplasm sources (7, 11, 12) and cultivars (1, 2). Within these lines and cultivars, plants typically show reaction levels varying from completely susceptible to highly resistant. Lu et al. (13) postulated that this variation in resistance may be explained by a monogenic model in which susceptibility is conditioned by a single tetrasomic gene with incomplete dominance.

Tests for resistance to *P. megasperma* in alfalfa have usually involved plants several weeks or months old (3, 6, 9, 13, 14). Erwin (3) reported that plants of all cultivars were eliminated if seeds were planted into infested soil, but Gray et al. (8) planted into infested soil and found resistance to damping-off expressed in seedlings less than 2 weeks old. They also found a positive correlation between resistance to damping-off in young seedlings and resistance to root rot in older plants.

We have discovered that when cotyledons are inoculated with zoospores, differential reactions to *P. megasperma* are evident. The purpose of work reported in this paper was to describe disease reactions which occur in cotyledons and to determine whether resistance in cotyledons of young seedlings is correlated with resistance in roots of older plants. Our results suggest that the cotyledon-inoculation technique may provide a simple means for evaluating the resistance and susceptibility of alfalfa lines to *P. megasperma*.

MATERIALS AND METHODS.—Zoospore inoculum was obtained from five isolates of *P. megasperma* baited from soils of alfalfa fields in five counties of Wisconsin (16). Each isolate was grown on 20% V-8 juice agar 9 days at 24 C in alternate light and darkness until colonies were approximately 7 cm in diameter. The agar surrounding each colony was cut away, and sufficient basic salt solution (BSS) (15) was added to fill the outer well and cover the colony to a depth of 2-3 mm. After 5 hours, sporangia developed and swimming zoospores were released. Zoospore suspensions of the five isolates were mixed together and counted with a Spencer hemacytometer. Dilutions of zoospores were made with additional BSS.

Alfalfa seed was scarified and germinated for 24 hours on moist filter paper in petri dishes. Germlings were planted in rows of 15 in flats ($42 \times 26 \times 5.5$ cm internal dimensions) containing steamed sand:loam (1:1, v/v) leveled to a depth of 4 cm prior to watering. The plants were grown in a growth chamber maintained at 24 C and with a 12-hour photoperiod under cool-white fluorescent lamps + 10% incandescent lamps (total intensity = 19,400-21,500 lx) and watered daily for 8 days.

Prior to inoculation, each flat was placed in a metal tray ($51 \times 29 \times 8$ cm) in which water was maintained at a depth of 2 cm. Upright plants with expanded, unwrinkled cotyledons were inoculated by placing a .01 ml droplet of zoospore suspension on the tip of each cotyledon. Immediately after inoculation, the top of each tray was covered with cellophane to prevent evaporation of droplets. During the infection period, trays were exposed to 18 hours of continuous light to prevent the cotyledons from folding up. The cellophane was then removed and the droplets on the cotyledons were removed by suction. Flats were removed to the 24-C growth chamber, and symptom development was observed daily for 1 week.

The same five isolates of *P. megasperma* were used to

provide cornmeal-sand (CS) inoculum for root rot tests. Cornmeal (9.0 g) and silica sand (374 g) were thoroughly mixed in 500-ml flasks, and 100 ml deionized water was added. Stoppered flasks were autoclaved 20 minutes, cooled, inoculated with infested agar blocks, sealed with aluminum foil, and shaken every 2 days to insure thorough infestation. After 2 weeks at 24 C in light and dark, CS of the five isolates was combined and mixed with unsteamed sand:loam at a ratio of 1/64 (v/v) (designated CSI/64).

Alfalfa seedlings for root rot tests were grown in steamed sand:loam in 296-ml (10-oz) wax-treated paper cups (three seedlings per cup) in a 24 C greenhouse. A thin layer of unsteamed soil was added to the top of each cup 10 days following planting to prevent colonization of the surface by fungal mycelia. After 6 weeks, cups were cut away and seedlings were washed and transplanted to 474-ml (16-oz) cups (three seedlings per cup) in CSI/64. Cups were watered daily, but not flooded. After 4 weeks at 20 C, the cups were cut away and root systems were washed free of soil and evaluated.

