Development of an Antiserum to Quantify *Aphanomyces euteiches* in Resistant Pea Lines

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ABSTRACT

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A polyclonal antiserum was developed that strongly reacted with antigens produced by mycelia and zoospores of *Aphanomyces euteiches*. The antiserum did not react with antigens produced by species of *Phytophthora, Fusarium*, and *Pythium* (except for a slight reaction with antigens produced by *P. ultimum*) and by *Rhizoctonia solani* AG-4. Resistant and susceptible pea (*Pisum sativum*) lines were compared for response to *A. euteiches* development by assaying inoculated roots using this antiserum. There was a slower buildup of *A. euteiches* in taproots of the resistant germ plasm lines 86-2236 and 90-2131 and PI 180693 when exposed to 100 zoospores per milliliter after 7 days. After 9 days and an inoculum level of either 100 or 1,000 zoospores per milliliter, only PI 180693 and line 90-2131 were resistant, as indicated by lower ELISA readings (A_{405nm}). The ELISA response was linear with increasing amounts of antigen. The results showed that in resistant pea roots, growth of *A. euteiches* within inoculated tissues was inhibited.

Additional keywords: common root rot, resistance

Common root rot of peas (*Pisum sativum* L.), caused by *Aphanomyces euteiches* Drechs. f. sp. *pisi* W.F. Pfender & D.J. Hagedorn, is the most important soilborne disease of peas worldwide (2, 25). This disease has been reported in most pea-growing areas of North America, northern Europe, Australia, New Zealand, and Japan (8,9,25). In the Great Lakes area and northeastern United States, overall yearly losses to common root rot have been estimated at 10%, and individual fields can be a total loss (8). Recently, common root rot has become a serious yield constraint in the Pacific Northwest (1,17).

Marx et al (22) reported that tolerance to Aphanomyces root rot was associated with dominant, undesirable alleles at three unlinked marker loci. Substitution of recessive alleles, which express horticulturally desirable traits at each of these loci, resulted in a reduction in tolerance to root rot (22). However, resistance to *A. euteiches* has been recovered in breeding lines with acceptable horticultural traits (12). Lewis and Gritton (19) reported that resistance to *A. euteiches* was quantitatively inherited with low heritability. A recurrent selection program was used where disease pressure was intense. The release of several public germ plasm lines with resistance/tolerance to *A. euteiches* should improve the prospect of developing commercial cultivars with enhanced resistance to common root rot (5,7,11,13,15,18).

Techniques to quantify populations of *A. euteiches* in field soil by use of selective media and dilution plating have not been developed. Consequently, baiting techniques have been used to determine field inoculum levels of *A. euteiches* (17). The pathogen can be readily isolated from young infected root tissue on a semiselective medium (26) but not from more mature roots. Scoring individual plants for disease severity and counting numbers of oospores per gram of root tissue has been the primary means of selecting pea lines with resistance to *A. euteiches* (24). Measuring differences in oospore numbers within infected root tissue is time-consuming, especially if large numbers of breeding lines are involved. Field screening of peas for resistance to *Aphanomyces* has relied on aboveground symptoms and seed yields (14).

Enzyme-linked immunosorbent assay (ELISA) is a valuable tool to identify a test organism and quantify pathogen numbers (4). The present investigation was conducted to develop a polyclonal antiserum against *A. euteiches*, to determine specificity and sensitivity of the antiserum, and to compare *Aphanomyces*-resistant and *Aphanomyces*-susceptible pea lines using the polyclonal antiserum. A preliminary report has been published (16).

MATERIALS AND METHODS

Antiserum production. A strain of *A. euteiches* (A-1) obtained from Jennifer Parke (University of Wisconsin, Madison) was used to produce the antiserum. The culture was maintained on cornmeal agar, and inoculum was increased by growth in oatmeal broth in the dark at 22 C for 21 days (27). Microscopic examination revealed copious numbers of oospores and zoospores at this time. Mycelial mats were washed three times in sterile distilled water to remove residual nutrients, submerged in distilled water, and ground with a mortar and pestle. The triturate was then sonified at the highest power setting in an ice bath to disrupt oospores. The sonified triturate was then air-dried, ground into a powder, and stored at −4 C until injected intramuscularly into 8-wk-old New Zealand female rabbits. Each injection comprised 1 ml of fungus triturate plus 1 ml of Freund's complete adjuvant. Booster injections of the preparation were made at 11, 16, and 56 days after the first injection. Bleedings were started 18 days after the last injection and continued on a weekly basis for 6 wk.

