Variability in Virulence of *Aphanomyces euteiches*

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ABSTRACT

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Host specificity of *Aphanomyces euteiches* was studied with 14 isolates from throughout the United States and Canada. Isolates were obtained by baiting from soil samples and by direct recovery from roots of field-grown plants. At least one of the 14 isolates of *A. euteiches* was pathogenic to alfalfa; red, berseem, and crimson clovers; white and yellow sweet-clovers; field pea; garden pea; snap bean; lima bean; and faba bean. Isolates of *A. euteiches* differed in the expression of virulence on specific hosts. None of the isolates were pathogenic to subterranean, arrowleaf, alsike, or ladino clovers; crown vetch; bird's-foot trefoil; soybean; cowpea; lupine; or peanut. The isolate hosts ranged from multiple hosts (isolate from pea) to host specific (isolate from snap bean). Nine of 27 isolates of *A. euteiches* were highly virulent on an alfalfa population (WAPH-1) selected for resistance to isolates MD433, MN122, and NY101. Isolates were recovered from alfalfa seedlings grown in soils that originated from North Carolina, Mississippi, Virginia, Idaho, Minnesota, and Wisconsin. An alfalfa accession, PI 468051, expressed a disease reaction intermediate between that of a susceptible alfalfa population (Saranac) and WAPH-1 for three isolates and lower than that of WAPH-1 for one isolate. This report provides evidence that *A. euteiches* is composed of host-specific subpopulations. In addition, two alfalfa populations reacted differentially to isolates of *A. euteiches*.

Seven species within the genus *Aphanomyces* cause root rot of economically valued plants (13–15,23,30,32). *Aphanomyces euteiches* Drechs. occurs throughout North America, Europe, Australia, Japan, and New Zealand (2,5,14,17, 33,34). Because crop rotation is an important means of controlling this soilborne plant pathogen, the host range of *A. euteiches* has been of interest to many investigators (23). Many early investigators conducted host range studies with *A. euteiches* and concluded that pea (*Pisum sativum* L.) was the primary host. However, only isolates obtained from pea were used in these studies. Linford (18) was the first to report a possible pathogenic relationship between *A. euteiches* and a crop other than pea. He observed the mortality of alfalfa (*Medicago sativa* L.) seedlings in fields with a history of pea root rot, but the cause was not confirmed. Schmitthenner (29) was the first investigator to recover *A. euteiches* from field-grown alfalfa plants and concluded that a specialized form of the fungus infected alfalfa. Since 1964, other investigators have recovered *A. euteiches* from alfalfa (1,5,6,12,20), faba bean (*Vicia faba* L.) (17), snap bean (*Phaseolus vulgaris* L.) (2,7,12,25), red clover (*Trifolium pratense* L.) (12), and subterranean clover (*Trifolium subterraneum* L.) (9) grown in naturally infested soil. Isolates expressed different degrees of specificity to crop species, but morphological traits were either identical or sufficiently similar to preclude new species designations. Pfender and Hagedorn (25) proposed a forma specialis designation for isolates of *A. euteiches* pathogenic to snap bean and pea. Amazon sword plant (*Echinodorus brevipedicellatus* (Kuntze) Buchenau) is also reported as a host of *A. euteiches*, but isolates are not specific to this aquatic plant (27).

Isolates of *Aphanomyces* that we have recovered from legume crops other than pea meet all the taxonomic criteria established for *A. euteiches*, except that many are not pathogenic on pea (12,30). Our objective was to explore the degree of host specificity that exists within populations of *A. euteiches* recovered from alfalfa, red clover, pea, snap bean, and faba bean. We also wished to further characterize variability in virulence within a group of isolates recovered from alfalfa. An improved understanding of the variation of virulence within *A. euteiches* would aid in the development of control strategies that employ crop rotation and host resistance.