Statistical significance of cotyledon and root disease data was determined by Duncan's multiple-range test (17).

RESULTS.—*Symptomatology of infected cotyledons.*—Symptoms were first evident on susceptible cotyledons 24 hours after inoculation as small, sunken "pock-mark" lesions. After 2 days, these had developed into larger, coalesced, sunken necrotic patches and the cotyledons had become flaccid and slightly curled. By the third day, cotyledons of many susceptible plants were completely necrotic and shriveled, and infection had progressed down the cotyledons and into the growing shoots. Cotyledons of most susceptible plants were dead by the third or fourth day, but some had not turned completely necrotic 1 week after inoculation.

In resistant plants, small, red-brown flecks first appeared after 2 days and did not enlarge, even after 1 week. Other plants were apparently highly resistant or immune, and developed no symptoms at all following zoospore inoculations. The cotyledons did not appear different from noninoculated controls which received only BSS. Plants intermediate between the susceptible and resistant types showed either a slight coalescing of flecks, to heavy coalescing leading to the development of large, but still restricted necrotic patches and networks surrounded by dark borders (Fig. 1). Some plants developed a resistant reaction on one cotyledon and a susceptible reaction on the other.

*Zoospore concentration-infectivity relationships.*—Similar numbers of Vernal seedlings became severely diseased (one or both cotyledons more than three-fourths necrotic) at inoculum dosages varying from 940 to 15,100 zoospores/ml (9.4 to 151 zoospores/cotyledon) (Table 1). Numbers of plants with severely diseased cotyledons declined when less than five zoospores were applied to each cotyledon. Infection developed rapidly in susceptible plants, and at least 81% of those that became severely diseased at inoculum levels of 9.4 or more zoospores per cotyledon did so within 4 days.

*Cotyledon reactions of different alfalfa lines and cultivars.*—Cotyledon reactions of Vernal and Saranac, two commercial cultivars which had not been developed for resistance to Phytophthora root rot, were compared with reactions of six lines and cultivars developed for resistance at the University of Minnesota (1, 6, 7) (Table 2). Cotyledons of 40 plants of each line or cultivar were inoculated with zoospores (58/cotyledon) and disease scores based on severity of symptoms were determined within 1 week (Table 3). Plants were scored with a rating of 1 if no symptoms or only small, localized lesions developed; 2, if lesions were coalesced into necrotic networks or patches covering less than one-fourth of a cotyledon; 3, if necrotic patches covered one-fourth to three-fourths of a cotyledon; and 4, if one or both cotyledons were more than three-fourths necrotic.

Mean disease scores of Vernal and Saranac were similar and both were significantly different ($P = 0.01$) from scores for the other six lines and cultivars. Differences among the latter were not significant, $P = 0.01$, but MnP-A3 was different from MnP-D1 and MnP-C3, $P = 0.05$. Disease development in susceptible seedlings of Vernal and Saranac was faster than in susceptible seedlings of the other six lines. Of the most severely diseased plants (scores of 3 and 4) (Table 3), more

