Ability of *Aphanomyces euteiches* to Cause Disease of Seedling Alfalfa Compared with *Phytophthora megasperma* f. sp. *medicaginis*

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ABSTRACT

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Four-day-old alfalfa seedlings were inoculated with zoospores of *Aphanomyces euteiches* or *Phytophthora megasperma* f. sp. *medicaginis* and incubated at 16, 20, 24, 28, or 32 C. Seedling survival was recorded at 3, 6, and 10 days after inoculation, and disease severity was rated on a four-class scale 10 days after inoculation. Both pathogens caused root discoloration and chlorosis of cotyledons, followed by advancing stem necrosis. Disease incidence was equivalent for seedlings incubated at 16–28 C. However, optimal temperatures for disease, measured by disease severity rating and area under a mortality progress curve, were 24–28 C and 16–28 C for *A. euteiches* and *P. m. medicaginis*, respectively. The reaction of alfalfa cultivars Vernal and Apollo II also was examined. Both cultivars are susceptible to *A. euteiches*, and Apollo II is resistant to *P. m. medicaginis*. Vernal reacted with higher disease ratings and greater area under a mortality progress curve than Apollo II when inoculated with either pathogen. However, the difference between cultivars was greatest against *P. m. medicaginis* at 16 and 20 C.

Cultivation of alfalfa (*Medicago sativa* L.) in slowly drained, wet soils often is restricted by pathogens that cause root disease. One such pathogen is *Phytophthora megasperma* Drechs. f. sp. *medicaginis* Kuan and Erwin (6,9,13,25). Resistant cultivars (8) and the fungicide metalaxyl (4,20) currently are used in strategies to protect alfalfa against this pathogen. However, these controls have not always provided satisfactory control of root rot in Wisconsin (10). Other factors may contribute to poor root health of alfalfa, including injury from flooding stress (1,29) and disease caused by additional pathogens (19,26).

Aphanomyces euteiches Drechs. also is reported to be a pathogen of alfalfa (5,26). The oomycete is known primarily for its destructiveness of pea (*Pisum sativum* L.) (12) and green bean (*Phaseolus vulgaris* L.) (23), but researchers who investigated Aphanomyces root rot of pea determined that alfalfa also was a host for *A. euteiches* (15,27). More recently, *A. euteiches* has been associated with seedling blight and poor seedling establishment of alfalfa (5,17) and has been recovered from field soils in which performance of alfalfa cultivars resistant to *P. m. medicaginis* was unsatisfactory (5). Unlike *P. m. medicaginis*, *A. euteiches* is insensitive to metalaxyl (22). *A. euteiches* also is distributed widely in North America, and the root disease it causes on pea is favored by wet soils (21). Thus, it is possible that *A. euteiches* could play a role in root disease of alfalfa grown in wet soils and may contribute to the unsatisfactory performance of cultivars resistant to *P. m. medicaginis*.

A direct comparison has not been made between the pathogenicity of *A. euteiches* on alfalfa and the pathogenicity of another economically important pathogen of alfalfa. Therefore, the objective of this study was to compare the ability of *A. euteiches* to cause disease of seedling alfalfa compared with *P. m. medicaginis* and to examine the effects of temperature, inoculum rate, and host cultivar on the reaction of alfalfa seedlings to each pathogen.

MATERIALS AND METHODS

A seedling assay developed by Irwin, Miller, and Maxwell (11) for studying postemergence resistance of alfalfa seedlings to *P. m. medicaginis* was modified for use with both pathogens. Our modifications included using the zoospores of each oomycete as inoculum, direct seeding the alfalfa to avoid wounding of roots, and providing a common water source for all treatments.

