Potential for Seedling Disease of Alfalfa Caused by *Aphanomyces euteiches* in a Kentucky Soil

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ABSTRACT

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Studies were initiated to investigate the potential for seedling disease of alfalfa under controlled conditions in a Kentucky soil naturally infested with *Aphanomyces euteiches*. Germinated seeds of 21 alfalfa cultivars or experimental lines were planted into pots containing a Heitt silty clay loam and placed in a growth chamber at 24 C for 3 wk. Pots were irrigated with either a 5-μg/ml metalaxyl solution (to control *Pythium* and *Phytophthora* spp.) or a solution of 5 μg/ml of metalaxyl + 10 μg/ml of hymexazol (to control *A. euteiches* also). In pots irrigated with metalaxyl alone, plants of cultivars susceptible to *A. euteiches* generally were killed or severely stunted. When *A. euteiches* was controlled with hymexazol, root rot was nearly eliminated, and dry weight and nodulation were greatly increased in all cultivars and experimental lines. The dry weight of seedlings treated with metalaxyl alone increased significantly with increasing resistance to *A. euteiches*. Given the disease potential observed in this study and the widespread distribution of the pathogen in the state, *A. euteiches* may be an important cause of seedling disease of alfalfa in Kentucky.

Additional keywords: *Medicago sativa, Phytophthora medicaginis, Rhizobium meliloti*, root health

The planting of alfalfa (*Medicago sativa* L.) in Kentucky has increased greatly in recent years. The expanding interest in this forage crop has fostered a greater need to identify constraints to alfalfa production in the state.

A variety of biotic and abiotic factors can interfere with the establishment of a uniform, dense stand of alfalfa seedlings. In Kentucky, failures in stand establishment are often associated with soils that are poorly drained. This condition may be due to poor surface drainage or to slow percolation resulting from a high clay content or soil compaction.

The role of *Pythium* spp. and *Phytophthora medicaginis* Hansen & Maxwell (= *Phytophthora megasperma* Drechs. f. sp. *medicaginis* T. Kuan & D. C. Erwin) (9) in causing seedling disease of alfalfa in poorly drained soils is well established (6,8,12,16,18). Recent research has shed light on the importance of the distantly related oomycete *Aphanomyces euteiches* Drechs. in causing seedling disease of alfalfa in some slowly drained soils in northern North America and Australia (1,2,13,15). Similar studies have not been conducted to date in southern areas of the United States.

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In an ongoing survey, *A. euteiches* has been detected in soils from 34 counties in Kentucky to date (P. C. Vincelli and W. C. Nesmith, *unpublished*). Given the apparent prevalence of *A. euteiches* in Kentucky, studies have been initiated to investigate the importance of this pathogen to alfalfa production in the state. The objectives of the present study were to develop a method for measuring seedling disease potential of *A. euteiches* under controlled conditions, to evaluate the potential for seedling disease due to *A. euteiches* in a naturally infested soil from Kentucky, and to evaluate the response of host resistance to the pathogen in a naturally infested soil from Kentucky under controlled conditions.

MATERIALS AND METHODS

Soil. A Heitt silty clay loam (pH 6.9) naturally infested with *A. euteiches* was collected in September 1990 from a field in Harrison County, Kentucky. The field was planted to an alfalfa-grass mixture. Soil was collected to a depth of 20 cm and stored at 16% moisture in a galvanized can at 5 C until use within 6 mo. The soil was screened (with 3-mm openings), mixed thoroughly, and allowed to equilibrate at room temperature prior to use. For experiments requiring autoclaved soil, 4-kg quantities of soil were autoclaved at 121 C for 1 hr on each of two consecutive days, then stored for 2 wk on a laboratory bench.

Toxicity of hymexazol to *A. euteiches*. The fungicide hymexazol has shown activity against *A. euteiches* in recent field tests (C. Grau, *personal communication*). A test was conducted to confirm the toxicity of hymexazol to local isolates of the pathogen and to determine an inhibitory concentration for subsequent experiments.

