Reaction of Red Clover to *Aphanomyces euteiches*

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ABSTRACT

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Aphanomyces euteiches is an economically important root rot pathogen of pea (*Pisum sativum*). Red clover (*Trifolium pratense*) is known to be a host but expresses low susceptiblity to *A. euteiches*. Recently, an isolate (Ae-572) of *A. euteiches* was observed to be highly virulent on red clover. A seedling assay was used to characterize the reaction of red clover to *A. euteiches* (isolate Ae-572). One-week-old red clover is most susceptible to *A. euteiches*. Two- and 3-wk-old seedlings were significantly less susceptible. A reduction in forage dry matter and root weight of inoculated plants was observed at 5 and 8 wk of age. An inoculum concentration of 333 zoospores per milliliter at 24 C was optimum to determine the reaction of red clover to *A. euteiches*. Tetraploid red clover developed by asexual or sexual methods was not significantly different from diploid red clover in its reaction to *A. euteiches*. No common cultivars of red clover adapted for the northern Midwest have a high level of resistance to the isolate Ae-572 of *A. euteiches*.

Aphanomyces euteiches Drechs. has been identified as a root rot pathogen of many plant species, primarily legumes (11). Early studies have reported that this oomycete was only slightly pathogenic on red clover (*Trifolium pratense* L.) (2,4,13). Recently, Holub et al (10) collected 157 isolates of *A. euteiches* from diverse locations and determined the pathogenicity of these isolates on four different host species, alfalfa (*Medicago sativa* L.), pea (*Pisum sativum* L.), green bean (*Phaseolus vulgaris* L.), and red clover. During this study, they recovered an isolate (Ae-572) that was highly virulent on red clover but did not further characterize its effect on red clover.

The objectives of this research were to determine the effect of seedling age, temperature, and inoculum concentration on the reaction of red clover seedlings to *A. euteiches* isolate Ae-572. Results from these experiments were used to identify resistance to *A. euteiches* in red clover germ plasm, determine the effect that ploidy level of the host has on the reaction to *A. euteiches*, and determine the effect of seedling age at inoculation on forage dry matter and root weight in two elite populations of red clover.

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MATERIALS AND METHODS

Inoculum preparation. Zoospores of *A. euteiches*, isolate Ae-572 (9), were produced using the method described by Mitchell and Yang (10). To determine the inoculum concentration, the zoospore suspension was first decanted from the mycelial mats and a 5-ml subsample of the suspension was agitated in a test tube with a Vortex Genie mixer (Scientific Products, McGaw Park, IL) to encyst the zoospores. The number of encysted zoospores per milliliter was determined with a hemacytometer.

Preparation of red clover seedlings for inoculation. Scarifed red clover seeds were planted into Jiffy cavity trays (Jiffy Products, West Chicago, IL) with seven rows of 14 cone-shaped cavities (2.5 × 2.5 × 7.0 cm) per tray. A 1-cm plug of absorbent sterile cotton was placed at the bottom of each cavity, and the cavity was filled to within 1 cm of the top with sterilized sand. Ten seeds were planted in each cavity and covered with 1 cm of moistened coarse vermiculite. The tray with seeds was placed in a flat plastic pan to which tap water was added to a depth of 3 cm. The water level in the pan was increased to 5 cm 1 hr before inoculation.

Inoculation. Three milliliters of a zoospore suspension (concentration of 333 spores per milliliter unless otherwise stated) was dispensed into each cavity using a Cornwell syringe 5 days after planting. Inoculated seedlings were placed in a growth chamber at 24 C (unless otherwise stated) with a 14-hr photoperiod (light intensity = 200 $\mu E \cdot m^{-2} \cdot s^{-1}$), and the water level in the flat pan was lowered to 2 cm. One week after inoculation, the water was replaced with full-strength Hoagland's solution (mineral nutritive solution) (6), and percent emergence was determined for each cavity (approximately 90% emergence per cavity).

Disease severity assessment. Two weeks after inoculation, disease severity was determined for each seedling. The disease severity index (DSI) ranged from 1 to 5 where 1 = no necrosis of root, hypocotyl, or cotyledon; 2 = minimal necrosis of lateral roots and hypocotyl; 3 = necrosis of roots and lower hypocotyl with minimal necrosis of cotyledons; 4 = extensive necrosis of roots, hypocotyl, and cotyledons, plants were stunted; and 5 = dead plant. Plant reaction was characterized as highly resistant (DSI = 1), resistant (DSI= 2), or susceptible (DSI = 3–5).