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Culture of alfalfa seedlings. Alfalfa seedlings were grown in plastic cavities (2.5 × 2.5 × 7 cm, Jiffy Corp., Chicago, IL). Two centimeters of vermiculite was placed in the bottom of each cavity and covered with a 3- to 4-cm layer of steam-pasteurized sand. The vermiculite prevented sand from sifting out through the drainage hole. A suction seed planter (Fig. 1A) was used to distribute 10 seeds per cavity, and seeds were covered with 1 cm of vermiculite. All seeds were inoculated with *Rhizobium meliloti*. Forty-eight cavities comprised a flat, and each flat was placed in a plastic box (10 × 20 × 28 cm). Tap water was added to each box to a level 5 cm from the top of the cavities (water potential estimated to be −4 millibars at the level of the seed), and the boxes were placed in a growth chamber. Fluorescent lights were positioned 20 cm from the top of the cavities (50 $\mu E\ m^{-2}\ sec^{-1}$). Photoperiod was a 16-hr day and 8-hr night, and the temperature was maintained at 24 C until seedlings were inoculated with zoospores.

Preparation of zoospores and inoculation. Methods reported by Mitchell and Yang (18) and Irwin, Miller, and Maxwell (11) were used to produce zoospores of *A. euteiches* and *P. m. medicaginis*, respectively. A 3-ml sample of a zoospore suspension was drawn in and forced out of a Pasteur pipette 10 times to encyst the zoospores before counting. The concentration of the initial zoospore suspension was estimated with the aid of a hemacytometer, then diluted with glass-distilled water to obtain zoospore concentrations.

Alfalfa seedlings were inoculated 4 days after planting (cotyledon stage) with zoospores. Flats were removed from boxes for inoculation and placed on shallow trays. Three milliliters of inoculum was dispensed onto the vermiculite surface with a Cornwall syringe (Becton-Dickinson, Rutherford, NJ) (Fig. 1B), and excess water was allowed to drain out after inoculation. Flats were returned to the boxes, and tap water was added 3–4 hr later to a level 5 cm from the top of the cavities. Thereafter, water was added to boxes every 24–48 hr to maintain a constant level.

Comparison of isolates of *A. euteiches*. To determine if alfalfa responded differently to various isolates of *A. euteiches*, seedlings of Vernal alfalfa were inoculated with isolates representative of several strains, including P-14 and Ae164. These isolates are typical of described formae specialis *A. e. pisi* Pfender & Hagedorn (23) (American Type Culture Collection [ATCC] 46690) and *A. e. phaseoli* Pfender & Hagedorn (collected by authors from Waushara County, WI). Other isolates tested were Ae349 recovered from alfalfa seedlings grown in a Barron County, WI, soil, AeNC1 recovered from alfalfa seedlings grown in a North Carolina soil, and isolate Ae572 from a red clover seedling grown in a Wood County, WI, soil and highly pathogenic to that host. Vernal alfalfa was grown as described above and inoculated with 500 zoospores of a single isolate per seedling. All combinations of isolate and host were grown together in a group of cavities. Isolates were completely randomized within a group and were replicated six times at constant temperatures of 16, 20, 24, or 28 C. The experiment was repeated once. Because most isolates of *A. euteiches* induced similar reactions in alfalfa (Fig. 2), isolate Ae349 was used as a representative isolate in the following comparison of pathogenicity.

Comparison of isolates of *A. euteiches* and *P. m. medicaginis*. Seedlings of Vernal and Apollo II alfalfa were grown as described above and inoculated 4 days after planting with 0, 10, 100, or 1,000 zoospores/seedling of either *A. euteiches* (Ae349) or *P. m. medicaginis* (isolate Pm2019 from Wood County, WI). Vernal and Apollo II alfalfa were selected because they differed in susceptibility to *P. m. medicaginis*, with 54 and <1% resistant plants, respectively (2). Neither cultivar is resistant to *A. euteiches*.

A split-plot design was used for this experiment within each temperature environment. Whole plots were split into two boxes, and all of the seedlings in one box were inoculated with the same pathogen. Subplot treatments were factorial combinations of inoculum rate and alfalfa cultivar. Each treatment combination consisted of a row of six cavities. The treatments were randomized within each box and were replicated three times at each temperature. The experiment was repeated once.