Antiserum selectivity and sensitivity. All sera were tested against dried, powdered mycelia, zoospores, and oospores of *A. euteiches*; mycelia of *Rhizoctonia solani* Kühn; conidia and mycelia of *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *pisi* (J.C. Hall) W.C. Snyder & Hanna races 1 and 2, *F. solani* (Mart.) Sacc. f. sp. *pisi* (F.R. Jones) W.C. Snyder & H.N. Hans.; mycelium, oospores, and sporangia of *Pythium irregulare* Buisman, *P. myriotylum* Drechs., *P. paroecandrum* Drechs., and *P. ultimum* Trow var. *ultimum*; and mycelium, oospores, and sporangia of *Phytophthora cinnamomi* Rands, *P. cambivora* (Petri) Buisman, *P. cactorum* (Lebert & Cohn) J. Schröt., and *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian.

R. solani was grown in potato-dex-

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trose broth, and *Pythium* and *Phytophthora* spp. in V8 juice broth, for 7 days in the dark at room temperature. All of the fungal mats produced by *Rhizoctonia, Pythium*, and *Phytophthora* were washed three times in glass distilled water and ground with a mortar and pestle, and the triturate was air-dried. *Fusarium* spp. were grown in Kerr's liquid medium (10) on a rotary shaker, under continuous fluorescent light, at room temperature for 5 days. The resultant mycelia and conidia were centrifuged, the supernatent was discarded, and the pellet was resuspended in glass distilled water, ground with a mortar and pestle, and air-dried.

All bleeds were tested against a previously prepared dried and powdered mycelial and oospore preparation of *A. euteiches*, which was diluted to final concentrations of 0.25, 0.10, 0.04, and 0.02 mg of fungus per milliliter of CEP buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3, 10 g polyvinylpyrrolidone, and 1 g/L egg albumin, pH 9.6). For each fungus, the mycelial preparation was diluted in CEP buffer to a final concentration of 0.1 mg/ml of buffer.

An experiment was conducted to determine if the test antiserum was specific for hyphae, oospores, or zoospores of *A. euteiches*. To produce oospores, the culture was grown on cornmeal agar in the dark for 7 days and then macerated in a blender for 30 sec. The homogenate was decanted and resuspended, and the supernatent was discarded over several cycles. A concentration of 3×10^5 oospores per milliliter was used, and the air dry weight of oospores was determined to the nearest 0.1 mg. This initial concentration was diluted with CEP buffer to give concentrations of 1×10^5, 5×10^4, and 1×10^4 per milliliter. To produce zoospores, the fungus was grown in autoclaved pea seed broth (10 g seed per 200 ml water) for 5 days in the dark. The resultant mycelial mats were washed three times with glass distilled water and placed in a mineral salts (3) solution (0.26 g CaCl, 0.5 g $MgSO_4/7\ H_2O$, 0.07 g KCl/L). The submerged mats were aerated overnight with filtered air. The zoospore suspension was centrifuged at low speed for 5 min, and the supernatant was discarded. A final concentration of 4×10^6 zoospores per milliliter was obtained. This concentration was diluted to 4×10^5, 4×10^4, and 4×10^3, and the zoospore weight was determined with air-dried samples of the initial concentration, weighed to the nearest 0.1 mg. Both oospores and zoospores were disrupted by sonification.

Mycelium free of oospores or zoospores was produced by growing the fungus in autoclaved pea seed broth for 5 days. Mycelial mats were air-dried, and 0.02 g was added to 5 ml of CEP buffer and macerated with a mortar and pestle. This initial triturate contained 4 mg/ml of mycelium and was diluted to give 0.4, 0.04, and 0.004 mg/ml of mycelium.

