
Production and Characterization of Antiserum to *Aphanomyces cochlioides*.

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ABSTRACT

Methods for the evaluation of root rot disease in sugarbeet caused by pathogenic fungi historically have relied on visual assessment. In an initial attempt to develop complementary means to detect and quantitate root rot disease caused by *Aphanomyces cochlioides*, antiserum was produced in rabbits that had been immunized with a cell-wall preparation of this organism. Specificity tests using enzyme-linked immunosorbant assay (ELISA) indicate that the antiserum is strongly reactive with both *A. cochlioides* and *A. euteiches*, but weakly with oomycetes non-pathogenic to sugarbeet, with filamentous fungi that infect sugarbeet, or with extracts prepared from healthy sugarbeet. A 1:2,000 dilution of the serum was sufficient to readily detect *A. cochlioides* in infected sugarbeet seedlings. Sugarbeet roots obtained from a piling station in Minnesota, USA that exhibited adult root rot symptoms characteristic of those caused by *A. cochlioides* tested negative for the presence of this pathogen. The antiserum provides an additional tool for the detection of *A. cochlioides* in field and greenhouse-grown sugarbeet and for immunochemical investigations of root rot disease.

Additional Key Words: *Beta vulgaris*, Black-root, ELISA, detection, oomycete

Root rot of sugarbeet (*Beta vulgaris* L.) caused by the oomycete *Aphanomyces cochlioides* Drechs. is a devastating disease of historical and re-emerging importance (Duffus and Ruppel, 1993, Beale et al., 2002). Significant yield losses due to *A. cochlioides* infection have occurred in Minnesota and North Dakota as a consequence of heavy mid-summer rains in recent years. In contrast to infection by *Rhizoctonia solani* which yields a dark-pigmented, penetrating rot in mature sugarbeet (Schneider and Whitney, 1986a), infection by *A. cochlioides* produces a more constricted and gnarled root phenotype accompanied by root surface russeting (Schneider and Whitney, 1986b). In addition to field losses to growers from the disease, roots infected by *A. cochlioides* that reach maturity may, in the case of moderate to severe infection, exhibit elevated respiration resulting in accelerated decline of stored beet quality (Campbell and Klotz, 2003).

Seedling damping-off caused by *A. cochlioides* can be controlled by the treatment of sugarbeet seed with hymexazol, the efficacy of which declines over a 5-6 week period (Payne and Williams, 1990). For this reason, control of the disease in adult plants to date has been provided by host resistance. Although much of the sugarbeet germplasm developed for resistance to *A. cochlioides* was selected after exposure of seedlings to the pathogen, many investigators have reported difficulty in detecting host resistance at the seedling stage (Coe and Schneider, 1966, Windels and Brantner, 2000). Current protocols for the selection of varieties with improved resistance to the chronic (adult) phase of black root disease include the inoculation of immature roots with zoospores of the pathogen in the greenhouse or the evaluation of accessions in field plots after treatment of the seed with hymexazol, which only controls the seedling phase of the disease. In both cases, harvested roots are examined and scored visually for the extent of damage by the pathogen (Windels and Brantner, 2000).

Evaluation of resistance to plant viruses, including the causal agent of sugarbeet Rhizomania, beet necrotic yellow vein virus, often includes an assessment of virus load in the plant using virus-specific antiserum (Agrios, 1988). Additionally, such antisera have been used in the immunocytochemical localization of virus within the infected plant or plant cell (Matthews, 2001). In an effort to develop a similar tool for use with *A. cochlioides* infections, antiserum was produced using mycelium of the pathogen.