MATERIALS AND METHODS

Isolates of *A. euteiches*. Fourteen isolates of *A. euteiches* were selected for the host range study based on host and geographic origins. In addition, 27 isolates recovered from alfalfa were used to study virulence on alfalfa. Seedlings of alfalfa, pea, snap bean, or faba bean were used to bait *A. euteiches* from soils collected in Idaho, Illinois, Kentucky, Maryland, Minnesota, Mississippi, New York, North Carolina, Oklahoma, Ten-

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nessee, Utah, Virginia, Wisconsin, and Manitoba, Canada. Seed was treated with metalaxyl (0.1 g/100 g of seed, formulated as Apron 25W) to deter species of *Pythium* and *Phytophthora*. Seedlings were grown in each soil for 7 days, after which root tissue was incubated on a semiselective medium for the recovery of *Aphanomyces* (24). Alternatively, the seedling roots were floated in sterilized lake water and sporangia of *Aphanomyces* spp. were removed by pipet and transferred to the medium. Isolate RC572 was recovered directly from roots of red clover plants collected from the field.

Inoculum production. Zoospores of *A. euteiches* were produced using a modification of the technique described by Mitchell and Yang (21). To allow enumeration with a hemacytometer, a 2-ml sample of each zoospore suspension was agitated in a vortex mixer to induce zoospores to encyst. The inoculum concentration for each isolate was adjusted to 333 zoospores per milliliter in sterile deionized water.

Plant growth system—Host range study. Seeds of each crop were planted in plastic trays with cavities (2.5 × 2.5 × 7 cm, Jiffy Corp., Chicago, IL). Vermiculite was used as the planting medium. Approximately 10 seeds of small-seeded legumes or one pregerminated seed of large-seeded legumes were planted per cell. Three cells comprised a replication. Seeds were covered with vermiculite, and cavity trays were placed in plastic trays. Deionized water was added to a depth of 1 cm to moisten the medium and promote germination. Seedlings were inoculated 5 days after planting by dispensing 1,000 zoospores suspended in 3 ml of deionized water per cell. The plastic trays were filled with deionized water to within 1 cm of the top of each cavity tray. In the first repetition of the experiment, seedlings were incubated in a growth chamber with 21-C days and 16-C nights for 2 wk, followed by 24-C days and 20-C nights for the last week of incubation. The light intensity of the growth chamber was 200 $\mu E \cdot m^{-2} \cdot s^{-1}$. For the second repetition of the experiment, the second temperature regime was maintained for the entire duration. Half-strength Hoagland's solution (10) was dispensed in the trays 7 days after inoculation. Crop species were randomized in one cavity tray, and one isolate was used per tray.

Plant growth system—Alfalfa study. Seeds were planted in vermiculite in paper cups (90 cm^3 capacity), 20 seeds per cup. The vermiculite was kept moist for 5 days and was flooded with deionized water 6 days after planting in preparation for inoculation. Six days after planting, 2,000 zoospores were added to each cup in 5 ml of sterile distilled water. Seedlings were incubated at 24 C during the day and at 20 C at night. The plants were fertilized with full-strength Hoagland's solution 1 wk after inoculation. Each cup contained one population of alfalfa and received one isolate of *A. euteiches*.

Legumes evaluated for reaction to *A. euteiches*. Twenty-one species of legumes were evaluated for their reaction to *A. euteiches* in two experiments. The legumes and cultivars used were as follows: alfalfa (cv. Saranac), common alsike clover (*T. hybridum* L.), arrowleaf clover (*T. vesiculosum* Savi 'Yuchi'), berseem clover (*T. alexandrinum* L. 'Bigbee'), crimson clover (*T. incarnatum* L. 'Chief'), common ladino clover (*T. repens* L.), red clover (cv. Arlington), subterranean clover (cv. Woogenellup), yellow sweet-clover (*Melilotus officinalis* (L.) Lam. 'Goldtop'), white sweet-clover (*M. alba* Medik. 'Denta'), bird's-foot trefoil (*Lotus corniculatus* L. 'Norcen'), crown vetch (*Coronilla varia* L. 'Emerald'), cowpea (*Vigna sinensis* (L.) Savi

Table 1. Disease severity index[a] ratings of 10 legumes to 11 isolates of *Aphanomyces euteiches* (experiment 1)