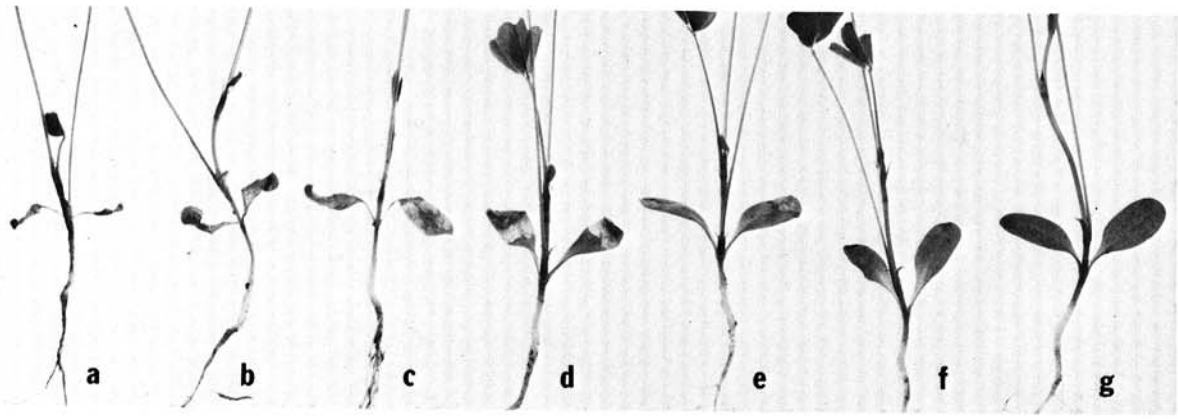


**Fig. 1-(A to G).** Cotyledons of alfalfa seedlings showing: **A to D)** susceptible, **E,F)** intermediate, and **G)** resistant disease reactions 7 days after inoculation with zoospores of *Phytophthora megasperma*.

TABLE 1. The effect of *Phytophthora megasperma* zoospore concentrations on disease severity in cotyledons of Vernal alfalfa seedlings 3 to 7 days after inoculation

| Mean number of zoospores per cotyledon[a] | No. of plants with severely diseased cotyledons[b] Days after inoculation | | | | | Plants with moderately diseased to healthy cotyledons 7 days after inoculation[c] |
|---|---|---|---|---|---|---|
| | 3 | 4 | 5 | 6 | 7 | |
| 151.0 | 10 | 5 | 1 | 0 | 0 | 4 |
| 75.5 | 9 | 9 | 1 | 1 | 0 | 0 |
| 37.8 | 11 | 3 | 3 | 0 | 0 | 3 |
| 18.9 | 6 | 7 | 2 | 1 | 0 | 4 |
| 9.4 | 6 | 7 | 1 | 0 | 0 | 6 |
| 4.7 | 1 | 3 | 1 | 0 | 0 | 15 |
| 2.4 | 1 | 2 | 1 | 1 | 1 | 14 |
| 1.2 | 1 | 2 | 1 | 1 | 1 | 14 |
| 0 | 0 | 0 | 0 | 0 | 0 | 20 |

[a]Zoospores in 0.01 ml of a basic salt solution. Twenty plants inoculated with each zoospore concentration.
[b]One or both cotyledons more than three-quarters necrotic.
[c]Moderately diseased = neither cotyledon more than 50% necrotic.

than 50% in Vernal and Saranac were so classified within 4 days after inoculation, but in the six resistant lines and cultivars, less than 50% of such plants were so classified within 4 days.

*Leaflet reactions.*—Six terminal leaflets from leaves on young, growing shoots of each of three plants known to have resistance to Phytophthora root rot and three susceptible plants were excised and inoculated with zoospore droplets (ca. 150 zoospores per leaflet) while incubated on moist filter paper in Petri dishes. Droplets of zoospores were removed after 18 hours as with cotyledons. After 10 days, all leaflets from the three susceptible plants were completely rotted. All leaflets from one resistant plant remained green and turgid, with localized necrotic patches surrounded by chlorotic halos in areas where the zoospores had been applied. Some leaflets of the other two resistant plants were decomposed while others remained green and turgid.

*Correlation between cotyledon reactions and root rot.*—Six-week-old seedlings of Vernal, Saranac, and the six resistant lines and cultivars were evaluated for resistance to Phytophthora root rot in a greenhouse test. After 4 weeks at 20 C in CS1/64, both taproots and lateral roots were scored according to the percentage of the total root(s) that showed root browning (decay) (Table 4). Where lower portions of taproots were rotted off, percentage decay was based on an estimate of the previous length of the taproot. Where taproots were completely girdled, all of the distal portions were considered rotted.

Control plants of all lines and cultivars had completely white taproots, and showed only small amounts of lateral root browning, when grown in uninfested CS, 1/64. In plants grown in infested soil, browning of lateral roots was darker and always more extensive. All plants of Vernal and Saranac had lateral root scores of 3 or 4 (i.e., more than 50% browning), while some plants of five of the six resistant lines had less than 50% lateral root browning (Table 4). The majority of plants of Vernal and Saranac had taproots with scores of 3 or 4, while the majority of plants in the resistant lines and cultivars had scores of 1 or 2 (i.e., less than 50% browning) (Table 4).