MATERIALS AND METHODS

Fungal cultures and pathogen and plant extracts

Isolates of *A. cochlioides* Drechs., *A. euteiches* Drechs.,

Saprolegnia parasitica Coker, *R. solani* Kuhn, *Cercospora beticola* Sacc., *Phoma betae* Frank, *Pythium aphanidermatum* (Edson) Fitzp., and *P. ultimum* Trow were cultured on commercial potato dextrose agar. The oomycetes *Phytophthora infestans* (Mont.) deBary and *P. erythroseptica* Pethybr. were cultured on V8 juice agar (Table 1). Cultures were maintained in the dark at 22°C. Isolates subsequently were grown in clarified V8 juice broth (*P. infestans*, *P. erythroseptica*), peptone glucose broth (*A. cochlioides*, *A. euteiches*, *S. parasitica*), or potato dextrose broth (*R. solani*, *C. beticola*, *P. betae*, *P. aphanidermatum*, *P. ultimum*) for 7 days in a dark, 22°C chamber (Singleton et al., 1992). Preparation of pathogen extracts involved rinsing of mycelial mats with distilled water and grinding the mycelium with a mortar and pestle in 3 ml distilled water per gram fresh weight tissue. After grinding, the liquid from each preparation was decanted into a vial and autoclaved for 20 min. at 120°C and 1.1 kg/cm² pressure. Sterilized extracts were stored at 4°C.

Seedlings of sugarbeet variety ACH 9369 (seed generously provided by Dr. John Kern, American Crystal Sugar Co., Moorhead, MN) were planted into soil naturally infested with *A. cochlioides*. At 7 days post-planting, dying seedlings were ground in a mortar and pestle in a similar manner and autoclaved. Finally, mature sugarbeet roots obtained from the beet piling station near Sabin, Minnesota in November of 2003 were divided into diseased and healthy groups (15 roots per group) based on typical Aphanomyces symptoms. Roots were washed and root surface tissue used to prepare an autoclaved extract as described above, except that acid-washed sand (Sigma

Table 1. Organisms used to characterize antiserum to *A. cochlioides*.

Organism	Source [†]	Contributor
<i>Aphanomyces cochlioides</i>	MN soil	J. Weiland
<i>Aphanomyces euteiches</i>	MN soil	C. Windels
<i>Pythium aphanidermatum</i>	MN soil	J. Weiland
<i>Saprolegnia parasitica</i>	ATCC#200015	S. Kamoun
<i>Phytophthora erythroseptica</i>	ND potato	N. Gudmestad
<i>Phytophthora infestans</i>	ND potato	N. Gudmestad
<i>Rhizoctonia solani</i> AG4	ND sugarbeet	W. Bugbee
<i>Rhizoctonia solani</i> AG2-2	CO sugarbeet	L. Panella
<i>Cercospora beticola</i>	MN sugarbeet	J. Weiland
<i>Phoma betae</i>	ND sugarbeet	W. Bugbee

[†] Isolates originated from Minnesota (MN) and North Dakota (ND) fields or from the American Type Culture Collection (ATCC).

Chemical, St. Louis, MO) was included during the grinding as a pulverizing agent.

A. *cochlioides* cell wall preparation

Three flasks containing *Aphanomyces cochlioides* grown in 50 mL of potato dextrose broth per flask for 15 days were filtered through 2 layers of Miracloth (Calbiochem, La Jolla, CA USA). The filtrate was discarded and the mycelia harvested had a wet weight of 4.51g. The mycelia were put in a 40 mL ultracentrifuge tube and 15 mL of nanopure water (Millipore Inc., Billerica, MA USA) was added. The contents of the tube were homogenized using a Tekmar tissuemizer (Tekmar Inc., Cincinnati, OH USA) for 1 minute and the tube cooled on ice. This was repeated three times with the contents cooled between pulsations. The suspension was centrifuged at 10,000 g for 25 minutes and the supernatant removed. The tissue was resuspended and the entire process was repeated 4 times finally giving 1.43 g (wet weight) of a cell wall preparation. The cell wall preparation was suspended in 7.5 mL of phosphate buffered saline (PBS, pH 7.2: 0.14 M sodium chloride, 3 mM potassium chloride, 2 mM potassium phosphate, 10 mM sodium phosphate) and stored at -20°C until used.

Antibody generation

Four female New Zealand White rabbits were obtained from Mytle's rabbitry, Inc. (Thompson Station, TN USA), were given water and food *ad libitum*, and were housed individually. The rabbits were injected with 200 μg of cell wall emulsified with Imject Alum (Pierce Biotechnology, Inc., Rockford, IL USA) as 2 intramuscular and 4 subcutaneous injections (0.5 mL emulsion/site).