Naturally infested soil was air-dried on a laboratory bench for 2 days, and 15 g of soil was dispensed into 10-cm polystyrene petri dishes. The soil was moistened by adding one of five solutions dropwise (four replicate plates per solution). Five solutions were used: distilled water, 5 μg/ml of metalaxyl, 5 μg/ml of metalaxyl + 1 μg/ml of hymexazol, 5 μg/ml of metalaxyl + 10 μg/ml of hymexazol, and 5 μg/ml of metalaxyl + 100 μg/ml of hymexazol. Metalaxyl and hymexazol solutions were prepared with appropriate concentrations of Ridomil 2E and Tachigaren 30% EC, respectively, in distilled water.

Three days after moistening, the soil was flooded by adding 25 ml of the corresponding solution to each dish. At that time, two 7-day-old alfalfa seedlings (cultivar Arc) were floated in each dish. Seedlings had been grown in autoclaved perlite at room temperature under a fluorescent light. Four days after flooding, seedlings were scored for decay at 10×, using the following scale: 0 = no decay, 1 = 1–25% of seedling tissue decayed, 2 = 26–75% decay, and 3 = >75% decay. Each seedling exhibiting decay was examined at 100–400× to identify fungi sporulating on infected tissues. In initial analyses, linear models of the relationship between hymexazol concentration and seedling decay had poor fits (nonrandom residuals, low R^2). The data were best fitted by a nonlinear model (14) of the form $Y = ae^{bX}$, where Y = decay rating and X = μg/ml of hymexazol.

Effect of metalaxyl and hymexazol on growth of alfalfa. Experiments were conducted to test whether metalaxyl or hymexazol exhibited any growth-regulating activity independent of disease control. Seeds of three alfalfa cultivars (Asset, WL-320, and A9008) were scarified, inoculated with *Rhizobium meliloti* Dangeard, and incubated at room temperature in moistened paper towels for 48 hr. Autoclaved soil was added to polystyrene greenhouse sheet pots (150 g of soil per pot at 16% moisture, with pots measuring 57 mm square and 70 mm deep). Ten germinated seeds of each cultivar were transferred to pots.

Pots were placed in 17- × 25-cm baking pans, put into a growth chamber (24 ± 1 C, 17-hr photoperiod at 160 μE

m^{-2} sec^{-1}), and irrigated with 5 μg/ml of metalaxyl, 10 μg/ml of hymexazol, or unamended distilled water. At each irrigation, solutions were added to baking pans to within 2 cm of the soil surface (approximately −0.2 MPa), and all pots were maintained near saturation for 5 days before being allowed to drain for 2 days. There were four replicate pots per cultivar × irrigation treatment combination.

After 3 wk, plants were harvested by gently washing soil from roots, drying them at 68 C for 4 days, and weighing them. Data were subjected to an analysis of variance (ANOVA) following a factorial treatment structure (17). The experiment was performed twice.

Root health and growth of seedlings in soil infested with *A. euteiches*. Seeds of 21 alfalfa cultivars and experimental lines, varying in resistance to *A. euteiches*, were germinated as above and transferred to pots containing naturally infested soil (five and 10 germinated seedlings per pot in first and second trials, respectively). Pots (four per treatment) were incubated and irrigated as above with either a solution of 5 μg/ml of metalaxyl or 5 μg/ml of metalaxyl + 10 μg/ml of hymexazol. In preliminary experiments, levels of seedling disease were equal in pots irrigated with distilled water or a 5-μg/ml metalaxyl solution, and only *A. euteiches* was associated with diseased tissues in both treatments. Although other oomycetes were not detected in preliminary tests, hymexazol has activity against *Pythium* spp. and *P. medicaginis* as well as against *A. euteiches*. To avoid possible confounding effects of *Pythium* spp. and *P. medicaginis*, metalaxyl was added to all pots to suppress possible activity of other oomycetes. This was considered to be especially appropriate, since metalaxyl is a common seed treatment fungicide for alfalfa. At each irrigation, all pots were maintained near saturation for 4–5 days before being allowed to drain for 2 days. Four days after transplanting, emerged seedlings were counted in each pot.

Symptomatic root segments were collected at random from dying plants during and at the conclusion of experiments. Tissue segments were washed with running tap water and placed in individual petri dishes containing 20–25 ml of a 5-μg/ml metalaxyl solution, along with two or three healthy 4- to 10-day-old Arc seedlings as a bait. The original symptomatic tissues and bait seedlings that developed decay were checked regularly at 100–400× for fungal sporulation.