Legume	WI139	MN122	WI1	IL11	NC1	NC3	MS12	MS13	RC572	P467	SB164	U[b]
Alfalfa	4.16[c]	4.31	4.27	3.80	4.59	4.63	4.68	4.50	3.56	4.33	2.95	1.00
Red clover	1.00	1.52	1.56	1.07	1.21	1.20	1.20	1.33	3.95	1.14	1.14	1.14
Subterranean clover	2.28	1.83	2.10	2.22	2.31	2.55	2.50	2.20	2.49	2.85	1.62	1.00
Crimson clover	1.98	2.49	2.21	1.46	2.07	1.55	1.77	1.61	2.58	3.61	1.16	1.00
Berseem clover	3.05	2.86	2.15	2.97	3.14	3.01	3.06	3.00	2.63	2.32	3.01	1.00
White sweeet-clover	2.96	3.19	3.70	2.93	3.44	3.50	3.33	3.12	3.13	2.51	2.51	1.00
Yellow sweet-clover	2.68	2.66	2.13	2.50	2.48	2.91	2.18	2.62	2.73	2.93	1.17	1.00
Field pea	1.00	1.00	1.00	1.22	1.00	1.00	1.11	1.00	1.00	4.89	1.00	1.17
Garden pea	1.00	1.00	1.11	1.11	1.11	1.11	1.11	1.11	1.00	4.89	1.00	1.00
Snap bean	1.11	1.89	2.44	2.00	2.25	2.56	1.67	2.39	1.34	3.00	4.00	1.00

[a] The following disease severity scale was used: 1 = no to very slight discoloration of roots and hypocotyls; 2 = slight necrosis of roots and hypocotyls; 3 = necrosis of roots and lower hypocotyl, slight chlorosis of cotyledons, and moderate stunting of stems; 4 = extensive necrosis of roots, hypocotyls, and cotyledons and severe stunting of stems; and 5 = seedling dead.
[b] Isolates recovered from alfalfa have a state abbreviation as a prefix. Additional isolates used in the experiment were recovered from red clover (RC572), pea (P467), and snap bean (SB164). An uninoculated (U) treatment was employed for each crop.
[c] LSD ($P = 0.05$) = 0.63 across columns and 0.61 within columns.

Table 2. Disease severity index[a] ratings of 11 legumes to 11 isolates of *Aphanomyces euteiches* (experiment 2)

Legume	WI139	MN122	MD433	IL11	ID46	NC1	MS13	RC572	P467	SB164	FB1	U[b]
Alfalfa	4.26[c]	4.75	4.82	4.85	4.68	4.83	4.61	3.44	4.45	2.01	4.59	1.25
Red clover	1.28	1.36	1.31	1.24	1.38	1.65	1.29	3.86	1.23	1.26	1.26	1.10
Subterranean clover	1.98	2.22	2.06	2.17	1.73	2.23	2.42	2.09	2.62	2.00	2.26	1.06
Crimson clover	1.98	2.29	2.13	1.57	2.26	2.18	1.81	2.07	3.50	1.42	1.69	1.36
Berseem clover	2.39	2.07	2.16	3.00	2.71	2.79	2.21	2.61	1.70	2.63	1.32	1.00
White sweeet-clover	3.94	3.39	3.72	3.87	4.00	3.87	3.49	4.00	3.72	2.49	4.00	1.29
Yellow sweet-clover	2.44	3.08	2.40	2.74	3.42	3.00	3.25	3.16	3.26	1.79	2.83	1.15
Garden pea	1.22	1.00	1.11	1.00	1.00	1.00	1.00	1.00	4.11	1.00	4.22	1.00
Snap bean	1.11	1.44	1.44	1.11	1.67	1.11	1.33	1.11	2.33	3.33	1.22	1.00
Lima bean	2.06	2.06	2.33	1.11	1.22	2.06	1.67	3.00	1.78	3.67	1.67	1.00
Faba bean	1.11	2.44	2.56	2.00	1.11	2.17	1.78	1.00	4.00	1.00	4.67	1.00