Rabbits were given monthly booster injections for 4 months total in addition to the initial immunization. Control samples were obtained prior to the immunization, and test samples collected from the 2nd boost, with all samples being taken from the marginal ear vein. After positively identifying rabbits that had a high titer of antibodies for *A. cochlioides* two more boost injections were made prior to the final blood draw. The final bleeding was done at 6 months post-immunization using cardiac puncture after the rabbits were anesthetized with halothane. The final sera collected ranged from 33-67 mL. The sera were stored at -80°C until used.

ELISA development

Extracts made from fungal biomass and infected or symptomatic sugarbeet tissue were used for characterization of the antiserum.

Extract (50 μ L) was pipetted into the wells of an Immulon 96-well plate (Immulon-Dynatech, Chantilly, VA USA) with each sample tested in triplicate. Samples were dried overnight at 60°C in order to permit the sample to adhere to the bottom of the well. Wells were pre-incubated for 1 hr with a solution containing 5% non-fat dried milk dissolved in TBS pH 8.0 (10 mM TRIS-HCl, 0.15 M NaCl) to block non-specific binding sites in the well. During this time, the anti-*Aphanomyces* antiserum was mixed to various dilution levels in ELISA buffer (2% [w/v] polyvinylpyrrolidone-40 and 0.2% ovalbumin in 1 liter of PBST). After rinsing the wells four times with PBST (10 mM NaPO₄ pH 7.4, 150 mM NaCl including Tween-20 at 0.05%), 100 μ L of the diluted antisera was added to the wells and allowed to react for 4 hr at room temperature. This was followed by four washes with PBST after which a 100 μ L of goat anti-rabbit IgG, alkaline phosphatase conjugate (GAR-AP; Sigma Cat. #A0418) diluted 1:5,000 in ELISA buffer was added to each well. Following one hour of incubation at room temperature, the secondary antibody was discarded and the wells were washed five times with PBST. After the final wash, 200 μ L of ELISA substrate buffer containing 1.0 mg/ml of para-nitrophenyl phosphate (pNPP) were added and the plate incubated at room temperature. At various times after substrate addition, the plate was read in a microplate spectrophotometer (Spectramax™, Molecular Devices, Sunnyvale, CA USA) with a wavelength setting of 405 nm.

Statistical analysis was performed using functions within the Microsoft® Excel™ spreadsheet software with the post-hoc calculation of Tukey's Least Significant Difference. Analysis was performed on three independent cultures for each organism tested and each culture was tested in duplicate by ELISA. Because antiserum from rabbit #114 exhibited the highest signal-to-noise ratio in ELISA tests, all subsequent characterization described below was performed with this serum at a 1:2,000 dilution level. A comparison of post-immune antiserum from rabbit #114 with pre-immune serum from that same animal in the ELISA was performed using the above method.

Western Blot analysis.

Extracts that were made for ELISA analysis were fractionated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred to a nitrocellulose membrane for subsequent reaction with antiserum. Gels were made using standard methods with a 12% concentration of acrylamide in the resolving gel (Ausubel et al., 2002). An extract sample (15 μ L) was mixed with 5 μ L of sample loading dye and the samples were electrophoresed at 15V/cm in a Bio-Rad Mini-

Protean II gel system. After electrophoresis, gels were placed into an Idea Scientific Mini-Genie Electrobloetter (Minneapolis, MN USA) for the transfer of proteins to nitrocellulose overnight at 6V. Nitrocellulose membranes then were transferred to a solution containing Tris-buffered saline (TBS, pH 8.0) and 5% (w/v) non-fat dried milk. The blots were subsequently incubated for 4 hr at room temperature with TBS containing 5% non-fat dried milk plus 1% TWEEN 20 (MTTBS) and included a 1:2,000 dilution of the anti-*Aphanomyces antiserum*. This was followed by three washes with MTTBS and a subsequent incubation with MTTBS containing a 1:5,000 dilution of GAR-AP. After three washes each with TBS and TBS, membranes were transferred to a solution of CDP-Star™ (Amersham Biosciences, Piscataway, NJ USA), sandwiched between cellophane sheets, and exposed to X-ray film. Film images were digitized with an Alpha-Innotech (San Leandro, CA USA) ChemImager™ 6000 system.