Plants were harvested three weeks after transplanting. Soil was removed by gently washing roots, and roots were rated for decay on a scale of 1 (no symptoms) to 5 (dead plant) (10). Root nodules were also counted. Harvested plants were dried at 65 C for 48 hr and weighed. The experiment was performed twice.

Since pots were the experimental unit, root rot ratings and numbers of nodules were averaged for each pot; dry weights were summed for each pot. Preliminary analyses indicated that a $\log_{10}$ transformation was necessary to meet the ANOVA assumption of equality of variances (17) for both root rot ratings and numbers of nodules. Emergence of plants in some pots was reduced in the second trial; this was thought to be due to injury during transplanting. In order to correct for this effect, the dry weight data were analyzed using an analysis of covariance (covariate = number of seedlings emerged 4 days after transplanting), after checking the assumption of a common covariate slope among cultivars (17). Paired *t* tests and linear regression were used to test hypotheses (17).

RESULTS

Toxicity of hymexazol to *A. euteiches*. Four days after flooding, seedlings were highly decayed in dishes treated with distilled water and metalaxyl (with mean decay ratings of 2.6 and 3.0, respectively). Hymexazol was somewhat inhibitory to *A. euteiches* at 1 μg/ml and was highly inhibitory at 10 and 100 μg/ml (Fig. 1). *A. euteiches* was detected in all symptomatic tissues; no other oomycetous fungi were detected in any seedlings, including those floated in distilled water.

Effect of metalaxyl and hymexazol on growth of alfalfa. Neither metalaxyl nor hymexazol affected growth of alfalfa seedlings at rates used in these experiments (Table 1). In the ANOVA, effects for irrigation treatment, cultivar, and irrigation treatment × cultivar were nonsignificant ($P > 0.10$).

Root health and growth of seedlings in soil infested with *A. euteiches*. In pots treated with metalaxyl alone, plants of cultivars susceptible to *A. euteiches* generally were killed or severely stunted. Roots of such plants generally were decayed, and nodule numbers were low (Table 2). Data on the incidence of resistant plants (with root rot ratings of 1 or 2) in pots treated with metalaxyl alone were pooled for cultivars having the same resistance rating to *A. euteiches*. Recognized resistance classes based on standardized tests (4) are: susceptible (S), with 0–5% resistant plants; low resistance (LR), with 6–14% resistance; moderately resistant (MR), with 15–30% resistance; resistant (R), with 31–50% resistance; and highly resistant (HR), with over 50% resistance. There were seven, three, five, five, and one entries representing the S, LR, MR, R, and HR resistance classes, respectively. Resistance ratings for the cultivars or experimental lines used in these tests were supplied by commercial breeders and university scientists. In cultivars having reported resistance ratings to Aphanomyces root rot of S, LR, MR, R, and HR, the proportion of resistant plants observed in these experiments was 0.06, 0.09, 0.28, 0.37, and 0.24, respectively. In all cultivars, root rot was nearly eliminated, and substantial increases in nodulation were observed ($P < 0.01$), when *A. euteiches* was controlled with hymexazol. There was a significant ($P < 0.01$) association between increasing root rot and decreasing nodulation (Fig. 2).

Dry weight of seedlings was increased ($P < 0.05$) in all cultivars and experimental lines when *A. euteiches* was controlled with hymexazol; a twofold increase or more was observed in most entries. Dry weights were pooled for entries having the same resistance rating to *A. euteiches*. In pots treated with metalaxyl alone, dry weight generally increased with increasing levels of resistance (Fig. 3). The one exception to this trend occurred in the HR category, although this category was represented by only one experimental line (Table 2). Dry weights were high in hymexazol-treated pots and generally were unaffected by the level of resistance to Aphano-

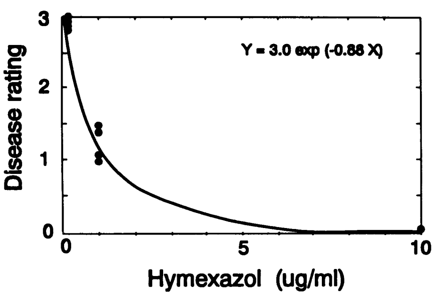

Fig. 1. Concentration of hymexazol inhibitory to *Aphanomyces euteiches*. Mean disease rating for 100 μg/ml of hymexazol = 0 (not depicted). Y = disease rating; X = concentration of hymexazol (μg/ml). Model statistics: $P < 0.01$, $R^2 = 0.99$.