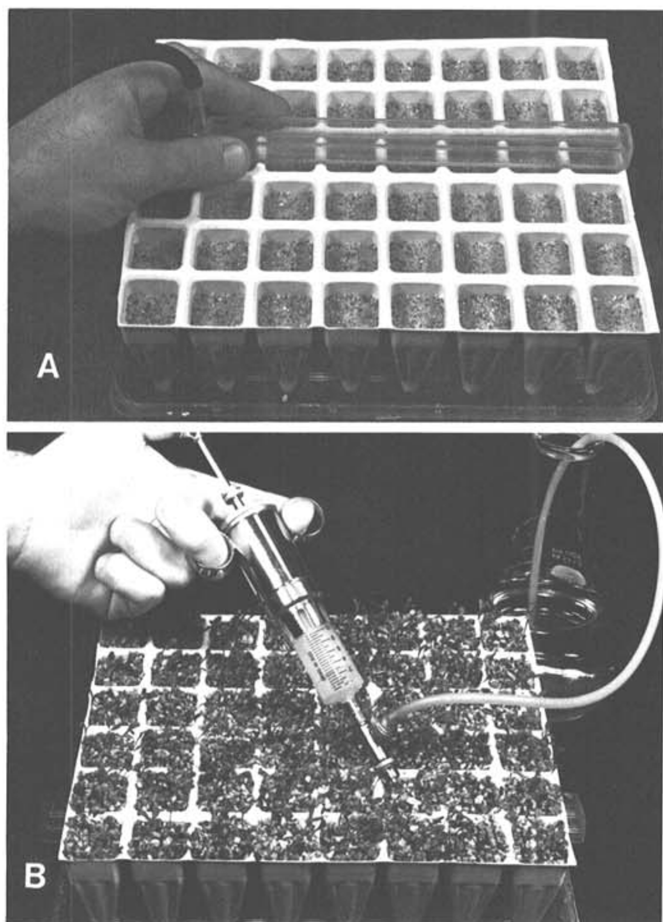

Fig. 1. Plastic flats with cavities and methods used in seedling assay for: **A,** planting seeds, and **B,** inoculating seedlings after emergence. Flats were divided into 48 cavities (2.5 × 2.5 × 7 cm, tapered to 1 cm at the bottom with a hole for drainage). A suction seed planter was used to place 10 alfalfa seeds per cavity, and a Cornwall syringe was used to dispense zoospore suspensions into each cavity (B).

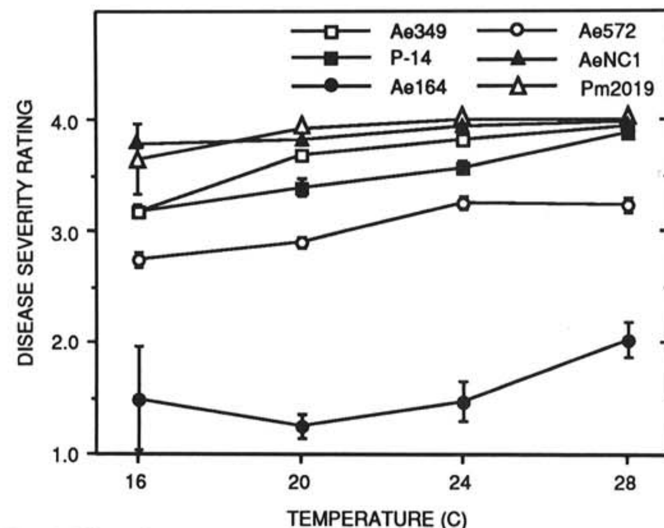

Fig. 2. Effect of temperature on seedling disease of Vernal alfalfa caused by several isolates of *Aphanomyces euteiches*, including Ae349, an isolate of *A. e. pisi* (P-14), an isolate of *A. e. phaseoli* (Ae164), an isolate recovered from a red clover seedling (Ae572), and AeNC1 recovered from a North Carolina soil. Isolate Pm2019 of *Phytophthora megasperma* f. sp. *medicaginis* was included for comparison. Figure shows mean disease severity ratings at 10 days after inoculation with 500 zoospores/seedling. Each point represents a mean of approximately 120 inoculated seedlings. The standard error of a mean is shown with each bar.

