# Factors Involved with the Reaction of Alfalfa to Root Rot Caused by Phytophthora megasperma

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ABSTRACT

The reaction of alfalfa to root rot caused by *P. megasperma* was examined by comparing the response of susceptible and tolerant varieties and selections to infection in solution culture and in soil. Considerably less root rot occurred in highly tolerant than in susceptible plants. Zoospores of *P. megasperma* encysted readily on both susceptible and tolerant plants. Resistance to infection in growing root tips was associated with a hypersensitive type of reaction to infection of young cortical cells. Roots with large-diameter central steles survived better in infested soil. Tolerant selections produced more lateral roots with large-diameter central steles than did those susceptible to root rot. Phytopathology 61:510-514.

The importance of *Phytophthora megasperma* Drechs. as a causal agent of root rot of alfalfa, *Medicago sativa* L., in Minnesota and Wisconsin has only recently been appreciated (5, 6, 12). The occurrence depends to a large extent on the environmental conditions during a particular season (1, 5, 6). The development of varieties resistant to this pathogen has been undertaken in California (3, 10), Minnesota (Frosheiser, *personal communication*), and Wisconsin (Bingham, *personal communication*). The response of alfalfa roots to this pathogen is the principal factor in determining whether a particular plant will be resistant or susceptible. Tests with seedlings of a series of varieties by methods reported previously (15) indicated that resistance to the pathogen was not expressed at germination or in early stages of seedling growth but appeared as the seedling developed. The nature of the resistant response during the development of the alfalfa plant was the object of this study. Growth, after inoculation, of resistant and susceptible selections was compared under conditions favorable for disease development. The factors associated with root survival in infested soils and solution culture were examined.

MATERIALS AND METHODS.—In preliminary experiments, the pathogenicity of five soil isolates of *P. megasperma* from Wisconsin and from Minnesota were compared on a series of varieties and selections of alfalfa. The inoculum was prepared from 8-day-old cultures grown on 60 ml of Campbell's V-8 broth in 900-ml capacity bottles. The method of Tsao & Garber (16) was used for harvesting and standardizing the amount of inoculum. The contents of four bottles was used to inoculate 15 pots. All soil inoculations were carried out by transplanting 18- to 21-day-old seedlings into infested soil. A soil mixture consisting of sandy loam, steamed for 45 min, mixed with sand (1:1) was used in all experiments. After inoculation, the soils were saturated with water for 3 days and then were alternately saturated for 2 days and allowed to dry for 2 days until the end of the experiments. Root development and damage were assessed visually after 25-28 days. All treatments contained four replicates; the experiments were repeated 3 times. Tests were performed in a growth room with 28 C, 15-hr day and 13-C night temperatures.

Solution culture tests were carried out with 3-week-old seedlings of root rot-tolerant and -susceptible varieties grown in a complete, aerated Hoagland's solution in darkened, 2-liter containers. Plants were grown by placing germinated alfalfa seeds in holes in 8-mm thick polystyrene sheets floating on the aerated nutrient solution. After 3 weeks' growth, the plants supported by the polystyrene were transferred to the inoculum suspension. The zoospore suspension used for inoculation was prepared from 20-40 Vernal alfalfa seedlings inoculated by placing them on the surface of an 8-day-old culture of *P. megasperma* on V-8 agar for 12-18 hr. The infected seedlings were then floated in a shallow layer of distilled water contained in a large flat dish to stimulate sporangial production. These seedlings were next tied up loosely in a muslin pouch and transferred to a container of cool (12 C) filtered, nonsterile lake water in which the roots of the test seedlings were also immersed. The test plants were exposed to the zoospores subsequently released for 8-24 hr, and were then either examined directly or transferred back to the aerated nutrient solution. Direct observations were made of 8-10 randomly selected root-tip samples of each variety after immersion for 8, 12, or 24 hr. The whole root system was inspected for root rot and oospores 3 days after inoculation. All experiments were carried out under long-day (15 hr) conditions at 28-C day and 13-C night temperatures and a relative humidity of 65-75%.