Effect of inoculum concentration. Five-day-old seedlings of the susceptible cultivar Arlington were inoculated with one of the following five zoospore concentrations: 33, 333, 3,330, 33,300, or 333,000 zoospores per milliliter. These concentrations were approximately 10–100,000 zoospores per seedling, respectively. A randomized complete block design with four replications was used. A column of three consecutive cavities (each containing 10 seeds) constituted one experimental unit for each concentration in each replication. The center row of 14 cavities contained uninoculated seedlings.

Effect of temperature on the reaction to *A. euteiches*. Seedlings of the cultivar

Arlington were planted into a four-row × four-cavity tray and inoculated. The trays were placed into five growth chambers that were set at temperatures of 16, 20, 24, 28, and 32 C. A row of four consecutive cavities, each with 10 seeds, constituted a replication, and three replications were used. An uninoculated row of four cavities was also included. A duplicate experiment was conducted 2 wk later.

Effect of seedling age on reaction to *A. euteiches*. Seedlings of the cultivar Arlington were inoculated at either 1, 2, or 4 wk after planting. A replicate for each seedling age was represented by two cavities with 10 seeds in each. The three age classes were replicated 12 times each. Two uninoculated cavities were randomized within each of the three age classes.

Evaluation of red clover germ plasm. Twelve red clover cultivars (Arlington, Atlas, Kenstar, Lakeland, Marathon, Mor Red, Persist, Prosper I, Reddy, Redland II, Ruby, and Starglo) and two experimental lines (C11 and C136) were inoculated to determine their reaction to *A. euteiches*. A randomized complete block design with three replications was used. A column of three cavities each with 10 seeds represented one experimental unit per cultivar. A duplicate experiment was conducted 3 wk later.

Effect of ploidy level on the reaction to *A. euteiches*. Tetraploid red clover can be produced either asexually (colchicine and nitrous oxide) or sexually (unilaterally [2*x*-4*x*] or bilaterally [2*x*-2*x*] via 2*n* gametes) (12). Diploid (2*n*=2*x*=14) germ plasm was compared with tetraploid (2*n*=4*x*=28) germ plasm to determine the effect of ploidy on the reaction to *A. euteiches*. The diploid germ plasm used in this study was represented by the cultivars Arlington, Kenstar, Homidori, Ultuna, Bombi, and an experimental line designated as C16 (F_1 population of a cross between Arlington and Kenstar). The tetraploid germ plasm was developed using nitrous oxide (Kenstar 4*x*) or colchicine (Homidori 4*x*, Hedda, St. 970, and WI-16). The experimental line WI-10 was derived from diploid × tetraploid (2*x*-4*x*) crosses using Arlington (2*x*) and Kenstar 4*x*. C137 was derived from diploid × diploid (2*x*-2*x*) crosses. Each treatment was replicated four times in a randomized complete block design. Three consecutive cavities, each containing 10 seeds, constituted one experimental unit per entry in each replication.

Effect of seedling age at inoculation on forage dry matter and root weight. The cultivar Arlington and C223 (an experimental population) were inoculated at 1, 2, and 3 wk after planting. C223 represents germ plasm in the second cycle of selection for resistance to *A. euteiches* from an elite population of red clover. C223 had a mean disease severity rating (DSR) of 2.8 (moderately resistant) compared with Arlington, which had a DSR of 3.7 (susceptible) (15). Plastic cones (4 cm diameter × 21 cm) (Ray Leach Cone-Tainers, Canby, OR) were filled with 4 cm of vermiculite and then covered with 14 cm of Jiffy Mix (mixture of sphagnum peat moss and vermiculite) (Jiffy Products, West Chicago, IL) within 3 cm from the top of the cone. Eight seeds of either Arlington or C223 were planted in each cone and covered with 3 cm of moistened vermiculite. Seven days after planting, the germinated seedlings were thinned to three seedlings per cone and inoculated with 5 ml of a 2,000 zoospores per milliliter suspension.

Each age group (1, 2, and, 3 wk after planting) had an inoculated and an uninoculated treatment that was replicated four times. Each treatment within an age group consisted of six cones (three cones of Arlington and three cones of C223) placed into a 12 × 16 cm plastic container. The plastic containers were filled with tap water and placed into a growth chamber at 24 C with a photoperiod (light intensity = 200 $\mu E \cdot m^{-2} \cdot s^{-1}$) of 12 hr. One week after inoculation, the water was replaced with full-strength Hoagland's mineral solution (6). Plants from each age group were harvested to the height of 3 cm with scissors at 5 wk after planting, and a second harvest was made 3 wk later (8 wk after planting). At each harvest, forage top growth was dried in a forced-air oven at 60 C for 3 days. After the second harvest, crowns and roots in each cone were also harvested and dried at 60 C for 3 days. Forage or crown/root dry matter yield was based on the total dry matter of three plants in each of the three cones in each treatment.

