Enzyme-linked Immunosorbent Assay for *Cercospora beticola* in Soil

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ABSTRACT

Naturally infected sugarbeet residues mixed with soil during tillage after harvest can serve as inoculum for the leaf spot fungal pathogen *Cercospora beticola* when a new sugarbeet crop is planted. An enzyme-linked immunosorbent assay (ELISA) was developed in an attempt to quantify *C. beticola* mycelial biomass in soil. Amounts as small as 0.38 µg of freeze-dried *C. beticola* mycelia dispersed in carbonate buffer were detected. Fungi from different classes (Ascomycetes, Basidiomycetes, and Hyphomycetes) showed negligible cross reactivity with the polyclonal antibodies, except for the isolates of Fusarium and Trichoderma. A pre-adsorption of the antibodies was used to decrease cross reactivity of the antibodies to these isolates. Evaluation of field soil naturally infested with *C. beticola* showed that the assay using pre-adsorbed serum augments current detection methods and is a potential diagnostic tool for quantifying the amount of pathogen antigens in soil, therefore the potential for incidence of leaf spot disease.

Additional Key Words: *Cercospora beticola*, ELISA, leaf spot disease, pre-adsorption, soil.

*C*ercospora beticola Sacc. causes leaf spot in sugarbeet (Bleiholder and Weltzien, 1972; Windels et al., 1998) and lesions in safflower (Lartey et al., 2005). The disease is reported wherever sugarbeet (*Beta vulgaris* L.) is grown. The pathogen over-winters as stromata in infected beet residue in the soil where it survives for up to 27 months (Nagel, 1938). Under conditions of relatively high humidity or heavy dew, conidiophores and conidia are produced on stromata. The current model

indicates that conidiophores and conidia are primary inoculum and are dispersed by wind, irrigation, rain, water, and insects to sugarbeet to initiate infections (Ruppel, 1986; Whitney and Lewellen, 1976). Weiland and Koch (2004) have postulated that sporulation may be preceded by saprophytic, vegetative growth of fungal mycelia. Indeed, Vestal (1933) suggested that *C. beticola* grows saprophytically on dead tissues of sugarbeet and several weed hosts in the soil. Recent research has shown that *C. beticola* may infect roots of sugarbeet seedlings (Vereijssen et al., 2004).

Even though *C. beticola* survives in the soil for years, it is considered a foliar pathogen. Cercospora leaf spot is mainly controlled with foliar applied chemicals (Jacobsen et al., 2000). Other control measures include clean plough down of crop residues, use of resistant cultivars and two-to-three year rotation with non-hosts (Ruppel, 1986). Recently, the possibility of controlling the pathogen with foliar applied bacterial agents has been documented by Jacobson et al. (2000). This is consistent with current practices in which control is applied upon the appearance of symptoms of the disease.

Understanding survival and longevity in the soil may suggest prospects for alternate approaches that focus on soil applied agents to control *C. beticola*. Such an approach certainly requires a technique to evaluate the efficacy of any applied biological agent. Several diverse indirect methods, such as soil-dilution and plate counts, continue to be used for qualitative and quantitative assessment of soil microorganism, including fungal propagules. These methods, however, are labor intensive, cumbersome and require considerable taxonomic expertise. Also they favor not only viable propagules and abundantly sporulating fungi but also fast growing non-sporulating fungi (Curl and Truelove, 1985). To our knowledge there is no semi-selective media for *C. beticola*. Enzyme-linked immunosorbent assay (ELISA) has been used to detect many fungal pathogens and non-pathogens in host tissues (Aldwell et al., 1983; Unger and Wolf, 1988) and in soil, such as *Phytophthora* (Klopmeyer et al., 1988), *Glomus* (Wright and Morton, 1989), *Thanatephorus* (Dusunceli and Fox, 1992), and *Rhizoctonia* (Thornton et al., 1993), however there is no report on the detection and quantification of *C. beticola* in soil, as there is for leaf tissues (Lartey et al., 2003). Direct detection in soil is difficult due to the problems encountered in extracting fungal antigens from soil and to interference from non-specific soil contaminants. Retention of the antigen may occur on components of the soil solid phase by a range of processes including electrostatic bonding by ion exchange or hydrogen bonding, in particular between N-H groups and organic compounds (Dewey et al., 1997).

To overcome these problems, many of the soil-based immunoassays employ a period of biological amplification, which usually involves baiting with host tissue (Klopmeyer et al., 1988) or enrichment in solid or aqueous semi-selective media (Yuen et al., 1993; Thornton et al., 1994) to allow growth of viable fungal propagules. Moreover, elimination of contaminant soil components is most important where the detection assay involves the immobilization of fungal antigen on to a solid support, for example a PVC microtitre well. Many techniques, such as increasing the dilution of the soil extract, the use of blocking agents or the use of detergents in the washing steps were attempted to minimize or reduce the amount of interference.

Because antisera raised against fungi often cross-react to related or unrelated fungi when tested by ELISA (Dewey et al., 1984), we assessed the possibility of using pre-adsorbed polyclonal antibodies to attain a degree of specificity that could practically emulate the effectiveness of monoclonal antibodies. The objective of this research was to develop an enzyme-linked immunosorbent assay (ELISA) using pre-adsorbed polyclonal antibodies for the detection and quantification of *C. beticola* in soil.