Seedling survival was recorded on the day of inoculation and 3, 6, and 10 days later. On the 10th day after inoculation, seedlings were rated for disease severity on a scale of 1 to 4, with 1 = no symptoms, 2 = major areas of discoloration on >5% of roots but hypocotyl and cotyledons were symptomless, 3 = extensive discoloration of roots and hypocotyl, and chlorosis or necrosis of cotyledons, and 4 = dead seedling. Disease incidence (%), mean disease severity rating, and area under mortality progress curves were used to compare seedling response to each pathogen at each temperature. Error variances from separate analyses of variance of data at individual temperatures were homogeneous; therefore, data were combined for overall analyses within temperatures. Variances were not homogeneous among temperatures. The percentage of plants in disease classes 1 and 2 is presented for those interested in a disease measurement that estimates the percentage of resistant plants.

RESULTS

The isolates of both *A. euteiches* and *P. m. medicaginis* caused indistinguishable symptoms on alfalfa seedlings. The earliest indication of disease was chlorosis of cotyledons, followed by necrosis progressing up the hypocotyl and into the cotyledons until the seedlings eventually collapsed. Both oomycetes caused root decay characterized by a generalized brown discoloration of lateral and tap roots.

Disease incidence. Disease incidence was nearly 100% for *A. euteiches* at all three inoculum rates at temperatures from 16 to 28 C (Fig. 3A). At the same temperatures, disease incidence caused by *P. m. medicaginis* was nearly 100% only at the two highest inoculum rates (Fig. 3B). Incidence was significantly less at 10 zoospores/seedling and was influenced by temperature such that *P. m. medicaginis* was less effective at 24 C than at 16, 20, or 28 C. Disease incidence was suppressed at 32 C for both pathogens.

The alfalfa cultivars differed in reaction to each pathogen with respect to disease incidence. Vernal and Apollo II expressed equivalent disease incidence when inoculated with *A. euteiches* (Fig. 4A), whereas incidence was slightly, but significantly, less for Apollo II at 16 and 20 C when inoculated with *P. m. medicaginis*. The interaction between inoculum rate and alfalfa cultivar was insignificant ($P = 0.05$) for *A. euteiches* at each temperature. However, this interaction was significant for *P. m. medicaginis* at 16 and 20 C. Under these conditions, the lowest percent incidence of disease on Vernal was 85% at 10 zoospores/seedling (Table 1), whereas percent incidence on Apollo II was <62% at the same inoculum rate.

Disease severity rating. Disease severity on alfalfa caused by *A. euteiches* was affected by inoculum rate and temperature (Fig. 3C). Lowest disease severity ratings occurred at 16 and 32 C at 10 zoospores/seedling, and severity increased stepwise with greater inoculum rates and increasing temperatures from 20 to 28 C. Maximum severity was observed at 28 C. These results contrast with incidence as a measure of disease because neither inoculum rate nor temperatures from 16 to 28 C affected disease incidence caused by *A. euteiches* (Fig. 3A). For *P. m. medicaginis*, the trends observed with disease severity ratings were similar to trends observed with percent incidence (Fig. 3B and D).

Vernal expressed more severe symptoms than Apollo II when inoculated with either pathogen (Fig. 4B). *A. euteiches* was more virulent on Vernal than on Apollo II at 16, 20, and 24 C, but this difference was less than the difference observed for the reaction of cultivars to *P. m. medicaginis* at 16 and 20 C. A significant interaction ($P = 0.05$) between inoculum rate of *P. m. medicaginis* and cultivars was observed at 16 and 20 C (Table 1). The reaction of each cultivar to the pathogens, with the percent seedlings in disease classes 1–2 used to measure disease (Fig 4C), was similar to trends observed when disease severity ratings were used (Fig. 4B).