Root disease severity was significantly correlated with cotyledon disease severity. Mean cotyledon disease scores of Vernal and Saranac plants were significantly different from those of all resistant selections, $P=0.01$, while mean lateral root scores of Vernal and Saranac were different from five of the six resistant selections, $P = 0.01$. The mean taproot score of Saranac was different from all others except Vernal, $P = 0.01$; that of Vernal was different from three of the six resistant selections. The selection GN1-Syn 2-F2 had the least lateral and taproot browning and ranked second to the least diseased in the cotyledon test.

The correlation coefficient for cotyledon disease and lateral root disease was .78; that for cotyledon and taproot disease was .80 (6 df).

TABLE 2. Sources or description of germplasms used in determinations of cotyledon and root reactions to *Phytophthora megasperma*[a]

| Alfalfa breeding line or cultivar | Source or description |
|---|---|
| Saranac | Commercial variety |
| Vernal | Commercial variety |
| MnP-B1 | Experimental line synthesized from *Phytophthora*-resistant selections from 29 winter-hardy varieties and lines |
| MnP-D1 | Experimental line synthesized from *Phytophthora*-resistant selections from 14 southern-grown varieties |
| Agate | Variety synthesized from *Phytophthora*-resistant selections from Ramsey and Vernal backgrounds |
| MnP-A3 | *Phytophthora*-resistant selection from Agate |
| MnP-C3 | *Phytophthora*-resistant selection from Lahontan |
| GN1-Syn 2-F2 | F2 from cross of two *Phytophthora*-resistant parents from Appalachee |

[a]The six lines and varieties developed for resistance to Phytophthora root rot were supplied by D. K. Barnes, Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul.

DISCUSSION.—This study shows that resistant, intermediate, and susceptible disease reactions occur in alfalfa cotyledons inoculated with zoospores of *P. megasperma,* and that severity of cotyledon disease is correlated with severity of root rot in the eight alfalfa lines and cultivars. Differential reactions also occur in detached leaflets inoculated with zoospores. These observations suggest that the same genetic factors which govern susceptibility or resistance in roots may also be operable in aerial plant parts. Susceptible reactions in roots, cotyledons, and leaflets are all characterized by collapse of tissue leading to complete necrosis. Resistant reactions in cotyledons are characterized by the development of small, localized necrotic flecks suggesting a hypersensitive reaction. Marks and Mitchell (14) similarly reported that hypersensitive reactions occur in fine roots of alfalfa plants resistant to Phytophthora root rot, but not in roots of susceptible plants. Some plants showed reactions in infected cotyledons which were clearly intermediate between resistant and susceptible types, and these might be similar to the intermediate reactions to zoospores which occur in some roots (14). Intermediate reactions in cultivated tetraploid alfalfa could be explained by a polygenic system, or by a simple genetic system where resistance is controlled by allelic dosage of an incompletely dominant gene. Lu et al. (13) presented the latter type of model to explain intermediate root disease reactions which were evident in some progeny of crosses between resistant and susceptible plants.

Keen and Horsch (10) criticized the use of "unnatural" host organ-parasite systems in the study of naturally occurring disease-resistance mechanisms in plants. They reported that the *Rps* gene for resistance to *P. megasperma* var. *sojae* Hildebrand in soybeans was expressed in hypocotyls, but not in roots. Our observations on differences in disease symptoms in alfalfa cotyledons and leaves clearly show that some mechanism for resistance to *P. megasperma* operates in these tissues. The correlations noted between disease severity in cotyledons and roots strongly suggest, either that the same system operates in both organs, or that different systems are present which produce the same effect.

Severity of Phytophthora root rot varies with inoculum level and seedling age (Pratt and Mitchell, *unpublished*), temperature (8, 13), and soil moisture level (5). Although the effects of these and other environmental factors on disease reactions in alfalfa cotyledons and leaflets have not been studied, it is apparent that severity in seedlings of the highly susceptible variety, Vernal, is similar over a wide range of inoculum concentrations (Table 1). This suggests that the cotyledon assay for resistance and susceptibility may produce more consistent results than root assays. It remains to be determined, however, whether differences in virulence of *P. megasperma* isolates would cause differences in disease reactions of cotyledons and leaflets as in roots (3, 8).