The pre-adsorbed antibody was tested against fungal organisms from different classes, including Ascomycetes, Basidiomycetes, and Hyphomycetes, for potential cross reactivity. This study also could indicate saprophytic growth of *C. beticola* in soil.

MATERIALS AND METHODS

Cell wall preparation. *Cercospora beticola* isolate C2 (Whitney and Lewellen, 1976) was used for the production of antibodies. Cultures were grown in potato dextrose broth (PDB, Difco, Detroit, MI) at 25°C for 4 weeks and centrifuged at 10,000 g at 4°C for 15 min using a Sorvall superspeed RC-2 centrifuge (Sorvall Instruments Division, DuPont Company, Wilmington, DE). Mycelium was homogenized once in distilled water and at least 3 times in saline phosphate buffer (PBS, 8.00 g NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4/liter, adjusted to pH 7.2) using a homogenizer with an 8 mm rotator (SDT Tissumizer, Tekmar Company, Cincinnati, OH). Each cycle of homogenization was performed on ice twice at 60 sec intervals. The fineness of the cell wall fragments was assessed by its ability to pass through a 20-gauge needle. Wet weight was used for mass estimation of the precipitated cell wall. The final cell wall preparation was divided into aliquots and stored at -20°C.

Polyclonal antibodies (PAbs) production. A female goat (Boer cross-breds) was obtained from Solberg farms, Wild Rice, North Dakota. One mg of cell walls, derived from a 2 week-old culture of *C. beticola* was injected into 6 subcutaneous sites for each immunization. Test bleeding was obtained from the jugular vein 10 to 14 days after each injection. After successfully identifying the presence of specific antibody, the third boost blood collection was done via jugular vein. After the blood was collected it was allowed to clot at 37°C for 2 h and the blood clot was further constricted at 4°C overnight. Serum was obtained by centrifuging clotted blood cells and stored in 2.0 ml portions at < -20°C until used.

ELISA. The antibodies were tested for cross reactivity with *Fusarium oxysporum* (ATCC # 9593), *Fusarium proliferatum* (ATCC # 201904), *Trichoderma harzanium* (ATCC # 52443), *Trichoderma* (*Gliocladium*) *virens* (ATCC # 48179), *Trichoderma aureoviride* (provided by Ken Conway, Oklahoma State University, Stillwater, OK), *Rhizoctonia solani* (provided by A. J. Caesar, USDA, ARS, Sidney, MT), *Peniophora nuda* (provided by R. G. Thorn, University of Western Ontario, Canada) , *Gaeumannomyces graminis,* var. *graminis* (provided by J. Henson, Montana State University, Bozeman, MT), *Botrytis cinerea* (ATCC # 48344), *Laetisaria arvalis* (provided by Ken Conway) and *Cercospora beticola* isolates C1, C2 and S1 (provided by J. J. Weiland, USDA-ARS, Fargo, ND and by A. J. Caesar, USDA, ARS, Sidney, MT). *Fusarium* species were chosen because they are commonly found in soils and cause major diseases in sugarbeet (Schneider and Whitney, 1986). *Rhizoctonia solani*, a common soil fungus, causes foliar blight and root and crown rot of sugarbeet (Ruppel, 1986). *Laetisaria arvalis, T. harzanium, T. aureoviride* and *T. virens* are being evaluated for biological control of *C. beticola* in soil. The other fungi were chosen as representatives of a variety of classes (Basidiomycetes, Ascomycetes, and Hyphomycetes). Pure cultures of fungi were grown in potato dextrose broth for 4 weeks (1 week for *Fusarium* and *Trichoderma* spp.) at 25°C then freeze-dried. Freeze-dried fungi (25 mg/ml) were homogenized in carbonate buffer (20 mM $NaHCO_3$, 28 mM Na_2CO_3, pH 9.6) then centrifuged for 1 min (14,000 g). The supernatant was serially diluted then 100 μl of each dilution was loaded in microtiter plate wells (Immulon 4HBX, Dynex Technologies Inc., Chantilly, VA) followed by incubation overnight at 25°C. After washing with phosphate buffer saline-Tween 20, (PBST, 0.01 M, Sigma, St Louis) a 1/20,000 dilution in PBS of the immune serum containing the antibodies of *C. beticola* was added (100 μl/well). Plates were incubated for 90 min at 25°C, and

then washed again with PBST. A 1/13,000 dilution in PBS of horserad-
ish peroxidase-conjugated (H + L) rabbit anti-goat immunoglobulins
(Jackson Immuno. Res. Lab., Inc. West Grove, PA) was then added (100
μl/well) and the plates were incubated for 1 h at 25°C. After washing,
substrate solution of 3,3', 5,5' tetramethylbenzidine (0.4 g/l) (Pierce,
Rockford, Illinois) and hydrogen peroxide (50 % in 3,3', 5,5' tetrameth-
ylbenzidine) was added, then the reaction was stopped with sulfuric
acid after 30 min. Absorbencies were collected at 455/655 nm using
a microplate reader (BioRad, Hercules, CA). The cross reactivity tests
were repeated 3 times. Standard curves, relating antigen extracts of *C.
beticola* freeze-dried (Labconco Free Zone™ freeze dryer, Kansas City,
MI) mycelia to absorbance value were constructed using pre-adsorbed
serum and non pre-adsorbed serum.