Prepared dilutions in CEP of all test fungi were loaded into micro ELISA plates (Immulon 2, Dynatech Labs, Inc., Chantilly, VA) with 0.1 ml per well. Plates were incubated for 1 hr in a moist chamber and then washed three times with Tris-Tween (1.2 g Tris base, 8.0 g NaCl, 0.2 g KCl, and 0.5 ml Tween 20 per liter, pH 7.4) for 5 min each. Antiserum was diluted 1:1,000 in Tris-EP (500 ml Tris-Tween, 10 g polyvinylpyrrolidone 40, 1 g egg albumin), and 0.1 ml was dispensed into each well and incubated for 1 hr. The plates were again washed three times with Tris-Tween for 5 min each. The goat antirabbit IgG conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) was diluted 1:1,000 in Tris-EP, and 0.1 ml was dispensed into each well. The plates were again incubated for 1 hr and washed three times for 5 min each with Tris-Tween. Substrate phosphatase (Sigma 104) was mixed with substrate buffer (97 ml diethanolamine, 800 ml H_2O, 0.2 g NaN_3 at 1 mg/ml) and pipetted into each well. Readings were made after a 60-min incubation (7520 Microplate Reader at 405 nm, Cambridge Technology, Inc., Cambridge, MA), and data were presented as an average of at least three wells.

Greenhouse studies. The culture of *A. euteiches* was maintained in slant tubes of cornmeal agar (23) and stored at 4 C. Zoospore inoculum was produced by incubating agar disks in pea seed broth as described earlier. Zoospore numbers were determined after violently shaking suspensions to induce encystment and then counting with a hemacytometer. For this study, zoospore inoculum levels were adjusted to either 100 or 1,000/ml.

Three pea breeding lines (86-2236, 90-2131, and 90-2316 from the USDA-ARS breeding program) with resistance/tolerance to Aphanomyces root rot (13,15), the resistant PI accession 180693 (21), and the susceptible, freezing pea cultivar Dark Skin Perfection were used. Seeds of all test lines were surface-disinfested in 0.5% NaOCl for 1 min and planted in coarse-grade perlite in the greenhouse. Two weeks later, emerged seedlings were inoculated with 50 ml of a zoospore suspension applied directly to the base of each plant. Inoculated pots were placed in plastic saucers equidistant from other pots within a treatment to prevent cross-contamination. The pots were incubated for 5, 7, and 9 days under

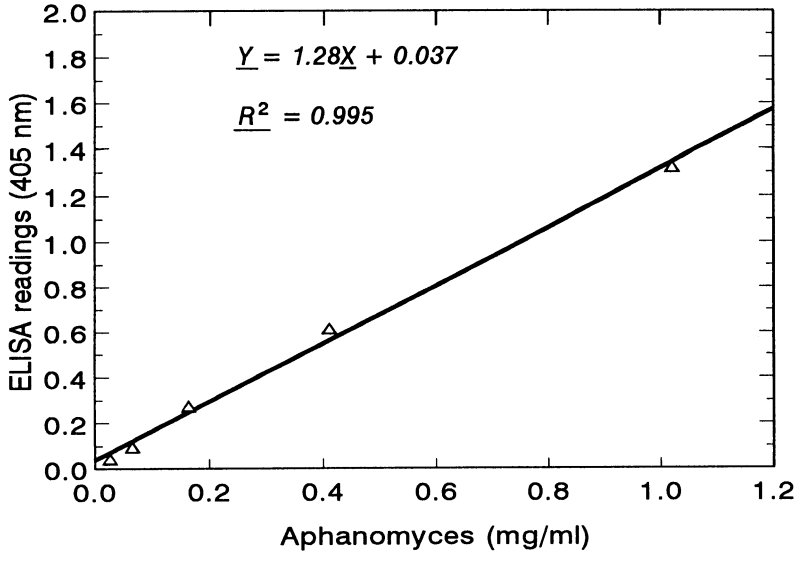

Fig. 1. Sensitivity of antiserum to *Aphanomyces* antigens. Values represent the average of four tests, with each test having at least three wells for each concentration of *Aphanomyces*. ELISA values were read 1 hr after the substrate was added. The antigen was attained by grinding hyphal mats grown in pea seed broth and diluting to final concentrations.

Table 1. Specificity of antiserum developed against antigens of *Aphanomyces euteiches* to antigens produced by several plant-pathogenic fungi

Comparison	Isolate[a]	ELISA reading[b]
1	Buffer	0.059
	Rhizoctonia solani AG-4	0.073
	Fusarium solani f. sp. *pisi*	0.091
	F. oxysporum f. sp. *pisi* race 1	0.118
	F. o. pisi race 2	0.118
	A. euteiches	1.762
2	Buffer	0.029
	Pythium irregulare	0.165
	P. myriotylum	0.117
	P. paroecandrum	0.127
	P. ultimum	0.331
	A. euteiches	1.430
3	Buffer	0.007
	Phytophthora cinnamomi	0.007
	P. cambivora	0.022
	P. cactorum	0.031
	P. citrophthora	0.045
	A. euteiches	1.671

[a]Tested at concentrations of 0.1 mg of mycelia per milliliter of buffer as determined by dilution.
[b]Readings were made at 405 nm and are the averages of at least three wells. Plates were read 1 hr after substrate was added.

greenhouse conditions (20–24 ± 2 C) and watered every day to maintain a saturated condition. There were four replications for each test line, with four seeds per replication arranged in a randomized block design. All data were statistically analyzed by an LSD test.