Material for cytological examination was fixed in Formalin, acetic acid, alcohol, and water (5:10:35:50), dehydrated in tertiary butyl alcohol, embedded in paraffin in groups of 50 roots/block, and sectioned at 12 μ. A safranin fast-green stain was used (8).

RESULTS.—Since no major differences were seen in the reaction of the varieties and selections of alfalfa to various isolates (Table 1), Wisconsin isolate, DC 1-6, was used in subsequent tests. The reaction of the different varieties and selections of alfalfa as rated on the basis of growth and survival ranged from highly toler-

TABLE 1. Reaction of 6-week-old seedling of alfalfa varieties and selections to five isolates of *Phytophthora megasperma*

| Variety | Isolate of P. megasperma | | | | |
|---|---|---|---|---|---|
| | DC 1-6 | MM 5/4 | SS 1 | DC 1-2 | FFPM[a] |
| Lahontan | HT[c] | HT | HT | HT | HT |
| P-42[a] | HT | HT | HT | HT | HT |
| P-41[a] | HT | HT | | | |
| P-38[a] | T | T | | | |
| WP-59[b] | T | | | | |
| Delta | S | S | S | S | S |
| Narragansett | S | S | S | S | ST |
| Iroquois | S | ST | S | S | S |
| Florida 66 | ST | S | S | ST | ST |
| Saranac | S | S | S | S | S |
| Vernal | S | S | S | S | S |
| Caliverde 65 | S | S | S | S | S |
| P-17 × 35[a] | S | S | S | S | S |

[a] Minnesota isolate or selection supplied by F. Frosheiser.
[b] Supplied by E. T. Bingham.
[c] S = susceptible; ST = slightly tolerant; T = tolerant; HT = highly tolerant.

ant to susceptible. No variety or selection showing evidence of true immunity was found. The reaction of the two varieties, Saranac and Florida 66, and the two selections, P-42 and WP-59, illustrate the range of response noted.

Top growth of inoculated and noninoculated Saranac and P-42 plants slowed considerably for 10-12 days (Fig. 1) after transplanting into infested soil. At the end of this period, the foliage of Saranac (rated susceptible) turned yellow; by the 25th day, most of the plants had died. In other tests, Florida 66 (rated slightly tolerant) showed a similar growth pattern, but there were a large number of survivors after 25 days. In contrast, 90% of the plants of P-42 (rated highly tolerant) retained their green color and resumed vigorous growth about 14 days after transplanting. Selection WP-59 (rated slightly tolerant) recovered normal rate of growth more slowly, and 10-40% of the plants failed to survive.

The factors associated with this tolerance were studied. Resistance of alfalfa to root rot could result from zoospores either being repelled by root secretions (14) or failing to penetrate the outer walls of the root epidermis. These possibilities were tested in solution culture by placing root-rot tolerant P-42 and susceptible Vernal alfalfa seedlings in the same container with a source of zoospores. Numerous and approx equal numbers of zoospores were seen swarming around the root tips of both varieties after 8 hr; there were no obvious differences in the accumulation of cysts on the root tips after 24 hr (Fig. 3-F).

There were striking differences between P-42 and Vernal in the amount of root rot at the end of 48 hr. The roots of the susceptible variety had lost their turgor, and many oospores were present in the decaying cortex by the end of 72 hr (Fig. 3-B). At this stage, the shoots began to wilt and the plants soon died. There was considerably less root rot on the tolerant variety, and only very small numbers of zoospores were seen in the rotted roots. These observations were consistent in all three experiments, and suggest a cellular basis for tolerance in P-42.