Table 1. Effect of metalaxyl and hymexazol on growth of alfalfa seedlings of three cultivars in autoclaved soil

	Dry weight of seedlings (mg/pot)		
	Cultivar		
Treatment	Asset	WL-320	A9008
Distilled water	169[a]	127	160
Metalaxyl, 5 μg/ml	135	139	174
Hymexazol, 10 μg/ml	142	151	157

[a]ANOVA effects for treatment, cultivar, and treatment × cultivar were nonsignificant ($P > 0.10$).

Table 2. Effect of controlling *Aphanomyces euteiches* with hymexazol on root rot, nodulation, and dry weight of 21 alfalfa cultivars and experimental lines

Entry	Reported level of *Aphanomyces* resistance[a]	Root rot rating[b,c]		Nodules per plant[c]		Dry weight per pot (mg)[d]	
		Metalaxyl	Metalaxyl + hymexazol	Metalaxyl	Metalaxyl + hymexazol	Metalaxyl	Metalaxyl + hymexazol
Agate	S	4.2	1.4**[e]	0.9	7.1**	37	179**
Apollo Supreme	S	4.0	1.3**	0.6	8.4**	54	165**
Arrow	S	4.3	1.5**	0.7	5.0**	19	148**
Dart	S	3.9	1.2**	0.8	8.2**	46	119**
Resistar	S	3.8	1.6**	1.0	4.7**	48	157**
Trident	S	4.3	1.3**	0.8	7.3**	36	131**
WL-320	S	4.3	1.2**	0.6	5.8**	35	159**
Apollo II	LR	4.0	1.3**	1.1	6.7**	46	134**
Magnum III	LR	4.0	1.4**	0.3	7.3**	47	187**
Target II	LR	3.9	1.4**	1.0	7.4**	54	129*
Asset	MR	3.3	1.4**	1.7	5.9**	95	188**
Cimarron VR	MR	3.6	1.4**	1.0	8.1**	73	157**
A8925	MR	4.1	1.7**	0.7	5.9**	41	140**
DS801	MR	2.7	1.4**	2.7	7.5**	98	161**
XAE92	MR	3.7	1.2**	0.5	7.3**	47	146**
ABI9033	R	3.4	1.3**	1.1	7.6**	65	126**
ABI9043	R	3.0	1.3**	1.5	6.0**	94	188**
A9008	R	3.1	1.4**	3.4	7.5**	75	176**
89-30	R	3.1	1.5**	1.5	6.3**	89	146*
89-31	R	3.3	1.4**	1.2	6.8**	68	149**
XAM93	HR	3.3	1.4**	1.1	6.7**	66	144**

[a] S = susceptible; LR = low resistance; MR = moderately resistant; R = resistant; HR = highly resistant.
[b] On a scale of 1–5, with 1 = healthy roots and 5 = dead plant (10).
[c] Statistics presented are based on $\log_{10}$-transformed data (to equalize variances). Arithmetic means are presented for ease of interpretation.
[d] Least-square means are presented based on an analysis of covariance.
[e] Asterisks indicate that the mean is significantly different from the mean of plants treated with metalaxyl alone (paired t tests; * = $P \leq 0.05$, ** = $P \leq 0.01$).

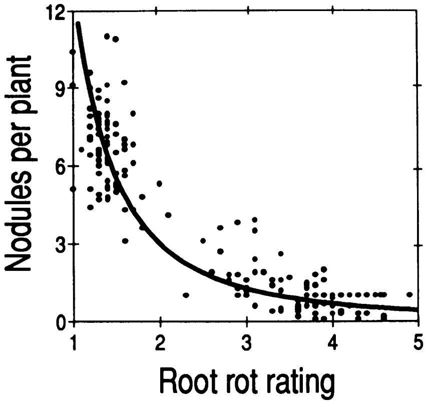

Fig. 2. Effect of Aphanomyces root rot on nodulation of alfalfa seedlings. Data were fitted to a linear model of the form $Y = 13.0 \log x - 2.11$ ($P < 0.01$, $R^2 = 0.88$).