At time of harvest, disease severity (based on a 0–5 scale, where 0 = healthy root and 5 = completely rotted root), fresh weight of plant tops (above cotyledonary attachment area), and roots (not including the cotyledons) were measured. Taproot tissue (0.3 g per sample) was ground with mortar and pestle in 3.5 ml of CEP, stored overnight at 4 C, and assayed in ELISA tests, which were repeated twice.

To determine if a positive linear correlation existed between ELISA and disease severity readings, all data for 5, 7, and 9 days for PI 180693 and cv. Bolero were used. Disease severity data for 0, 100, and 1,000 zoospores per milliliter were plotted against corresponding ELISA readings.

RESULTS

The reaction of the antiserum from all bleeds to *A. euteiches* antigens was linear, with increased ELISA readings obtained with increased concentrations of antigens produced by *A. euteiches* (Fig. 1). The reactions of all bleeds were similar. The polyclonal antiserum was tested against several plant-pathogenic fungi (Table 1). The only strong absorbance reading was with *A. euteiches*; a moderate reaction occurred against *Pythium ultimum*. The average disease indices for 5-, 7-, and 9-day incubation periods for both inoculum levels and all test pea lines were plotted against absorbance readings for those same incubation periods, inoculum levels, and test lines. A strong, positive linear relationship was evident in that as disease severity increased, so did ELISA readings (Fig. 2).

Mycelium, oospores, and zoospores of *A. euteiches*, used either intact or ruptured, were also tested against the antiserum (Fig. 3). Mycelium reacted strongly with the antiserum, whereas zoospores were moderately reactive and oospores were the least reactive.

Pea breeding line 90-2131 and PI 180693 appeared resistant when inoculated with either 100 or 1,000 zoospores per milliliter during a 5- to 9-day incubation period (Fig. 4). Dark Skin Perfection, which is susceptible under greenhouse and field conditions, exhibited higher absorbance readings than either 90-2131 or PI 180693. Pea breeding lines 90-2316 and 86-2236 had significantly lower absorbance readings than Dark Skin Perfection at an inoculum concentration of 100 zoospores per milliliter at 7 days. However, at 9 days, absorbance readings equaled those of Dark Skin Perfection.

DISCUSSION

The results reported here show that antigens produced by mycelia and zoospores of *A. euteiches* in infected pea roots can be readily detected. The correlation between increased amounts of antigen and increased ELISA readings was linear, as was the correlation between disease severity readings and ELISA readings. The ELISA technique was sensitive enough to allow quantitative measurements of *A. euteiches* in infected root tissue, even in homogenates of taproot extracts. In contrast to infected root tissues, ELISA values of noninoculated roots were very low. As the incubation time increased, so did disease severity readings, pathogen growth within infected root tissue, and corresponding ELISA readings. Our results were similar to those reported for other microbial-plant systems (4,6). El Nashaar et al (6) reported that antigens of *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *tritici* J. Walker could be readily detected by ELISA with use of a polyclonal antiserum, and quantitative measurements of pathogen invasion were obtained.

Our results indicate that ELISA may be used for rapid field diagnosis of *A. euteiches* in seedling pea roots where mycelial growth is prevalent. The antisera can readily detect antigens produced by mycelium and zoospores of *A. euteiches*. In addition, little or no reaction occurred from antigens produced by other common pea root pathogens,