Pre-adsorption technique. *Fusarium oxysporum, F. proliferatum, T.
harzanium, T. virens,* and *T. aureoviride* were grown in PDB for 9 d on
a gyratory shaker (100 rpm, 2.5 cm circular orbit) at 25°C. The cultures
of *F. oxysporum* and *F. proliferatum* were mixed (0.3 ml of each) then
transferred into a 2.0 ml-volume micro centrifuge tube and subjected
to centrifugation at 14,000 g for 10 min at 4°C. The supernatant was
discarded and the pellet containing the mycelia was placed in a vacuum
desiccator (Speed Vac Concentrator, Savant Inst. Inc., Hicksville, NY)
for 30 min to remove excess moisture. The immune serum containing
the antibodies of *C. beticola* antibodies (100 μl) was added to the myce-
lia, (100 rpm, 2.5 cm circular orbit), and incubated for 1 h at 4°C on a
shaker (100 rpm, 2.5 cm circular orbit). After incubation, the mycelial
suspension was centrifuged at 14,000 g for 20 min at 4°C; the pellet
was discarded and the supernatant stored at 4°C. The supernatant then
was subjected to another adsorption with a mixture of cultures of *T.
harzanium, T. virens*, and *T. aureoviride* following the same protocol
as above. The final supernatant is referred to in this study as the "pre-
adsorbed serum" versus the "non pre-adsorbed serum" that was not
treated. A new batch of "pre-adsorbed serum" was produced and used
at each repeated cross reactivity experiment.

Gel electrophoresis and Western blotting. Fungal crude extracts were
separated by SDS-PAGE on 10% polyacrylamide gels. The gels were
either stained with Coomassie brilliant blue or transferred to membranes
(Sequi-blot PCDF membrane, Bio-Rad, Hercules, CA). Membranes
were blocked for 1 h with 5% bovine serum albumin in PBST. After
washing the membranes with PBST, a dilution 1:100 in TBST of the
primary antibody was incubated with the membranes for 90 min. A

dilution 1:10,000 in TBST of alkaline phosphatase-conjugated anti-goat IgG (Sigma A-4187) was added as secondary antibody and reacted with the membranes for 90 min. Addition of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT; BCIP 0.15 mg/mL, NBT 0.3 mg/mL, 100 mM Tris buffer, and 5 mM MgCl$_2$, pH 9.5) generated the color. All steps were performed at room temperature.

PCR. To confirm the detection by ELISA, *C. beticola* isolates C1 and S1 and the two *Fusarium* species in soil were subjected to PCR detection (Lartey et al., unpublished). First the genomic DNA was extracted from the soil using the PowerSoil DNA Kit (MO BIO Laboratories, Inc, Carlsbad, CA) as recommended by the manufacturers.

The purified DNA was subjected to PCR amplification as described by Lartey et al. (2003). The PCR reaction was carried out with Extract-N-Amp Plant PCR Kit (Sigma Aldrich, St Louis, MO). Controls consisted of blank reaction (extract without template), purified genomic DNA from pure *C. beticola* and *F. oxysporum* cultures. The 20 μl PCR reaction consisted of 10 μl Extract-N-Amp PCR mix, 1.5 μl each of the forward and reverse primers in deionized water, 4 μl DNA extract from soil, and deionized water. The PCR primers for amplification of approximately about 915 bp fragment of the actin gene of *C. beticola* were CBACTIN915L (5' GTAAGTGCTGCCACAATCAGAC 3') and CBACTIN915R (5' TACCATGACGATGTTTCCGTAG 3') (Lartey et al., 2003). Additionally, the primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (Weiland and Sundsback, 2000) also were used. Amplification was carried out over 35 cycles using a Mastercycler gradient thermocycler (Eppendorf Scientific Inc., Westbury, NY) at 94°C for 1 min denaturation, 52°C for 30 sec annealing, 72°C for 1 min extension and a 5 min final extension at 72°C. The amplified products were resolved by electrophoresis in 1% agarose gels in Loening E buffer (Loening, 1969). The PCR product sizes were determined by comparing the relative mobility of the amplified fragments to the 1 KB ladder (New England Biolabs Inc., Beverly, MA) in adjacent lanes.

Experimental procedure using artificially infested soil. A Lihen sandy loam soil (classified as sandy, mixed, rigid, entic and haphustolls) from the USDA-ARS Agricultural Experimental Station located approximately 40 km east of Williston, ND was used for the experiments. Soil had a pH of 7.0, sand, silt, and clay percentage of 65, 20, and 15 % respectively, organic matter of 2 to 3 %, at 0 to 15 cm depth.