Progress of seedling mortality. The influence of temperature on disease potential of each pathogen was most evident when the progress of seedling mortality was used for measuring disease. Seedlings inoculated with *P. m. medicaginis* usually began expressing symptoms within 3 days after inoculation when incubated at 16–28 C, and most plants collapsed and died 1–3 days later (Fig. 5). This was especially true for Vernal seedlings. By contrast, *A. euteiches* caused symptoms that were expressed more gradually when inoculated seedlings were incubated at 16–20 C. At these temperatures, symptoms usually began to appear 4–5 days after inoculation, and most symptomatic seedlings eventually collapsed 2–4 days later. However, some seedlings remained upright even after death (that is, standing corpses), indicating that the structural integrity of the hypocotyl was insufficiently decayed to cause seedling collapse. At 24–28 C, seedlings inoculated with *A. euteiches* died rapidly in a manner similar to those infected by *P. m. medicaginis*.

Comparisons were simplified by calculating an area under a mortality progress curve for each combination of pathogen, cultivar, and temperature (Fig. 4C). The influences of temperature, inoculum rate, and cultivars that were observed when disease severity was used were even more pronounced when the areas under the mortality progress curves were used. For example, the change in disease caused by *A. euteiches* from 16 to 28 C (Fig. 4B) was greater when the areas under the mortality progress curves were used than when severity ratings were used (Fig. 4D).

DISCUSSION

Maximum disease incidence and severity caused by *P. m. medicaginis* occurred at temperatures from 16 to 20 and 28 C

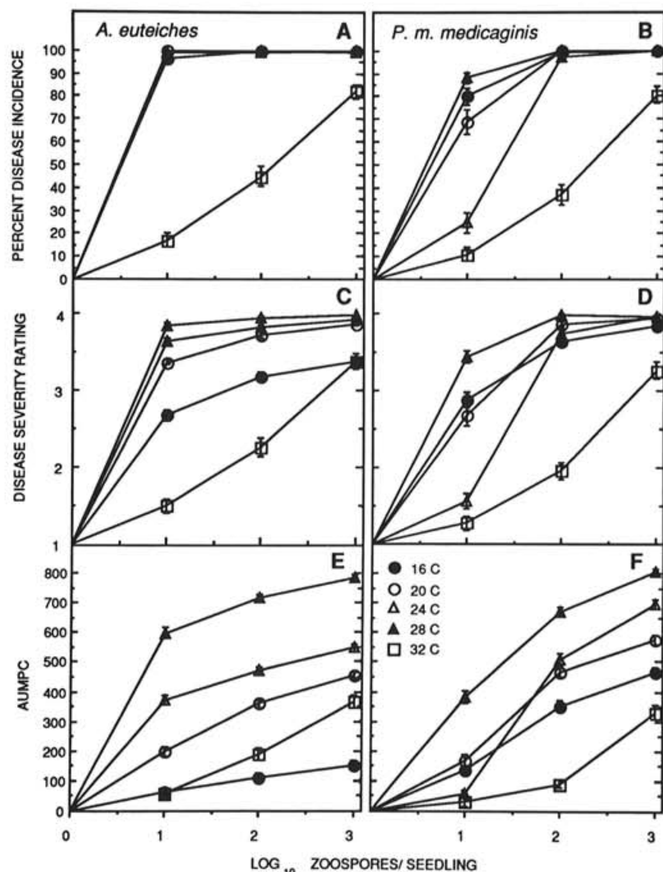

Fig. 3. Disease reaction between alfalfa seedlings and *Aphanomyces euteiches* (**A,C,E**) or *Phytophthora megasperma* f. sp. *medicaginis* (**B,D,F**) as affected by temperature and inoculum rate. The reaction was quantified by percent disease incidence (A and B) and mean disease severity rating (C and D) on the 10th day after inoculation, and also by the area under mortality progress curves (AUMPC) (E and F). Each point represents a mean of approximately 720 seedlings from two alfalfa cultivars (Apollo II and Vernal). The standard error of a mean is shown with each point. Statistically significant interactions ($P = 0.05$) were observed between inoculum rate of *P. m. medicaginis* and alfalfa cultivar at 16 and 20 C.