The cellular reaction to infection was studied by

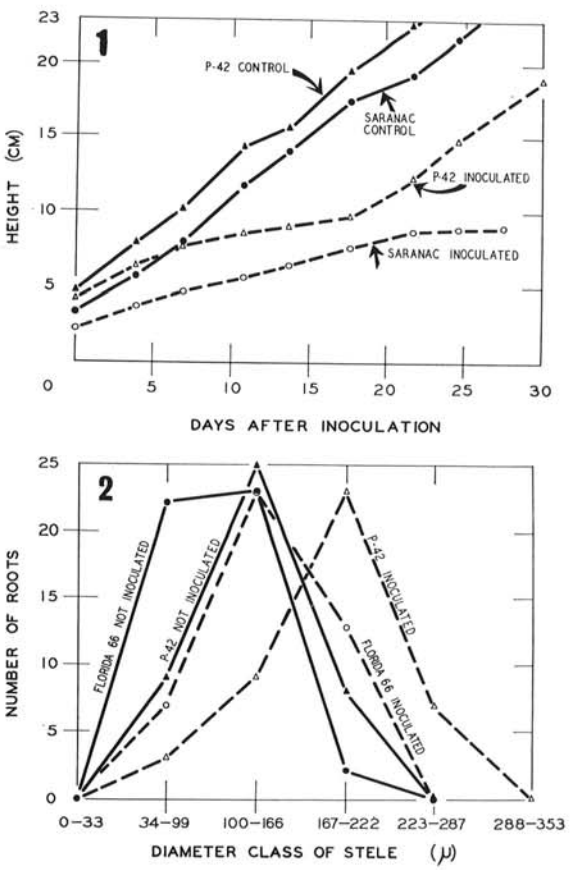


**Fig. 1-2. 1)** Cumulative height of a highly root rot-tolerant (P-42) and a susceptible (Saranac) variety of alfalfa growing in soil infested with *Phytophthora megasperma*. Nearly all the Saranac seedlings were killed at the end of four weeks. Note recovery in the growth rate of P-42. **2)** Frequency distribution of stelar diameters, measured 1-1.5 cm behind the growing apex, of 50 randomly sampled second- and third-order roots of slightly root rot-tolerant (Florida 66) and highly root rot-tolerant (P-42) selections of alfalfa after 25 days' growth in soils infested with *P. megasperma*.