In a first experiment, ELISA was used to quantify growing fungal mycelia in artificially infested soil. Pots (6 cm in diameter and 7 cm deep) containing air-dried soil (100 g) infested with growing mycelia (1 g) and moistened with distilled water (15 % soil moisture), were incubated for 14 days at 25°C in plastic boxes (50.8 x 15.2 cm). Mycelia were previously tested for viability on PDA and maintained at 4°C before soil infestation. Every other day during the course of incubation, a volume of water equivalent to water evaporation was added to each pot. Soil collected from the field at a depth of approximately 60 cm was used to avoid contamination with *Cercospora*. During incubation, three pots were used for ELISA, each pot representing a replication. Soil (200 mg) was air-dried, pulverized with a mortar and pestle, homogenized for 30 sec with carbonate buffer then processed for ELISA using pre-adsorbed serum. The experiment was repeated three times. To quantify the amount of fungi in the soil, ground freeze-dried mycelia of *C. beticola* were thoroughly mixed with soil (1 g) at concentrations of 0, 2, 4, 8, 10, and 12 % (wt/wt). Distilled water (volume equivalent to 0.15 g) was added to obtain a soil moisture of 15 %. By hydrating soil after spiking, we allowed water interactions between soil and the fungus to happen, and therefore potential binding of soil particles to fungal cells. Soil samples were air-dried at 25°C then pulverized with a mortar and pestle to avoid uneven distribution of fungal mycelia before being subjected to ELISA using pre-adsorbed serum. The experiment was repeated three times.

Description of field naturally infested with *C. beticola* and soil sampling. Two sites were selected because of a high incidence of *Cercospora* leaf spot on sugarbeet since the 1980's. Site A was a research site located at the State Experimental Station at Sidney, Montana and site B was a commercial production field 2 km north of the Experimental Station. Average annual precipitation was 200 to 300 mm. Site A is a Savage silty clay, fine montmorillonitic Typic Argiboroll and cropped to safflower (2003), corn (2002), and sugarbeet (2001) under flood irrigation. Soil was tilled twice to incorporate herbicide (TripleK™) to a depth of 7 to 10 cm. Site B is Venda clay and was cropped to barley (2003) and sugarbeet (2002) under sprinkler irrigation. Soil was tilled (moldboard plowing) once in mid-April. Herbicides (Betamix™, Betanex™, UpBeet™, Stinger™ and Select™) were applied post emergence for weed control and foliar applications of fungicides (SuperTin™, and Gem™) were applied mid July to late August to control *Cercospora*. Barley seeds were treated with fungicides (Raxil™ and Lindane™). Soil samples were collected in the spring of 2003 at site A and in the spring

and fall of 2003 at site B. At each site, four randomized located plots (20 m^2 with a distance of 15 m between each other) were established and soil was collected randomly along a transect within each plot. For both sites, three soil cores were collected from a 0 to 8 cm depth using a step-down probe (5 cm diameter), then separated onto 0 to 1 cm (layer 1), 1 to 4 cm (layer 2) and 4 to 8 cm (layer 3) lengths, composited within a length to represent a particular depth. Soils were air-dried, ground and stored at $4°C$ before being subjected to ELISA, using pre-adsorbed serum. To obtain a control soil without Cercospora infestation, soil was collected from approximately 60 cm deep and the 50 to 60 cm portion was used for ELISA. The amount of *Cercospora* in infested soil was expressed in absorbance values (OD). SigmaStat software was used for the analysis of variance. Comparison of means was done using the Honestly Significant Difference (HSD) procedure of Tukey-Kramer on data collected from the absorbance readings of soil samples. We used $P < 0.05$ to establish significance of data.

RESULTS

Standard curves and cross reactivity tests. Pre-adsorption of antibodies from the immune serum obtained from a goat immunized with cell walls of *C. beticola* (isolate C2) allowed detection by ELISA of freeze-dried mycelial antigens of isolate C2 at concentrations as low as 0.38 μg/ml with absorbance value of 0.040 (Table 1). Non pre-adsorbed antibodies resulted in overall higher ELISA absorbance readings than with the pre-adsorbed serum.

In cross reactivity tests (Table 2), the non pre-adsorbed antiserum was highly specific to isolate C2 and also to the other *C. beticola* isolates C1 and S1. There was negligible reactivity of the antibodies with mycelia of *R. solani, P. nuda, G. graminis,* var. *graminis, B. cinerea,* or *L. arvalis,* however the serum showed reactivity with the *Fusarium* and *Trichoderma* spp. When the serum was pre-adsorbed with a mixture of mycelia from *Fusarium* and *Trichoderma* spp., there was an immediate reduction in cross reactivity (< 0.1 absorbance reading at 455 nm using 98 μg/ml of freeze-dried fungal mycelia). The isolates of *C. beticola* (C2, C1 and S1) were recognized specifically by the pre-adsorbed serum; however signals were reduced when compared to signals detected with the non pre-adsorbed serum (reduction of 53.5 % for C2, 57.30 % for C1 and 20.9 % for S1).

Western blot analysis. When non pre-adsorbed serum was used for probing the Western blots of SDS-PAGE gels of fungal crude extracts

Table 1. Enzyme-linked immunosorbent assay (ELISA) for a dilution series of freeze dried mycelium of *C. beticola* (isolate C2) using non pre-adsorbed and pre-adsorbed[†] antiserum.