[a] The following disease severity scale was used: 1 = no to very slight discoloration of roots and hypocotyls; 2 = slight necrosis of roots and hypocotyls; 3 = necrosis of roots and lower hypocotyl, slight chlorosis of cotyledons, and moderate stunting of stems; 4 = extensive necrosis of roots, hypocotyls, and cotyledons and severe stunting of stems; and 5 = seedling dead.
[b] Isolates recovered from alfalfa have a state abbreviation as a prefix. Additional isolates used in the experiment were recovered from red clover (RC572), pea (P467), snap bean (SB164), and faba bean (FB1). An uninoculated (U) treatment was employed for each crop.
[c] LSD ($P = 0.05$) = 0.73 across columns and 0.71 within columns.

ex Hassk. 'Queen Anne Blackeye'), common faba bean, field pea (*P. sativum* var. *arvense* (L.) Poir. 'Trapper'), garden pea (cv. Perfection 8221), lima bean (*Phaseolus limensis* Macfady. 'Henderson's Bush'), snap bean (cv. Early Gallatin), lupine (*Lupinus albus* L. 'Sweet White'), peanut (*Arachis hypogaea* L. 'Florigiant'), and soybean (*Glycine max* (L.) Merrill 'McCall').

Nine legume species and eight isolates of *A. euteiches* were common to both experiments. Alsike, arrowleaf, and ladino clovers; bird's-foot trefoil; crown vetch; lupine; field pea; and soybean were included in repetition 1 but not in repetition 2. Lima bean, cowpea, faba bean, and peanut were added as new crops in repetition 2.

Alfalfa populations for virulence variation study. The alfalfa populations for this study were selected on the basis of previously observed reactions to isolates of *A. euteiches*. The cultivar Saranac was determined to be susceptible, the experimental population WAPH-1 expressed resistance, and the accession PI 468051 expressed a low level of resistance to isolates of *A. euteiches* originating from Wisconsin, Minnesota, and Maryland (C. R. Grau, *unpublished*).

Disease severity index. Seedlings were evaluated for disease severity 14 days after inoculation on a 1–5 scale where 1 = no to very slight discoloration of roots and hypocotyls, 2 = slight necrosis of roots and hypocotyls, 3 = necrosis of roots and lower hypocotyl, slight chlorosis of cotyledons, and moderate stunting of stems, 4 = extensive necrosis of roots, hypocotyls, and cotyledons and severe stunting of stems, and 5 = seedling dead. Disease severity indices were used to characterize the phenotype of each legume species for reaction to specific isolates of *A. euteiches*. Isolates were characterized as being highly virulent (>4.0), virulent (3.0–4.0), or having low virulence (<3.0). An isolate was characterized as nonpathogenic if it caused a host reaction rated ≤2.00.

Statistical analysis. Host range experiments were conducted in a split-plot arrangement of a randomized block design with isolates as whole plots and crop species as subplots. There were three replications of each whole and subplot. The alfalfa study was arranged as a randomized complete block design with three replications. Data were analyzed through analysis of variance and Fisher's least significant difference (LSD) test for mean comparisons.

RESULTS

Host range study. Each isolate of *A. euteiches* was highly virulent on the crop from which it was isolated originally (Tables 1 and 2). However, isolates differed from each other by the number of additional hosts and the degree of virulence expressed on a mutual host. For example, all isolates of *A. euteiches* from alfalfa were highly virulent (>4.0) to alfalfa and caused a lesser severity of disease (3.0–4.0) on white sweet-clover. Yellow sweet-clover and berseem clover were the only other hosts that expressed any degree of susceptibility to isolates originating from alfalfa. Isolates MN122, ID46, MS13, NC1, and NC3 caused an intermediate level of disease severity on yellow sweet-clover, and isolates WI139, IL11, NC1, NC3, MS12, and MS13 caused intermediate levels of severity on berseem clover (Tables 1 and 2). Isolates of *A. euteiches* recovered from alfalfa were not pathogenic to all other crop species evaluated in this study.