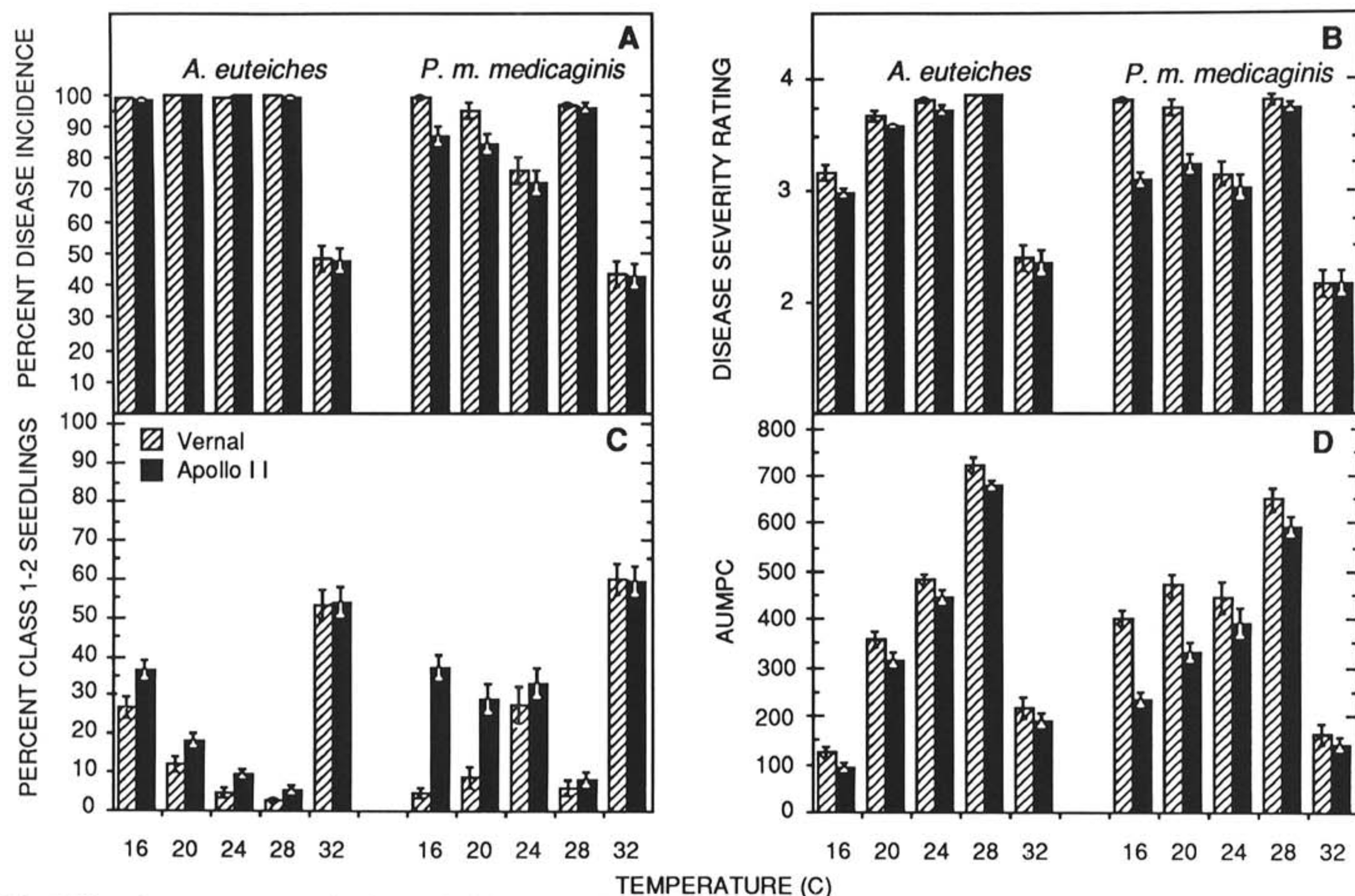

Fig. 4. Effect of temperature on seedling disease of alfalfa cultivars Vernal and Apollo II caused by *Aphanomyces euteiches* or *Phytophthora megasperma* f. sp. *medicaginis*. **A,** Percent incidence of symptomatic seedlings 10 days after inoculation. **B,** Mean disease severity ratings 10 days after inoculation. **C,** Percentage of seedlings in disease classes 1 and 2 10 days after inoculation. **D,** Area under mortality progress curves (AUMPC). Each bar represents a mean of approximately 1,000 inoculated seedlings that was averaged over all inoculum rates (10, 100, and 1,000 zoospores/seedling). The standard error of a mean is shown with each bar. Statistically significant interactions ($P = 0.05$) were observed between the inoculum rate of *P. m. medicaginis* and the alfalfa cultivar at 16 and 20 C.

on Vernal, the cultivar susceptible to this pathogen (Fig. 4A and B). Erwin (7) and Wilkinson and Millar (30) also studied the effect of temperature on Phytophthora root rot of alfalfa and found that disease severity was not affected by temperature from 17 to 27 C but that disease suppression did occur above 30 C. In the current study, however, disease incidence and severity declined at 24 C. The cause of this reduction was not determined, but the same trend was observed when the experiment was repeated. A likely explanation would be the sensitivity of Pm2019 to environmental conditions and may not be characteristic of the species.

A. euteiches responded differently to temperature than did *P. m. medicaginis*. Maximum severity and progress of mortality caused by *A. euteiches* was observed at 28 C (Fig. 4B and D). No previous research has been reported for the effect of temperature on root disease of alfalfa caused by *A. euteiches*, but the current results agree with those reported for Aphanomyces root rot of pea (3,16). Smith and Walker (28) also