Mycelial dry weight[‡] (μg/ml)	Absorbance at 455 nm[§]	
	Non pre-adsorbed antiserum	Pre-adsorbed antiserum
98.00	0.805[¶] ± 0.032[‡‡]	0.530[††] ± 0.085
49.00	0.843 ± 0.085	0.337 ± 0.130
24.00	0.787 ± 0.110	0.225 ± 0.085
12.00	0.667 ± 0.128	0.158 ± 0.102
6.00	0.539 ± 0.101	0.105 ± 0.088
3.00	0.413 ± 0.085	0.073 ± 0.058
1.50	0.277 ± 0.062	0.059 ± 0.050
0.78	0.183 ± 0.043	0.051 ± 0.033
0.38	0.140 ± 0.080	0.040 ± 0.025

[†]   Antiserum was pre-adsorbed with *F. oxysporum* and *F. proliferatum*, *T. harzanium, T. aureoviride* and *T. virens* mycelia.

[‡]   Isolate C2 was grown in liquid media, freeze-dried, homogenized by sonication and dilutions made in carbonate buffer (20 mM $NaHCO_3$, 28 mM Na_2CO_3, pH 9.6).

[§]   Absorbance values were mean values of 2 experiments, each with 3 replicate wells per antigen concentration. Values were absorbance readings after substraction of carbonate control values.

[¶]   Correlation between absorbance readings (y) and mycelial concentrations (conc) using non pre-adsorbed serum (y = 0.107 + (0.104 x conc) ; linear regression r^2= 0.994; n = 4) and antigen concentrations from 0.38 to 98 μg/ml.

[††] Correlation between absorbance readings (y) and mycelial concentrations (conc) using pre-adsorbed serum (y = 0.0677 + (0.00498 x conc) ; linear regression r^2= 0.975; n = 9) and antigen concentrations from 0.38 to 98 μg/ml.

[‡‡] ± standard deviation.

Table 2. Enzyme-linked immunosorbent assay (ELISA)[†] for mycelial preparations of various fungi.

Species[‡]	Absorbance at 455 nm[§]	
	Non pre-adsorbed antiserum	Pre-adsorbed antiserum
Cercospora beticola (isolate C2)	0.878 ± 0.052[ⁱ]	0.408 ± 0.062
Cercospora beticola (isolate C1)	0.986 ± 0.145	0.421 ± 0.095
Cercospora beticola (isolate S1)	0.582 ± 0.098	0.460 ± 0.120
Rhizoctonia solani	0.021 ± 0.008	NA
Peniophora nuda	0.019 ± 0.006	NA
Gaeumannomyces graminis, var. *graminis*	0.000 ± 0.001	NA
Botrytis cinerea	0.013 ± 0.004	NA
Laetisaria arvalis	NA	0.014 ± 0.006
Fusarium oxysporum	0.681 ± 0.152	0.087 ± 0.052
Fusarium proliferatum	0.579 ± 0.125	0.083 ± 0.043
Trichoderma aureoviride	0.161 ± 0.062	0.000 ± 0.001
Trichoderma harzanium	0.283 ± 0.120	0.061 ± 0.036
Trichoderma virens	0.363 ± 0.084	0.000 ± 0.000

[†] Pre-adsorbed antiserum with *F. oxysporum, F. proliferatum, T. harzanium, T. aureoviride*, and *T. virens* was used.

[‡] Pure cultures of fungi were grown in potato dextrose broth for 4 weeks (1 week for *Fusarium* and *Trichoderma* species) at 25°C, freeze-dried, homogenized by sonication and dilutions were made in carbonate buffer (20 mM $NaHCO_3$, 28 mM Na_2CO_3, pH 9.6). Dried weight fungal mycelia were diluted to a concentration of 98 μg/ml in carbonate buffer.

[§] Absorbance values were means of 3 experiments, each with three replicate wells per fungal antigen. Values were absorbance readings values after subtraction of carbonate buffer control values. NA, not analyzed.

[ⁱ] ± standard deviation.

of *C. beticola* isolate C1, C2, S1, *F. oxysporum*, and *F. proliferatum*, antibodies were reactive against a wide range of soluble proteins of all tested fungi (Fig. 1A). However, when pre-adsorbed serum was used, the antibodies were reactive with the *C. beticola* isolates C1, C2 and S1 but the reactivity was considerably reduced with *F. oxysporum* and *F. proliferatum* (Fig. 1B).

Performance of ELISA for the detection of *Cercospora* in soil. The *in vitro* experiments with mycelia from C2 inoculated artificially in soil (at final concentration of 1%) (Table 3) showed an increase in absorbance readings over 14 days when pre-adsorbed serum was used in ELISA at soil concentration of 195 μg/ml in carbonate buffer. After 2 days of incubation, absorbance increased 3.6 times compared to the initial absorbance readings (cut off OD 0.105 ± 0.019 at soil concentration of 390 μg/ml). Values of detection fluctuated slightly for the next 4 days, and by 7 days after incubation, the absorbance reading was 4.8 times higher than the initial reading. At 14 days of incubation, there was very little change in the values of absorbance compared to 7 days. When Cercospora mycelia were spiked to soil at concentrations of 2, 4, 8, 10, and 12 % (Fig. 2), spiked soil samples showed higher absorbance values than the control soil at soil dilutions of 25, 49, 98, 195, 390 and 780 μg/ml carbonate buffer.