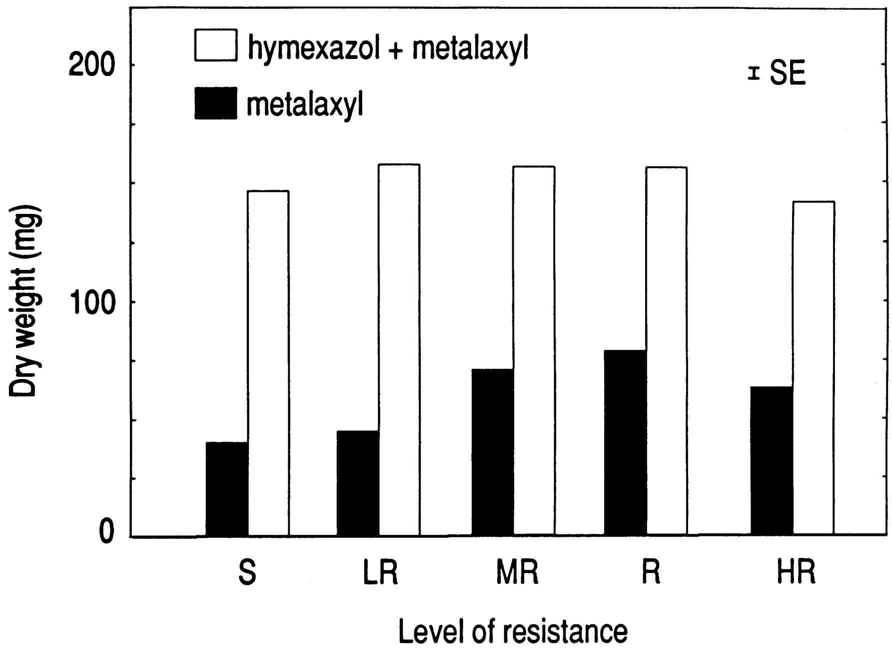

Fig. 3. Effect of *Aphanomyces* resistance and hymexazol on dry weight of alfalfa seedlings. Data represent mean dry weight of seedlings (milligrams per pot), pooled for all cultivars in each resistance class. Resistance classes are: S (susceptible), LR (low resistance), MR (moderately resistant), R (resistant), and HR (highly resistant). The standard error (P = 0.05) for the experiment is indicated in the upper right hand corner.

myces root rot. The lowest dry weight in hymexazol-treated pots occurred in the HR category.

A. euteiches was detected consistently (in 66 of 68 tissue segments) sporulating either directly from symptomatic test seedlings or from bait seedlings. Limited growth of *Rhizoctonia solani* Kühn was detected on a few (<5%) seedlings, which were highly colonized by *A. euteiches*. No other fungi were detected in the assay.

DISCUSSION

A. euteiches caused substantial deterioration of root health and growth of alfalfa seedlings grown under controlled conditions in a naturally infested soil from Kentucky. In cultivars with no known resistance to this pathogen, roots were decayed, and nodulation and seedling dry weight were severely reduced. Use of hymexazol to control *A. euteiches* resulted in improved seedling health and growth, as did the use of host resistance to *A. euteiches*.

Although improvements in seedling health from host resistance to *A. euteiches* were significant, a more dramatic response was observed with hymexazol. Significant improvements in seedling health by using hymexazol were observed even in cultivars having some resistance to *A. euteiches*, although the magnitude of the response was less than in susceptible cultivars. These observations are consistent with the hypothesis that *A. euteiches* was the sole cause of poor seedling health in pots treated with metalaxyl alone. As an autotetraploid, the inheritance of disease resistance in alfalfa is complex, and alfalfa cultivars with pest resistance are a mixed population of resistant and susceptible plants (3). In the experiments reported here, hymexazol suppressed infections by *A. euteiches* in all plants, regardless of the level of resistance. Consequently, all seedlings treated with hymexazol—susceptible seedlings as well as resistant—were relatively free of disease. The resistant cultivars tested here were all composed of a significant proportion of susceptible plants, with most reported to have less than 50% resistant plants (R rating or lower). Consequently, in pots treated with metalaxyl alone, diseased seedlings infected with *A. euteiches* were observed even in resistant cultivars. This resulted in higher mean levels of seedling disease in all pots not treated with hymexazol, as compared to pots treated with it. The consistent isolation of only *A. euteiches* from symptomatic tissues throughout these experiments provides strong evidence that this pathogen alone caused the symptoms observed.