RESULTS AND DISCUSSION

In the present work, antiserum raised in rabbits against a cell-wall preparation made from *A. cochlioides* was shown to be highly reactive to two members of this genus, *A. cochlioides* and *A. euteiches* in the ELISA test (Figure 1A). Some cross-reactivity of the antiserum was observed with extracts prepared from other oomycetes and other fungi pathogenic to sugarbeet. Antiserum reactivity with *A. cochlioides* and *A. euteiches*, however, was significantly higher ($p \leq 0.01$) than that observed for the other organisms tested. Reactivity of pre-immune serum with antigen from *A. cochlioides* and *A. euteiches* was minimal, indicating that serum reactivity observed required exposure to antigen ($p \leq 0.01$; Figure 1B). The significantly higher signal obtained for reaction with *A. euteiches* as compared to *A. cochlioides* in Figure 1A may indicate higher production of a specific antigen in the preparations of *A. euteiches* used in the study, or the production of a novel antigen lacking in *A. cochlioides*, that reacts with the antiserum. Irrespective of the basis for this effect, additional methods, such as polymerase chain reactions methods (PCR; Weiland and Sundsbak, 2000) would be needed to distinguish *A. cochlioides* from *A. euteiches*.

Differences were clearly noted in the signal generated by ELISA of extracts prepared from healthy sugarbeet seedlings as compared to infected seedlings produced in the greenhouse ($p \leq 0.05$; Fig. 2). As shown in Fig. 2, the antiserum possesses low background binding to extract from healthy sugarbeet even after 4 hr of incubation with substrate. Since the level of reactivity with *A. cochlioides* can readily be distinguished from that produced by interaction with other potential