TABLE 3. Disease severity in cotyledons of seedlings of eight alfalfa lines and cultivars following inoculation with zoospores of *Phytophthora megasperma*[a]

| Alfalfa line or cultivar | No. plants with indicated disease score[b] | | | | Mean disease score[c] |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | |
| Vernal | 4 | 1 | 3 | 33 | 3.60 |
| Saranac | 7 | 1 | 3 | 29 | 3.56 |
| MnP-C3 | 11 | 8 | 3 | 18 | 2.70 |
| MnP-D1 | 12 | 7 | 4 | 17 | 2.65 |
| MnP-B1 | 14 | 5 | 4 | 17 | 2.60 |
| Agate | 15 | 6 | 1 | 18 | 2.55 |
| GN1-Syn 2-F2 | 17 | 7 | 1 | 15 | 2.35 |
| MnP-A3 | 21 | 7 | 2 | 10 | 2.03 |

[a]Fifty-eight zoospores in 0.01 ml solution per cotyledon. Disease readings taken 3-7 days after inoculation.

[b]1 = no symptoms, to small, red-brown, isolated lesions; 2 = lesions coalesced into necrotic network or small necrotic patches, less than one-quarter of the cotyledon affected; 3 = lesions coalesced into necrotic patches, one-quarter to three-quarters of the cotyledon affected; 4 = 1 or both cotyledons more than three-quarters necrotic. Plants scored according to cotyledon with greatest disease score. Forty plants scored per line or cultivar.

[c]Means = means of scores of 40 seedlings of a line or cultivar. Mean scores of Vernal and Saranac significantly different from those of all others, $P = 0.01$.

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TABLE 4. Severity of root rot caused by *Phytophthora megasperma* in seedlings of eight alfalfa lines and cultivars grown in artificially infested soil

| Alfalfa line or cultivar | No. plants with indicated disease score[a] | | | | | | | | | Mean score, all roots |
|---|---|---|---|---|---|---|---|---|---|---|
| | Lateral roots | | | | | Taproots | | | | |
| | 1 | 2 | 3 | 4 | mean[b] | 1 | 2 | 3 | 4 | mean[b] | |
| Saranac | 0 | 0 | 0 | 29 | 4.0 | 3 | 4 | 3 | 20 | 3.3 | 3.6 |
| Vernal | 0 | 0 | 3 | 27 | 3.9 | 8 | 4 | 6 | 12 | 2.9 | 3.3 |
| MnP-B1 | 0 | 0 | 7 | 23 | 3.8 | 12 | 5 | 4 | 9 | 2.3 | 3.1 |
| MnP-A3 | 1 | 4 | 9 | 16 | 3.3 | 18 | 2 | 3 | 7 | 2.1 | 2.7 |
| MnP-D1 | 0 | 5 | 14 | 11 | 3.2 | 17 | 2 | 3 | 8 | 2.1 | 2.7 |
| Agate | 0 | 3 | 11 | 16 | 3.4 | 20 | 5 | 0 | 5 | 1.7 | 2.6 |
| MnP-C3 | 0 | 4 | 14 | 12 | 3.3 | 22 | 3 | 2 | 3 | 1.5 | 2.4 |
| GN1-Syn 2-F2 | 1 | 9 | 10 | 10 | 2.9 | 26 | 0 | 1 | 3 | 1.4 | 2.2 |

[a]1 = 0-25% of root(s) browned, 2 = 25-50%, 3 = 50-75%, 4 = 75-100%. Thirty plants were scored/line or cultivar.

[b]Laterals: means of Saranac and Vernal significantly different from all others except MnP-B1 at $P = 0.01$. Taproots: mean of Saranac different from all others except Vernal at $P = 0.01$., Vernal different only from Agate, MnP-C3 and GN1-Syn 2-F2.

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