None of the isolates of *A. euteiches* were characterized as pathogenic to alsike clover, arrowleaf clover, ladino clover, bird's-foot trefoil, crown vetch, cowpea, lupine, peanut, or soybean. The isolates from pea (P467), red clover (RC572), and faba bean (FB1) were pathogenic to more hosts than the isolates from alfalfa. Each isolate was highly virulent to the host of origin. In addition, isolate P467 was pathogenic to crimson clover, alfalfa, faba bean, snap bean, and both sweet-clovers. Isolate RC572 was pathogenic to alfalfa, both sweet-clovers, and lima bean; isolate FB1 was pathogenic to alfalfa, pea, and white and yellow sweet-clover (Tables 1 and 2). The isolate from snap bean, SB164, expressed a high degree of host specificity to snap bean and lima bean. Isolates of *A. euteiches* were identified as pathogenic to subterranean clover (≥2.00) but were regarded as weakly virulent (<3.0) on this forage legume.

Variation for virulence to alfalfa. Isolates of *A. euteiches* were characterized on the basis of the reaction they caused on the cultivar Saranac (Table 3). One isolate from Minnesota (MN53) was characterized as nonpathogenic to alfalfa and an isolate from Maryland (MD74) expressed intermediate virulence on all three populations (2.85–3.55). Isolates ID45, MN52, MN58, MS13, NC1, NC61, VA63, VA71, and WI98 were highly virulent (>4) to WAPH-1. Most of the isolates were as virulent or almost as virulent to PI 468051 as to Saranac. Isolate TN94, however, was less virulent on PI 468051 than on either Saranac or WAPH-1.

DISCUSSION

Results from this study provide additional evidence that *A. euteiches* is composed of subpopulations that differ in pathogenicity to plant species. Although the formae speciales concept was proposed to distinguish between two types of *A. euteiches* (25), (pathogenic to pea, *A. euteiches.* f. sp. *pisi* W. F. Pfender & D. J. Hagedorn; pathogenic to snap bean, *A. euteiches* f. sp. *phaseoli* W. F. Pfender & D. J. Hagedorn), only *A. e. phaseoli* seems to be supported by our current study. The isolate from pea, P467, had the broadest host range in the collection of isolates studied. Except for a consistent pathogenic reaction on white sweet-clover, the isolates recovered from alfalfa seem more deserving of a forma specialis designation than the isolate from pea. This concept is further supported by work of Holub et al (12). They found that 50% of isolates from alfalfa were pathogenic on pea, whereas 95% of all isolates in their collection (regardless of host origin) were pathogenic on alfalfa. In our current study, none of the 32 isolates of *A. euteiches* from alfalfa were pathogenic on pea. All isolates recovered from alfalfa in our study originated from soils with no immediate history of pea production. This result is supported by the findings of Holub et al (12), who found that only 20% of their isolates from alfalfa fields were pathogenic to pea. It has been our experience that isolates of *A. euteiches* recovered from pea tend to be pathogenic to both pea and alfalfa, but isolates from alfalfa tend to be pathogenic only to alfalfa (6,12).

Table 3. Disease severity index[a] rating of three alfalfa populations to 27 isolates of *Aphanomyces euteiches* recovered from alfalfa

Isolate	Saranac	WAPH-1	PI 468051
ID45[b]	4.64[c]	4.60	4.54
KY51	4.56	2.27	4.27
MD66	4.27	3.84	3.96
MD74	3.55	2.85	3.40
MD76	4.36	2.87	4.04
MD77	4.31	2.86	4.39
MD80	4.65	2.82	4.20
MD433	4.42	2.65	3.98
MN52	4.48	4.35	4.41
MN53	1.82	1.49	1.29
MN58	4.35	4.36	4.40
MN122	4.39	2.79	4.48
MS13	4.72	4.42	4.42
NC1	4.57	4.42	4.11
NC61	4.35	4.12	4.17
NY101	4.10	2.20	4.26
OK87	4.31	2.58	4.26
TN94	4.14	3.72	3.24
UT54	4.38	1.86	4.25
VA60	4.04	3.81	4.23
VA63	4.43	4.37	4.43
VA68	4.30	2.74	3.89
VA71	4.37	4.19	4.27
VA81	4.46	3.17	4.21
WI1	4.57	2.68	4.59
WI97	4.52	3.29	4.17
WI98	4.83	4.39	4.42
U[d]	1.28	1.04	1.11