Seedling dry weight increased with increasing resistance to *A. euteiches* in pots treated with metalaxyl alone. The only exception to this trend was observed in the HR class, in which dry weight declined slightly, as compared to the R class (Fig. 3). However, the HR class was represented by only one experimental line, XAM93, which produced a low dry weight under the conditions of these experiments even when *A. euteiches* was controlled with hymexazol. A decline in dry weight in the HR class might not have been observed, had a larger selection of cultivars with an HR rating been available at the time these experiments were conducted. The trends of decreasing root rot and increasing seedling dry weight with increasing resistance to *A. euteiches* in pots treated with metalaxyl alone suggest that improved vigor of alfalfa seedlings may be observed where *Aphanomyces*-resistant cultivars are grown in soils in Kentucky naturally infested with this pathogen. Experiments are necessary to determine whether the use of *Aphanomyces*-resistant alfalfa cultivars under field conditions will result in improvements in stand establishment and seedling health in Kentucky.

The experiments reported here confirm that host resistance in alfalfa to *P. medicaginis* does not confer resistance to the distantly related oomycete *A. euteiches* (10). All of the commercial cultivars tested were either resistant or highly resistant to Phytophthora root rot, yet those without resistance to *A. euteiches* were highly susceptible to this pathogen.

Recent studies have revealed an unexpectedly great amount of pathogenic variability among isolates of *A. euteiches*. Isolates have been found that can cause severe disease in an alfalfa population selected for resistance to Aphanomyces root rot (5). Such isolates have been detected in soils from numerous states, even though alfalfa cultivars resistant to *A. euteiches* reportedly have never been grown in those soils. These naturally occurring, new pathotypes of *A. euteiches* may represent a long-term threat to current genotypes resistant to Aphanomyces root rot. Clearly, studies are needed to determine whether deployment of currently available *Aphanomyces*-resistant cultivars will result in selection for isolates of *A. euteiches* capable of overcoming those resistance genes.

The use of hymexazol and metalaxyl in these studies provided selective tools for testing the effects of *A. euteiches* separately from those of *Pythium* spp. and *P. medicaginis*. The initial laboratory experiments confirmed two observations made by other investigators: that the fungicide hymexazol is active against *A. euteiches* (C. Grau, *personal communication*), and that metalaxyl has no appreciable activity against *A. euteiches* at normal use rates (11; C. Grau, *personal communication*). The use of metalaxyl in these experiments suppressed activity of *P. medicaginis* and *Pythium* spp. without supressing *A. euteiches*. Although some *Pythium* spp. are relatively insensitive to metalaxyl, none was detected in this study. Comparison of plants treated with metalaxyl + hymexazol to plants treated with metalaxyl alone permitted a direct assessment of the effects of *A. euteiches* without confounding effects from other oomycetes. No growth-regulating effect was detected for either fungicide at the rates used in these studies, providing further evidence that improved growth in hymexazol-treated plants was due to control of seedling disease caused by *A. euteiches*.

Although treatment of alfalfa seed with metalaxyl is becoming a common practice, evidence collected to date indicates that metalaxyl is not active against *A. euteiches*. The activity of hymexazol against *A. euteiches* suggests that this fungicide may have promise for use as a seed treatment against *Aphanomyces*-induced seedling diseases of alfalfa.

There was a statistical association in these studies between increasing root rot caused by *A. euteiches* and decreasing nodulation. This suggests that the impact of this pathogen on the growth of alfalfa may include interference with nitrogen fixation. *P. medicaginis* has also been found to cause a reduction in nodulation of alfalfa seedlings (7). Soilborne fungal pathogens could reduce nodulation by several mechanisms. In causing root rot, they may simply reduce the amount of root tissue available for nodulation. Conversely, they may act directly on nodulation sites to suppress nodule formation or to destroy nodules after formation (7). In this study, even low levels of root rot were associated with greatly decreased nodulation (Fig. 2), which suggests that a lack of available root tissue was not an important mechanism in these experiments. Studies to determine how *P. medicaginis* and *A. euteiches* reduce nodulation would be of interest.

To date, *A. euteiches* has been found in soils from 34 counties in Kentucky in an ongoing survey (P. C. Vincelli and W. C. Nesmith, *unpublished*). Given its widespread occurrence and the disease potential observed in this study, *A. euteiches* may be an important cause of seedling disease of alfalfa in slowly drained soils in the state.

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