Table 1. Frequency of Arlington red clover plants in respective disease severity index (DSI) classes and mean disease severity rating (DSR) when inoculated with five zoospore concentrations of *Aphanomyces euteiches* isolate Ae-572

Concentration (zoospores/ml)	Percent plants with DSI[y]				
	1	2	3	4-5	Mean DSR
33	0	9	22	69	4.0 a[z]
333	0	0	14	86	4.5 b
3,330	0	0	0	100	4.9 c
33,300	0	0	2	98	4.9 c
333,000	0	0	2	98	5.0 c

[y] Disease severity index scale: 1 = no symptoms; 2 = minimal necrosis of hypocotyl; 3 = necrosis of root; 4 = extensive root necrosis, stunted plant; and 5 = dead plant.
[z] Means followed by same letter are not significantly ($P = 0.05$) different using Fischer's least significant difference for comparison of treatment means.

Table 2. Frequency of Arlington red clover seedlings in respective disease severity index (DSI) classes and mean disease severity rating (DSR) when inoculated with *Aphanomyces euteiches* at five temperatures

Temperature (C)	Percent plants with DSI[y]				
	1	2	3	4-5	Mean DSR
16	0	11	63	26	3.2 b[z]
20	0	5	70	25	3.2 b
24	0	2	56	42	3.6 c
28	2	5	62	31	3.3 bc
32	66	12	21	1	1.6 a

[y] Disease severity index scale: 1 = no symptoms; 2 = minimal necrosis of hypocotyl; 3 = necrosis of root; 4 = extensive necrosis, stunted plant; and 5 = dead plant. Data are means of three replications in two experiments.
[z] Means followed by same letter are not significantly ($P = 0.05$) different using Fischer's least significant difference for comparison of treatment means.

RESULTS AND DISCUSSION

Inoculum concentration. The disease severity rating (DSR) increased as the amount of inoculum increased from 33 to 3,333 spores per milliliter (Table 1). No symptomless or highly resistant (DSR = 1) plants were observed, and essentially all plants were classified as susceptible (DSR = 3–5) at a concentration of ≥ 3,330 spores per milliliter. A range of phenotypic expression was evident at the concentration of 33 spores per milliliter. This level of inoculum may be useful for determining resistance in red clover; however, 33 spores per milliliter did not appear to be a high enough inoculum concentration to ensure consistent results. Therefore, the 333 spores per milliliter concentration was chosen as the optimum concentration for other experiments. In alfalfa, an inoculum density of 100 spores per seedling (approximately 333 spores per milliliter) of *A. euteiches* is an effective optimum inoculum concentration (5,7).

Effect of temperature on the reaction to *A. euteiches*. The results for two experiments on the effect of temperature on the reaction of red clover to *A. euteiches* were similar. In the analysis of

variance, the interaction of temperature × experiment was not significant. Therefore, the two sets of data were combined (Table 2). Severity of disease caused by *A. euteiches* was maximum at 24 C with a mean DSR of 3.6. However, this rating was not significantly different than the DSR at 28 C. In red clover, 24 C was chosen as the optimum temperature (temperature levels analyzed in this experiment) to select for resistance to *A. euteiches* because the frequency of plants with a DSR of 1 and 2 was lowest at this temperature. In experiments with alfalfa (8), disease severity was greatest at 28 C. However, 24 C is the optimum temperature used when screening for resistance in alfalfa. In the current study, there was no significant difference among 16, 20, and 28 C for mean DSR. No highly resistant plants (DSI = 1) were observed at temperatures between 16 and 24 C. Resistant plants were observed at temperatures of 28 C (2%), and a dramatic increase in the percentage of symptomless plants was observed at 32 C (66%). This significant increase in symptom-free plants at 32 C has also been observed in alfalfa and pea (1,8,11).

Effect of seedling age on the reaction to *A. euteiches*. Red clover seedlings were most susceptible when inoculated at 1 wk of age (mean DSR of 4.0) (Table 3). Seedlings inoculated at 2 and 4 wk exhibited little or no reaction to *A. euteiches*. Limited information is known about what physiological or anatomical process may be involved that make older seedlings more resistant to diseases. However, this reaction is not uncommon in red clover and alfalfa in relation to other seedling pathogens such as species of *Pythium* (3).