ELISA using pre-adsorbed serum was used to evaluate the biomass of *C. beticola* in naturally infested field soil. In site A, significantly higher absorbance readings were found with ELISA for naturally infested soil samples than for the control 60 cm depth samples, in the all three soil layers tested (Fig. 3). For each soil concentration used, there was no statistically significant difference in the amount of Cercospora found in the three soil layers. For example, fungal amounts in layer 1, 2 and 3 of naturally infested soil at soil concentration of 0.39 mg/ml carbonate buffer, were 4.0, 4.7, and 4.4 μg/mg of soil respectively compared to none found in non-infested samples. These data were calculated using the linear regression formula generated from a standard curve based on pre-adsorbed serum (Table 1). Samples collected in the spring and in the fall from site B showed higher absorbance readings in Cercospora-infested soils than in non-infested soils (Fig. 4), in the all three soil layers. However, in both spring and fall infested samples, absorbance readings were significantly higher in the deepest layer (layer 3, 4 to 8 cm in depth) than in the two surface layers at soil concentration of 1.56 and 3.13 mg/ml for the spring samples and at all soil concentrations tested for the fall samples. For example, Cercospora amounts of 2.8, 7.1, and 8.2 μg/mg of soil were present in layer 1, 2, and 3 respectively in spring samples at soil concen-
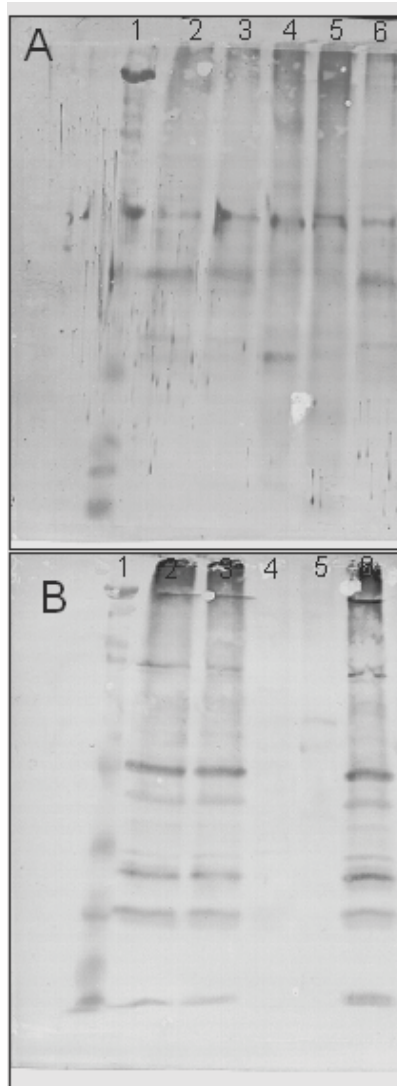
Fig. 1: Western blots from SDS polyacrylamide gel of proteins precipitated from mycelial extracts of *Cercospora beticola* isolate C1 (from left to right, lane 2), isolate C2 (lane 3), *Fusarium oxysporum* (lane 4), *Fusarium proliferatum* (lane 5), *C. beticola* isolate S1 (lane 6) probed with non pre-adsorbed serum (A) and with pre-adsorbed serum (B). Protein standards of known molecular masses ranging from 195 kDa to 6.5 kDa are shown on the left (lane 1).

tration of 0.39 mg/ml carbonate buffer, and no Cercospora was detected in the control. Fungal concentrations in fall samples was slightly different from spring samples in all three layers (3.4, 4.0, and 7.6 μg/mg soil in layer 1, 2 and 3 respectively).

Detection of *Cercospora beticola* in soil by PCR. Results of PCR based detection of *C. beticola* isolates C1 and S1 are presented in Figure 5. Using primers CBACTIN915L and CBACTIN915R the expected actin gene segment of *C. beticola* was amplified from both purified control cultures (5A lanes 3 and 4 respectively) and from soil purified C1 and S1 DNA (lanes 9 and 10). No amplified fragments were detected from pure cultures of *F. oxysporum* and *F. proliferatum* (lanes 5 and 6) nor from

Table 3. Enzyme-linked immunosorbent assay (ELISA)[†] for the detection of growing mycelia of *Cercospora beticola* (isolate C2)[‡] in soil.

Incubation Time (Days)	Absorbance at 455 nm[§]	
	Fungal Non-Amended Soil [⁋]	Fungal Amended Soil [⁋]
0	0.009 ± 0.004[††]	0.025 ± 0.011
1	0.006 ± 0.002	0.026 ± 0.019
2	0.012 ± 0.005	0.090 ± 0.015
3	0.000 ± 0.001	0.081 ± 0.041
4	0.001 ± 0.002	0.109 ± 0.032
5	0.000 ± 0.000	0.107 ± 0.045
6	0.000 ± 0.000	0.107 ± 0.032
7	0.000 ± 0.000	0.120 ± 0.053
14	0.005 ± 0.003	0.129 ± 0.052

[†]   Pre-adsorbed antiserum with *F. oxysporum*, *F. proliferatum*, *T. harzanium*, *T. aureoviride*, and *T. virens* was used.