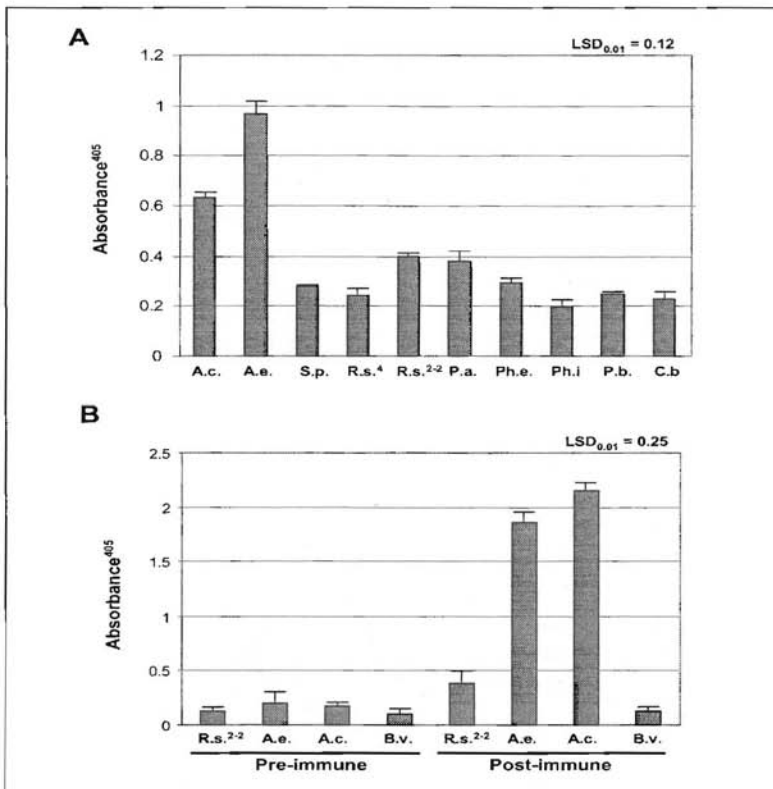


Fig. 1. Figure 1 Discrimination between *Aphanomyces* and other fungi and oomycetes by ELISA (A) and demonstration of reactivity of post-immune serum (B). In A the organisms *Aphanomyces cochlioides* (A.c.), *Aphanomyces euteiches* (A.e.), *Saprolegnia parasitica* (S.p.), *Rhizoctonia solani* AG4 (R.s.⁴), *Rhizoctonia solani* AG2-2 (R.s.²⁻²), *Pythium aphanidermatum* (P.a.), *Phytophthora erythroseptica* (Ph.e.), *Phytophthora infestans* (Ph.i), *Phoma betae* (P.b.), and *Cercospora beticola* (C.b.) were tested with the antiserum. Bars reflect the means of testing three independent cultures of each organism where each culture was tested in duplicate wells (i.e. six wells per organism tested). Absorbance at 405 nm denotes the relative extent of reactivity (y-axis) after 2 hr incubation with substrate. In B, the reactivity of a 1:2000 dilution of pre-immune serum was compared to that for post-immune serum using R.s.2-2, A.e., A.c., and an extract from healthy sugarbeet seedlings (B.v.) as antigen targets. Four hr after substrate addition the absorbance of the samples at 405 nm was recorded. Error bars indicate standard deviation.

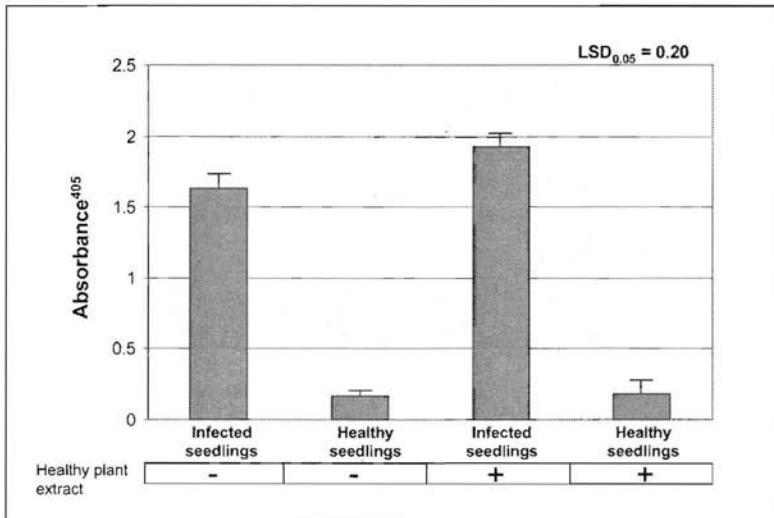


Fig. 2. Detection of *A. cochlioides* in infected seedlings by direct ELISA using anti-*Aphanomyces* antiserum. Extracts were prepared from symptomatic and healthy seedlings. Similar mass amounts of symptomatic and healthy tissue were processed for the ELISA. An aliquot (50 μ l) of extract from healthy seedlings was added (+) to the diluted primary antibody (10 ml) prior to use or diluted antiserum was used without added extract (-). Absorbance at 405 nm is indicated after incubation with the substrate for 4 hr. Error bars indicate standard deviation.

pathogens, the assay should be applicable to field-grown seedlings. Although in some regions sugarbeet is grown in fields known to contain *A. euteiches*, the recovery of *A. euteiches* from diseased sugarbeet seedlings is uncommon (Larsson, 1994), reducing the probability of false positive reactions due to the presence of this organism. Using the methods described, serum pre-absorption with extracts from healthy beet seedlings did not reduce background reactivity between the serum and healthy beet antigen (Figure 2), obviating the need to pre-absorb the antisera before each use.

In complementary tests, roots obtained from a sugarbeet storage pile that exhibited characteristic symptoms of chronic phase *Aphanomyces* root rot tested negative for presence of the fungus (not shown). This suggests that although these roots came from beet plants that were attacked by *A. cochlioides* during their growth, little mycelium was present at the time of harvest. The tested roots may still have

harbored oospores of *A. cochlioides*, but it is not known what quantities of oospores might exist in these samples or whether the antiserum possesses affinity for this phase of the organism. Although sugarbeet exhibiting significant symptoms of attack by *A. cochlioides* exhibit accelerated decline in storage relative to healthy beets (Campbell and Klotz, 2003; Alan Dyer, pers. comm.), data here supports the possibility that *A. cochlioides* growth per se in stored beets is at most a minor component of storage decline. This is consistent with the observations of Kjoller and Rosendahl (1998) who showed that *A. euteiches* is a poor saprophyte on dead roots of pea.