[a] The following disease severity scale was used: 1 = no to very slight discoloration of roots and hypocotyls; 2 = slight necrosis of roots and hypocotyls; 3 = necrosis of roots and lower hypocotyl, slight chlorosis of cotyledons, and moderate stunting of stems; 4 = extensive necrosis of roots, hypocotyls, and cotyledons and severe stunting of stems; and 5 = seedling dead.

[b] Letters before each isolate number represent state of origin.

[c] LSD ($P = 0.05$) = 0.42.

[d] U = Uninoculated.

The pathogenicity of *A. euteiches* to *Trifolium* species was explored further in this study. Previous reports (6,12,31) have described *A. euteiches* as being weakly pathogenic to red clover. However, isolate RC572 of *A. euteiches* was pathogenic to red clover and illustrates the importance of using isolates recovered from the host to evaluate for susceptibility to *A. euteiches*. Isolates with virulence phenotypes represented by RC572 are not frequently isolated from other legumes. In only one case was an isolate (MD74) recovered from alfalfa that also was virulent to red clover (C. R. Grau, *unpublished*). In addition, this study is the first report of berseem and crimson clovers as hosts of *A. euteiches*. None of the isolates tested were highly virulent to subterranean clover. Isolates that are virulent to this important forage legume are present in Australia (9).

A. euteiches has become recognized as a pathogen of alfalfa (1,6,12,20,28). Because breeding for resistance to the pathogen is being attempted (11), it is important to determine whether strains of the pathogen are present that are capable of overcoming resistance. Resistance to *A. euteiches* has been readily identified within alfalfa populations (11). Resistance was expressed against isolates from alfalfa and pea (11) and was stable against isolates within each group. However, in this current study, nine of 27 isolates of *A. euteiches* were highly virulent (>4.0) to both Saranac and WAPH-1. WAPH-1 was selected for resistance against isolate phenotypes such as MD433, WI139, and MN122. The concept of intraspecific variation for virulence has been established for populations of *A. euteiches* pathogenic to specific genotypes of peas (3,19,33). Isolates of *A. euteiches* that differed by two DSI classes were characterized as being phenotypically different for virulence against WAPH-1. It was not determined if differences in virulence between isolates constitute a race reaction, supervirulence, or a different species of *Aphanomyces* (4). Attempts are being made to identify resistance to the phenotypes of *A. euteiches* that are highly virulent on WAPH-1.

Similarities exist between the pathogenic diversity of *A. euteiches* and that of *Phytophthora megasperma* Drechs. *P. megasperma* has been divided into formae speciales primarily on the basis of host specificity (16,26). The formae speciales *glycinea* has been divided into races based on differential reactions of soybean cultivars to isolates (29). Because isolates of *A. euteiches* frequently are not limited to one host, the formae speciales distinction may not be appropriate in all cases. We support the taxon *A. e. phaseoli* but do not support the taxon *A. e. pisi*. Biochemical techniques have been used to characterize isolates of *P. megasperma* and should be used to supplement morphological and physiological characterization of *A. euteiches* (8,22). Biochemical techniques should be used to characterize isolates of *A. euteiches* that cause differential reactions on alfalfa populations. It is critical to ascertain if isolates that are highly virulent to WAPH-1 are races within *A. euteiches* or different species of *Aphanomyces*. As with *P. megasperma* f. sp. *glycinea* T. Kuan & D. C. Erwin, race distinctions can become very complicated if new isolate/cultivar combinations continuously reveal new races.

Our studies indicate that considerable variability exists within *A. euteiches* for pathogenicity and virulence. The concept that the pathogenic activity of *A. euteiches* is restricted to peas and snap beans should be dismissed. Research is needed to determine if crops and cultivars within a crop influence the virulence characteristics of the populations of *A. euteiches* that are present in a field.

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