[‡]   Mycelia were air-dried at 25°C, then mixed with soil to a final concentration of 1%. The mixture was divided into 100 g samples in small pots and kept at water moisture (15%) during incubation. Field soil collected from approximatly 60 cm deep was used as the fungal non-amended soil (control).

[§]   Absorbance values were means of 3 experiments, each with 3 replicate wells per each sample. Values were absorbance readings after substraction of carbonate control values.

[⁋]   Soil at concentration of 195 μg/ml in carbonate buffer was tested by ELISA.
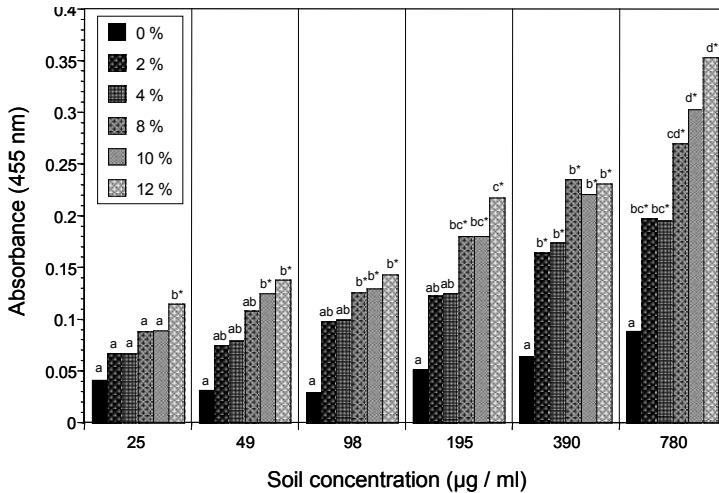
[††]   ± standard deviation

Fig. 2: Detection of *Cercospora beticola* antigens in spiked soil. Soil was spiked to mycelia to give concentrations of 2, 4, 8, 10, and 12 % (wt/wt). Data are the means of three replicates; values followed by the same letters within each soil dilution are not significantly different at $P < 0.05$ (HSD test). * indicates $P < 0.05$ difference from un-inoculated soil.
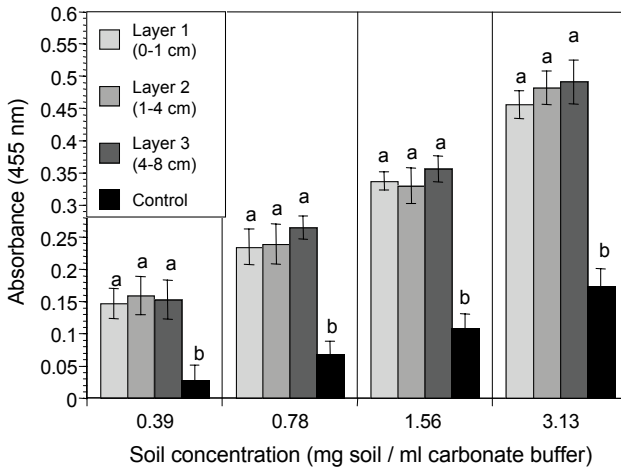

Fig. 3: Detection of *Cercospora beticola* in naturally infected soils at field site A. Data are the means of four replicates. Values followed by the same letters within each soil dilution are not significantly different at $P < 0.05$ (HSD test).
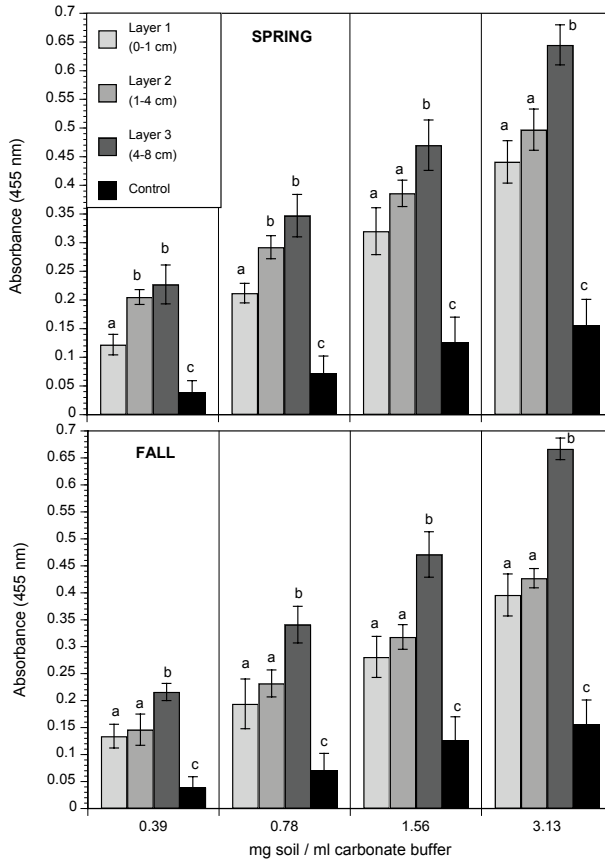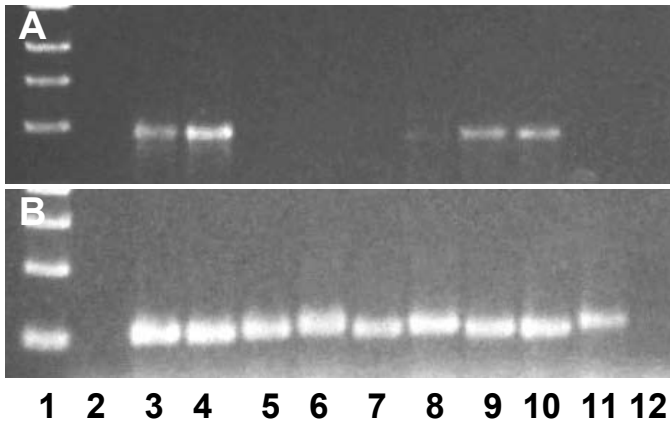
Fig. 4: Detection of *Cercospora beticola* in naturally infected soils at field site B in the spring and the fall. Data are means of three replicates. Values followed by the same letters within each soil dilution are not significantly different at $P < 0.05$ (HSD test).