reported maximum severity of Aphanomyces root rot of pea at 24–28 C. These results suggest that the greatest loss from seedling disease caused by *A. euteiches* could occur if alfalfa is planted after the soil has reached temperatures greater than 20 C. However, the potential for disease caused by *A. euteiches* also could be important in cooler soil because the incidence of disease caused by *A. euteiches* was not affected by temperatures from 16 to 28 C. For example, infection of alfalfa that occurs in soil cooler than 20 C may not be apparent under field conditions, but a subsequent increase in soil temperature eventually may lead to more severe expression of disease. Hence, the relationship between temperature and the progress of disease beyond the seedling stage of alfalfa should be considered in future research.

TABLE 1. Means for percent disease incidence (DI), disease severity rating (DSR), and area under mortality progress curves (AUMPC) that involved significant interactions between alfalfa cultivar and inoculum rate (zoospores per seedling) of *Phytophthora megasperma* f. sp. *medicaginis* at 16 and 20 C

Temperature (C)	Cultivar	Inoculum rate	Measurement of disease[z]		
			DI	DSR	AUMPC
16	Vernal	10	98 a	3.5 c	197 e
		100	100 a	4.0 a	461 b
		1,000	100 a	4.0 a	552 a
	Apollo II	10	62 b	2.3 d	72 f
		100	99 a	3.3 c	244 d
		1,000	100 a	3.7 b	379 c
20	Vernal	10	85 B	3.2 B	231 D
		100	100 A	4.0 A	547 B
		1,000	100 A	4.0 A	645 A
	Apollo II	10	53 C	2.1 C	105 E
		100	100 A	3.7 A	388 C
		1,000	100 A	3.9 A	507 B

[z] Means within a column and temperature followed by a different letter were significantly different ($P = 0.05$) according to Fisher's protected least significant difference (LSD) test. Means were calculated from the reactions of approximately 360 seedlings. LSD values for DI, DSR, and AUMPC were 8, 0.2, and 37 at 16 C, respectively, and 10, 0.3, and 54 at 20 C, respectively.