Effect of ploidy level on the reaction to *A. euteiches*. In red clover, increased resistance to particular diseases has been influenced by an increase in the number of chromosomes from the diploid to the tetraploid level. Tetraploid red clover has a greater degree of resistance to northern anthracnose (*Aureobasidium caulivorum* (Kirchn.) W. B. Cooke) (syn. *Kabatiella caulivora* (Kirchn.) Karakulin) (14) and crown rot (*Sclerotinia trifoliorum* Eriks.) (16) than does the diploid counterpart. In the current study, there was no significant difference in the overall mean rating between the diploid and tetraploid groups (Table 4). However, there was a significant difference in reaction between two of the five ploidy level comparisons (Bombi 2*x* [4.5] vs. St. 970 4*x* [4.8] and C16 2*x* [4.4] vs. WI-10 4*x* [4.9]). An explanation of why diploid red clover has a less severe reaction to *A. euteiches* than tetraploid red clover is unknown.

When colchicine-derived tetraploids were compared with sexually derived tetraploids (2*x*-4*x* and 2*x*-2*x*), no significant difference in mean DSR was observed (*data not presented*). Distribution of the disease severity index (DSI) classes was also similar for the different types of tetraploids.

Reaction of red clover germ plasm to *A. euteiches*. The results for two experiments evaluating the reaction of red clover germ plasm to *A. euteiches* were similar. The genotype × experiment interaction between the two experiments was not significant. Therefore, the two experiments were combined and only the combined data are presented. Significant differences ($P = 0.05$) for the reaction to *A. euteiches* were observed between the cultivars and experimental populations (Table 5). However, no cultivars or experimental populations expressed a high level of resistance as indicated by a high frequency of plants in the DSI classes 4 and 5. Of the red clover germ plasm tested, the most susceptible cultivar was Atlas, with a mean DSR of 4.2. The experimental line C11 had the lowest reaction level to *A. euteiches* (mean DSR = 3.6) and the highest percentage of plants in DSI classes 1, 2, and 3 (56%). C11 was selected as a base population to select for resistance against *A. euteiches* in red clover.

Effect of seedling age at inoculation

Table 3. Frequency of Arlington red clover seedlings in respective disease severity index (DSI) classes and mean disease severity ratings (DSR) when inoculated with *Aphanomyces euteiches* at three ages of red clover seedlings

	Percent plants with DSI[y]				
Age (wk)	1	2	3	4-5	Mean DSR
1	4	1	32	63	4.0 a[z]
2	89	1	4	6	1.3 b
4	91	4	2	3	1.2 b

[y] Disease severity index scale: 1 = no symptoms; 2 = minimal necrosis of hypocotyl; 3 = necrosis of root; 4 = extensive necrosis, stunted plant; and 5 = dead plant.
[z] Means followed by same letter are not significantly ($P = 0.05$) different using Fischer's least significant difference for comparison of treatment means.

Table 4. Mean disease severity rating (DSR) of diploid (2*x*) and tetraploid (4*x*) red clover germ plasm inoculated with *Aphanomyces euteiches*

Cultivar	Ploidy	Mean DSR[x]	Cultivar	Ploidy	Mean DSR[x]
Kenstar	2*x*	4.7	Kenstar	4*x*	4.7 NS[y]
Homidori	2*x*	4.8	Homidori	4*x*	4.8 NS
Ultuna	2*x*	4.9	Hedda	4*x*	4.8 NS
Bombi	2*x*	4.5	St. 970	4*x*	4.8 *[z]
C16	2*x*	4.4	WI-10	4*x*	4.8 *
Mean		4.6	Mean		4.8 NS

[x] Disease severity index scale: 1 = no symptoms; 2 = minimal necrosis of hypocotyl; 3 = necrosis of root; 4 = extensive necrosis, stunted plant; and 5 = dead plant.
[y] Means are not significantly (NS) ($P = 0.05$) different using Fischer's least significant difference for comparisons of a diploid and a tetraploid treatment mean.
[z] Means are significantly (*) ($P = 0.05$) different using Fischer's least significant difference for comparison of a diploid and a tetraploid treatment mean.