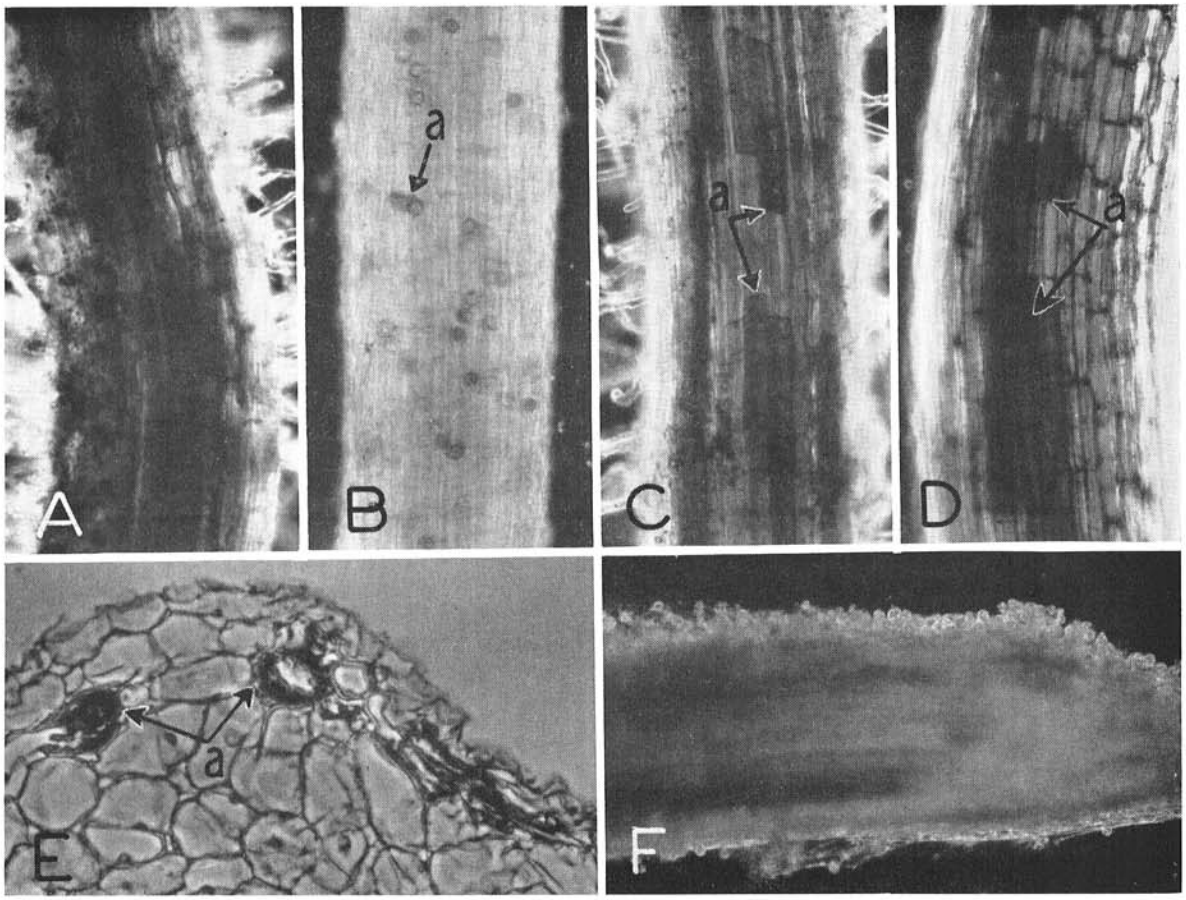


**Fig. 3.** Reaction of alfalfa root tips to infection. **A)** Infected root of susceptible variety (Vernal) in soil, ×75. **B)** Infected root of Vernal in solution culture. Note oospores (a) and the absence of granular material. **C)** Root of highly root rot tolerant variety P-42 infected in soil, ×75. Note accumulation of granular materials in some cells (a). **D)** Same as in (C) except that infection was in solution culture. **E)** Section of cortical cells containing granular material, ×400. Note localization of the reaction. **F)** Typical accumulation of cysts on the tip of roots of both P-42 and Vernal ×60.

examining both whole roots and fixed and stained sections of roots that had been inoculated in solution culture and in infested soil. The effects of exposure to both methods of inoculation were similar. The most obvious differences were noticed in the region of the root tips. In the susceptible varieties, Saranac and Vernal, there were slight changes in the color; small amounts of yellow-colored, granular material could be seen in several cortical cells. This was more obvious in roots obtained from inoculated soils (Fig. 3-A). In the tolerant selections, P-42 and WP-59, an orange-yellow, granular material densely filled either individual cells or small groups of cortical cells immediately behind the growing apex (Fig. 3-C, D). Adjacent cells were not affected (Fig. 3-E), and the damage was restricted. A similar but less intense reaction was observed in the roots of the slightly tolerant variety, Florida 66. Very few granular deposits were seen in the infected cells of the older parts of the primary cortex of either tolerant or susceptible varieties. These observations suggest that in the root rot-tolerant variety, a hypersensitive reaction to infection occurred at the root tips of the 3-week-old seedlings. This reaction took place between 8-24 hr after inoculation, and could be expected to contribute to the root rot resistance shown by plants in solution culture and soil.

Examination of root structure showed a distinct relationship between stele diam and plant survival in infested soil. The nature of this relationship was ascertained by a detailed analysis of the root systems produced in infested and uninfested soil. At the end of 6 weeks' growth, all plants produced third-order (17) roots that were slightly narrower and shorter than the second-order roots. Both types were covered with persistent root hairs for a greater part of their length. The second-order roots varied in length from 10-20 cm; in the young seedlings, it appeared that they were important water- and nutrient-absorbing structures. Measurements of the diam of individual intact roots of either category, at 2-cm intervals, showed that there was little variation from the root tip to the point of origin. The fact that the diam of the central stele gradually increased basipetally in many of the second-order roots was not evident externally because shrinkage of the ag-