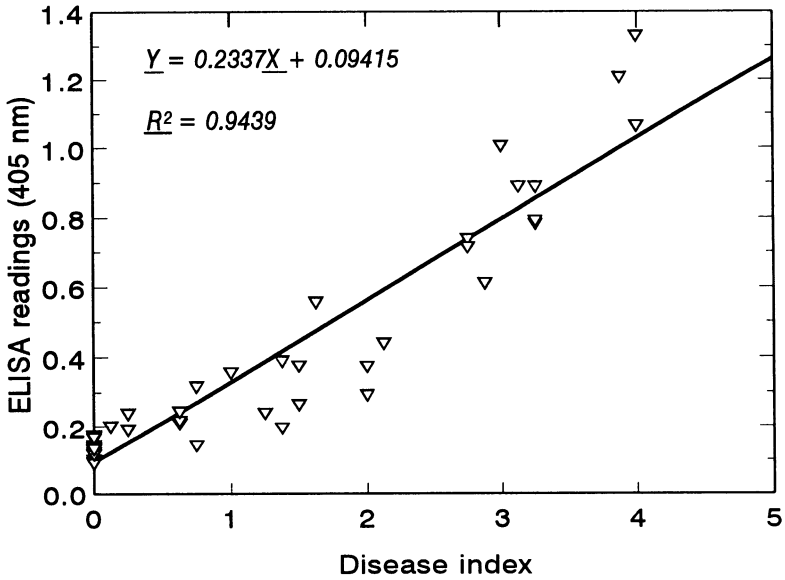

Fig. 2. ELISA readings plotted against disease index. The 45 points on the graph represent the values for harvest dates 5, 7, and 9. Each point is the average of four replications, two wells per replication, and two tests. ELISA readings were taken 1 hr after substrate was added. Disease index is based on a scale of 0–5, with 0 = no infection and 5 = 100% infection of the roots.

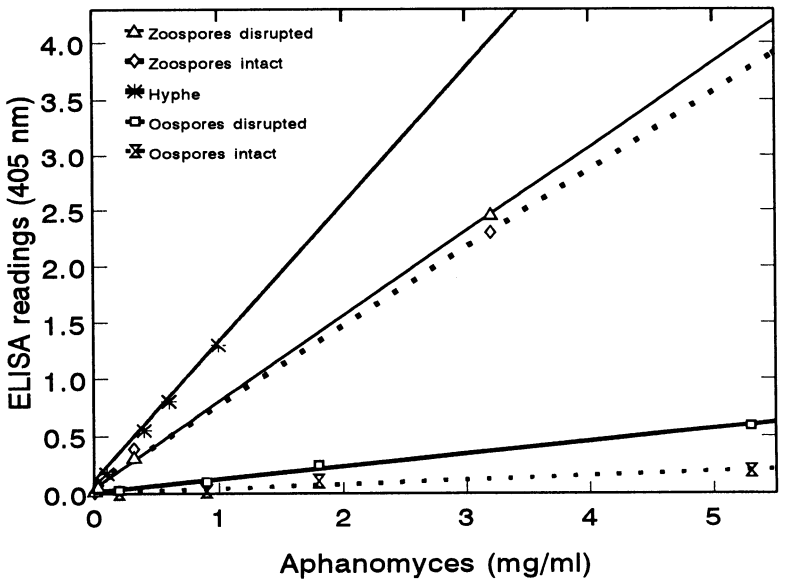

Fig. 3. Reactivity of hyphae, zoospores, and oospores of *Aphanomyces euteiches* to the antiserum.