Fig. 5: Detection of *Cercospora beticola* in naturally infected soils at field site A. Data are the means of four replicates. Values followed by the same letters within each soil dilution are not significantly different at *P* < 0.05 (HSD test).

soil extracts without fungi added (lanes 7 and 8). Also no amplicons were detected from control soil (lane 11 and 12) without fungus added. The blank control lane 2 did not indicate any amplification. Result of ITS primers based amplifications are presented in 5B. With exception of blank control (lane 2) and soil without fungus added (lane 12), amplicons were detected in all of the samples.

DISCUSSION

We generated polyclonal antibodies which could detect *Cercospora beticola* in soil by ELISA. Experiments performed on soils spiked with *Cercospora* mycelia and on naturally infested soils indicated that antigens are not degraded in soil and a proportion of immobilized antigens can be extracted from soil and detected by ELISA. The assay detected the pathogen in two naturally infested soils under different management practices. The non-significant difference in the amount of antigens of *C. beticola* in samples from site A from 0 to 8 cm depths (0 to 1 cm, layer 1; 1 to 4 cm, layer 2; 4 to 8 cm layer 3) likely occurred because the field was moldboard plowed to a depth of 8 to 10 cm. This practice is commonly used to incorporate herbicides before planting and this would mix fungal inoculum in the top soil. In site B, however, ELISA showed noticeably lower amounts of antigens of *C. beticola* in the two

upper surface layers (0-1 cm and 1-4 cm depth) compared to layer 3 (4-8 cm depth). A possible explanation is the practice of light tillage (to less than 5 cm of depth) to incorporate barley seeds that were treated with fungicides such as Raxil™, Lindane™ and Thiran™ in the spring of 2003. This could affect Cercospora survival and growth and ultimately their number in the two upper soil surface layers.

Although the polyclonal antibodies as generated exhibited reactivity to *Fusarium* and *Trichoderma* spp., a pre-absorption technique reduced this effect. It is not unexpected that the goat was previously exposed to these fungi commonly found in the environment and its serum possessed antibodies toward these fungi before immunization with *C. beticola*. We elected to expose the serum to fungi which would bind the undesirable antibodies leaving only antibodies binding the target (pre-adsorbed serum). The pre-adsorbed serum demonstrated relatively specific binding for *C. beticola* C1, C2 and S1 isolates with only trace interactions toward the other fungi.

Fusarium spp., in particular *Fusarium oxysporum* f sp. *betae* (Schneider and Whitney, 1986) cause Fusarium yellows diseases on sugarbeet in greenhouse test (Hanson & Hill, 2004), and the disease is common on approximately half of Montana sugarbeet acreage (Montana Agric Experiment Station, 2003). Therefore, the use of pre-adsorption technique to eliminate the antibodies from the antisera that recognize *Fusarium* was a necessary step to avoid cross reactivity when soil from sugarbeet fields is tested. This approach improved specificity for the ELISA and western blots because it minimizes the non-Cercospora-specific binding. The pre-adsorbed serum demonstrated relatively specific binding for *C. beticola* C1, C2 and S1 isolates with only trace interactions toward *Fusarium*.

Studies in the ecology and management of *C. beticola* in soil have been hampered by difficulties in detecting and quantifying the amount of inoculum of this pathogen. Even though the techniques of pre-adsorption and ELISA are not new, the application of these techniques have demonstrated specific binding of the pre-adsorbed immunoserum to *Cercospora* mycelia, thus enabling quick screening and estimation of the relative concentration of the pathogen in soil. The method also could be extended to derive monoclonal antibodies to better differentiate *Cercospora* isolates and provide an "infinite" source of defined antibodies.

Cercospora beticola in field soil detected by ELISA also was detected by PCR thus confirming the ELISA by itself as a viable technique for detection of *C. beticola* in soil. The PCR by itself could also be practical in detecting *C. beticola* in soil, however unlike Real-Time PCR, it could not be used to quantify the pathogen in soil. Certainly,

where there is need to quantify *C. beticola*, for example to determine the effect of applied biological agents or follow up changes in potential inoculum level over a period of time, the ELISA protocol will be more appropriate.

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