Table 5. Frequency of seedlings in respective disease severity index (DSI) classes and mean disease severity rating (DSR) for 14 red clover cultivars or experimental populations when inoculated with *Aphanomyces euteiches*

	Percent plants with DSI[y]				
Cultivar or line	1	2	3	4-5	Mean DSR
C11	1	8	47	44	3.6 a[z]
Marathon	2	0	50	48	3.7 ab
Starglo	0	8	44	47	3.7 ab
C136	0	9	40	51	3.8 a-c
Reddy	1	4	38	62	3.8 a-c
Arlington	0	5	34	61	3.9 b-d
Ruby	0	9	34	57	3.9 b-d
Persist	0	2	49	48	3.9 b-d
Lakeland	0	0	44	56	3.9 b-d
Prosper I	0	0	38	82	3.9 b-d
Redland II	1	6	23	70	3.9 b-d
Kenstar	0	3	33	64	3.9 b-d
MorRed	0	1	38	61	4.0 cd
Atlas	0	3	26	71	4.2 d

[y] Disease severity index scale: 1 = no symptoms; 2 = minimal necrosis of hypocotyl; 3 = necrosis of root; 4 = extensive necrosis, stunted plant; and 5 = dead plant. Data are means of three replications in two experiments.
[z] Means followed by same letter are not significantly ($P = 0.05$) different using Fischer's least significant difference for comparison of treatment means.

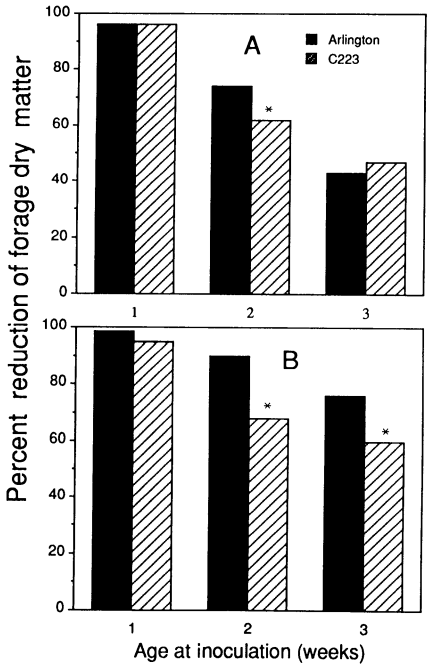

Fig. 1. Percent reduction (from uninoculated) of forage dry matter when red clover seedlings were inoculated at 1, 2, or 3 wk of age at **(A)** harvest one and **(B)** harvest two. Forage dry matter at each inoculation age differing by 11% within a population is significantly different ($P = 0.05$), and forage dry matter for a population (Arlington and C223) within an age differing by 9.3% (*) is significantly different ($P = 0.05$) using Fisher's least significant difference.

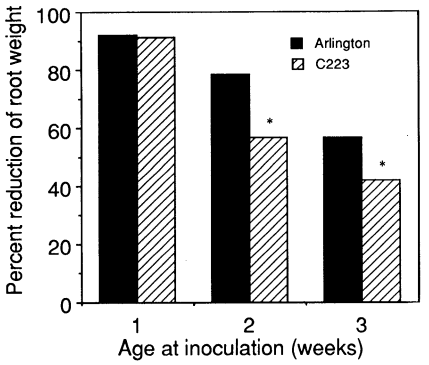

Fig. 2. Percent reduction of root weight when red clover seedlings were inoculated at 1, 2, or 3 wk of age. Root weight at each inoculation age differing by 13% within a population is significantly different ($P = 0.05$), and root weight for a population (Arlington and C223) within an age differing by 10% (*) is significantly different ($P = 0.05$) using Fisher's least significant difference.

on forage dry matter and root weight. The percent reduction in forage dry matter was calculated as the difference between the inoculated and the uninoculated treatments for both Arlington and C223. The first harvest (Fig. 1A) had a lower percent reduction in forage dry matter than the second harvest (Fig. 1B). In both harvests, the percent reduction in forage dry matter was significantly ($P = 0.05$) greater when seedlings were inoculated at 1 (approximately 95% reduction of forage dry matter) than at 2 or 3 wk of age. Although there were significant differences in percent reduction in forage dry matter between ages at inoculation, there was not always a significant difference in the percent reduction in forage dry matter between the two populations (Arlington and C223). Generally, the percent reduction in forage dry matter did not decline as rapidly for C223 as for Arlington when inoculated at a later seedling age. These results indicate that resistance to *A. euteiches* can improve red clover productivity.

Root weight (Fig. 2) was also most severely affected when seedlings were inoculated at 1 wk old and least affected at 3 wk old. As with percent reduction in forage dry matter, C223 seedlings inoculated at 2 and 3 wk of age had a significantly lower percent reduction in root weight than did Arlington seedlings.

Improved germ plasm (C223) and the selection parameters (time of inoculation, inoculation density, and temperature) reported in this paper are being used to improve the performance of red clover cultivars that are grown in wet soils where *A. euteiches* could cause yield reduction (15).

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