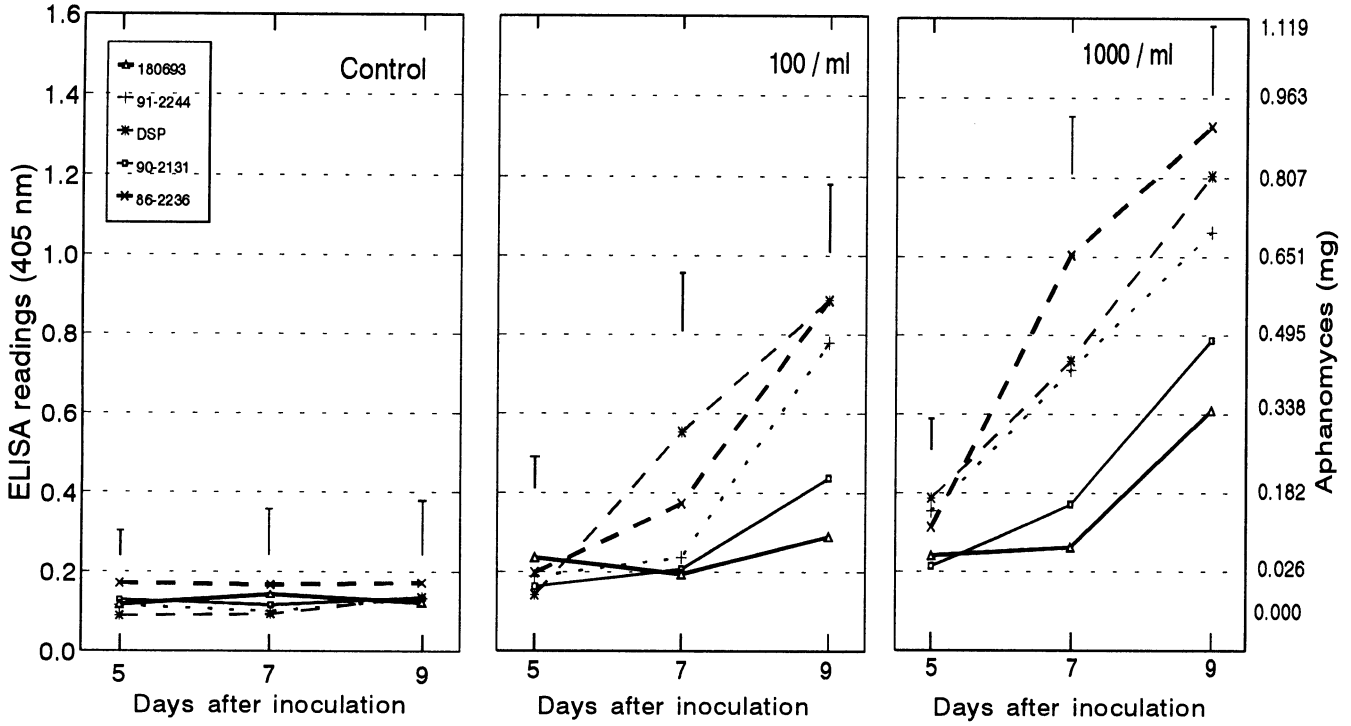

Fig. 4. Comparison of pea cultivars and time after inoculation at three inoculum levels. Each point is the average of four replications and two tests. ELISA readings were taken 1 hr after the substrate was added. Error bars are at the $P = 0.05$ level determined by LSD analysis. Values found on the right Y axis are from Figure 1.

including *F. s. pisi, F. o. pisi* races 1 and 2, *R. solani*, and *Pythium* spp. In addition, there was no reaction of the antiserum with antigens produced by another root pathogen in the Peronosporales, *Phytophthora*. However, detection of *A. euteiches* in senescent, infected pea root tissue, where oospores are abundant, was not possible using this antiserum.

Previous research has shown that resistance to *A. euteiches* in peas results in lower oospore numbers in excised root tips, perhaps related to less fungal multiplication (24). Counting oospores in excised root tips is very time-consuming, particularly if large numbers of lines are to be assessed. Quantitative measurement of vegetative growth of *A. euteiches* on and within inoculated root tissue is possible in seedling roots by using this antiserum. Changes in absorbance readings were correlated with inoculum level, time of incubation, and host genetics.

Use of ELISA has demonstrated that resistance to multiplication of *A. euteiches* occurs within pea root tissue. This resistance was illustrated, especially by PI 180693 and 90-2131, and was evidenced by reduced disease indices and pathogen multiplication within the taproot after a 5- to 9-day incubation period. Breeding lines 90-2316 and 86-2236 were intermediate in their disease responses. Inoculum level was previously shown to be important in screening pea lines for resistance to *A. euteiches* (20, 21), and this was reconfirmed in this report. At the lower inoculum level, PI 180693, 90-2131, and 90-2316 had significantly less fungal antigen than Dark Skin Perfection. At the higher inoculum level, only 90-2131 and PI 180693 were less infected than Dark Skin Perfection.

The ARS breeding program at Prosser, Washington, has been developing peas resistant to Aphanomyces root rot for the last 17 yr (11–16). It is encouraging that quantitative resistance to this serious cortical root pathogen is inheritable, can be quantified, and exists in the pea genome. PI 180693 has a high level of resistance to *A. euteiches* (21), and line 90-2131, which is quite resistant to *A. euteiches*, has PI 180693 in its pedigree (15).

This is the first report of the development of an antiserum that can detect antigens of *A. euteiches* with little or no reaction with antigens produced by other pea root pathogens and nonpathogens. In addition, cellular resistance to *A. euteiches* was demonstrated and quantified.

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