TABLE 2. Comparison of the diameters of roots of six surviving plants of susceptible, slightly tolerant, tolerant, and highly tolerant alfalfa varieties and selections after 25 days' growth in soils with and without *Phytophthora megasperma*

| | | Root diameter (μ)[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Nondisinfested soil | | | Infested soil | | |
| Variety | Reaction | Min | Mean | Max | Min | Mean | Max |
| Saranac[b] | Susceptible | 200 | 265 | 430 | 135 | 245 | 500 |
| Florida 65[c] | Slightly tolerant | 200 | 290 | 460 | 165 | 245 | 400 |
| WP-59[c] | Tolerant | 200 | 320 | 575 | 200 | 290 | 530 |
| P-42 | Highly tolerant | 200 | 310 | 460 | 160 | 310 | 460 |

[a] Measurements of 75 roots.
[b] Uncertain whether all survivors were viable. Means differ significantly at 5% level.
[c] Means differ significantly at 10% level.

ing cortical cells compensated for this change. The size distribution curve of 75 roots sampled at random was approx normal, with a skew towards the larger sizes. There was no relationship between total root diam and tolerance to root rot (Table 2), although it appeared that after 25 days' growth in infested soil, the surviving roots on tolerant plants were narrower than those produced in pasteurized soil. This could have been caused by infection and collapse of the cortical cells. An examination of roots of susceptible and tolerant selections indicated, however, that there was a marked shift in infested soil toward the production or survival of roots with larger-diam central steles. This was most marked in the highly tolerant P-42. Comparisons of stelar diam (measured 1-1.5 cm behind the apical meristem) of 50 randomly sampled root tips (Fig. 2) showed that in infested soil both Florida 66 and P-42 produced a larger number of roots with steles in the larger diam classes. The number of roots with smaller steles was reduced, suggesting that they were more susceptible to root rot. Since all plants were of the same age, it appears that these trends represented a response to the soil inoculum. In these tests, the susceptible variety Saranac could not be used because of excessive root mortality.

DISCUSSION.—Jones (9) observed that "non-cambial" roots were more susceptible to decay than those that produced some secondary growth. Yet, in the initial stages of development the two root types were indistinguishable. He assumed that the phellem produced during secondary growth protected the roots. The observations reported above show an association between root survival in soils infested with *P. megasperma* and the size of the central stele (Fig. 2). The larger steles usually had a clearly defined endodermis. However, cytological examination showed that this barrier was only partially effective in stopping infection of the decay sensitive stele (12). Although the endodermis provided only limited protection to individual roots, in a large root mass it could contribute significantly to root rot tolerance as it does in pine (7, 11). This may account for the observation that initial growth after inoculation was slow in the root rot-tolerant variety (Fig. 1). During this period the smaller roots decayed (Table 2), and renewed growth occurred only after there was an increase in the number of roots with larger steles that were better able to tolerate infection.

Marx & Bryan (13) showed that zoospores of *Phytophthora cinnamoni* Rands constitute the predominant inoculum in a nonsterile environment. The solution culture experiments showed no difference in the degree to which the zoospores were attracted to, and encysted on, the roots of root rot-tolerant and -susceptible varieties. It was apparent, however, that while penetration took place within 24 hr on the roots of susceptible varieties, only after 48 hr was appreciable penetration evident on roots of tolerant selections (12). Thus, in the aerated solution cultures it is unlikely that root secretions hindered encystment and penetration. The striking difference between root rot-tolerant and -susceptible selections was the mass of dark, orange-yellow colored granular material produced when the young, living, primary cortical cells of tolerant varieties were infected. The cytological appearance of the cells and the limited area of infection were similar to the hypersensitive reaction produced by *P. megasperma* var. *sojae* Hild. on soybeans (*Glycine max* [L.] Merr.) (4). It is possible that phenolic compounds were produced inside the infected cells (2) that slowed fungal growth in the tissue.

In alfalfa roots, there was no immunity to infection even in the most root rot-tolerant variety tested. It appeared that tolerance was associated with at least two factors: (i) the structure of the central stele; and (ii) a hypersensitive-type of reaction observed in the young cortical cells of the growing root tips. The latter appears to have greater importance because zoospores aggregate preferentially in this region (Fig. 3-F) and, consequently, this is the zone most in need of protection. It is uncertain, however, that this mechanism will operate as effectively in heavy, poorly aerated soils where root growth is slow.

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