Because disease caused by *A. euteiches* and *P. m. medicaginis* was only distinguishable by differences in their response to temperature, it would be difficult to diagnose visually which pathogen was responsible for disease in the field, except, perhaps,

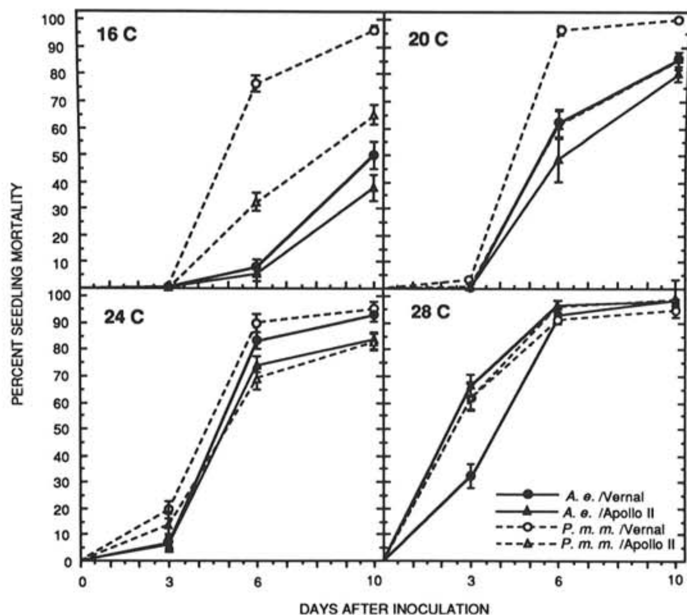

Fig. 5. Effect of temperature on the progress of seedling mortality over a 10-day period. Seedlings of alfalfa cultivars Vernal and Apollo II were inoculated with zoospores of *Aphanomyces euteiches* or *Phytophthora megasperma* f. sp. *medicaginis*. Each point represents a mean of approximately 1,000 inoculated seedlings that was averaged over all inoculum rates (10, 100, and 1,000 zoospores/seedling). The standard error of a mean is shown with each bar. Statistically significant interactions ($P = 0.05$) were observed between the inoculum rate of *P. m. medicaginis* and the alfalfa cultivar at 16 and 20 C.

by the common occurrence of standing corpses with disease caused by *A. euteiches*. However, this difference occurred only at 16–24 C under controlled conditions and would be difficult to observe in the field. These results differ from the common description of Phytophthora root rot on older plants as a disease that can be diagnosed visually through characteristic symptoms on roots (6,8).

Our study did not examine the possibility of a disease complex between the two pathogens on alfalfa. However, *A. euteiches* interacts synergistically with *Pythium* spp. to cause severe root rot of green bean (24). For this reason, *A. euteiches* may be more effective than expected, regardless of temperature, if a combination of these oomycetes acts synergistically on alfalfa. Such a complex might reduce the agronomic performance of alfalfa cultivars resistant to *P. m. medicaginis*. A disease complex of alfalfa involving both oomycetes should be investigated.

Further research is needed to evaluate the influence that environmental conditions have on the susceptibility of alfalfa to root pathogens. Kuan and Erwin (14) suggested that flooding of soil could modify the expression of resistance to *P. m. medicaginis*. Temperature also may condition such resistance. For example, Apollo II and Vernal reacted equivalently to *P. m. medicaginis* at 24 and 28 C when disease severity caused by each pathogen was compared (Fig. 4B). It was not possible to determine whether the similarity between the cultivars at higher temperatures was due to a modification of resistance in Apollo II or susceptibility in Vernal, or both. It seems noteworthy, however, that Apollo II was consistently less diseased than Vernal after inoculation with *A. euteiches* (Fig. 5). Neither cultivar has been bred intentionally for resistance to *A. euteiches*, but a low level of resistance to *A. euteiches* may occur in Apollo II.

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