Since the antiserum was produced against a cell-wall preparation of *A. cochlioides*, it was anticipated that the serum would recognize high molecular weight components in extracts of this pathogen due to formation of covalent complexes between proteinacious or other epitopes recognized by the antiserum with cell wall fragments of heterogeneous molecular size. Nevertheless, it is possible that the serum also

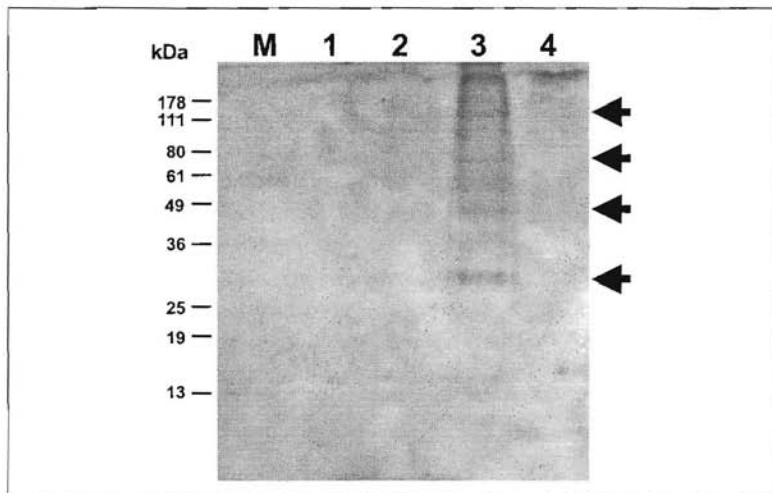


Fig. 3. Western Blot of *A. cochlioides* extract. Extracts were fractionated by SDS-PAGE and blotted to nitrocellulose. After reacting the blot with anti-*Aphanomyces* antiserum, chemiluminescent detection revealed reactive bands. Molecular weight markers were run as size standards (lane M) and extracts from healthy seedlings (lane 1), infected seedlings (lane 2), cultured *A. cochlioides* (lane 3) and cultured *R. solani* AG2-2 (lane 4) were applied in the experiment. At least 4 distinct *A. cochlioides* proteins are observed to react with the antiserum (lane 3, indicated by arrows).

would recognize proteins secreted by *A. cochlioides*, such as protease (JJW, submitted), that may play a role in pathogen virulence. A Western immunoblot shows that the bulk of the cross reacting material in extracts prepared from *A. cochlioides* mycelium is indeed of a medium to high molecular weight nature (Figure 3). The possibility that proteins or factors secreted by *A. cochlioides* can be detected by the antiserum remains under investigation. Although the ELISA data in Fig. 2 indicated that extracts from infected seedlings react strongly with the antiserum, the antiserum reacted with components of this extract on a Western blot only poorly. This could be explained by the possibility that the distinct immunoreactive proteins produced in culture by *A. cochlioides* (Fig. 3, lane 3) become degraded in the total plant extract, thereby reducing the apparent reactivity in this detection system.

In addition to visual keys and rating scales, proposed means by which *Aphanomyces* root rot can be diagnosed and quantified include the use of specific antisera in quantitative ELISA or specific DNA probes that could exploit quantitative DNA detection methods (Vandemark et al., 2002). Although both methods have advantages, use of ELISA represents a robust technique familiar to the majority of crop industries and plant disease diagnostic laboratories and has already been documented for the detection of *A. euteiches* in legumes (Kraft and Boge, 1994). Based on the results described here, use of the antiserum in the detection of *Aphanomyces* in both field and laboratory grown sugarbeet should prove useful, in addition to applications to immunomicroscopy and biochemistry of the *Aphanomyces* cell wall. Modification of protocols reported here further promise to produce new methods for the evaluation of sugarbeet germplasm or breeding material for resistance to *Aphanomyces* via quantitation of oomycete mycelia in synchronously inoculated plants.

ACKNOWLEDGEMENTS

The authors acknowledge Gary Nielsen and Amy McGarvey for technical assistance, Dr. Carol Windels, University of Minnesota at Crookston for the *A. euteiches* culture, Dr. Sophien Kamoun, Ohio State University at Wooster for the *S. parasitica* culture, and Dr. Neil Gudmestad, North Dakota State University, for the cultures of *P. erythroseptica* and *P. infestans*. This work was supported by USDA-ARS CRIS# 5442-22000-033-00D (J.W.) and CRIS # to 5442-32000-009-00 (W.S.).

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