A Sensitive Dot Immunoassay Employing Monoclonal Antibodies for Detection of *Sirococcus strobilinus* in Spruce Seed

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ABSTRACT

Mitchell, L. A. 1988. A sensitive dot immunoassay employing monoclonal antibodies for detection of *Sirococcus strobilinus* in spruce seed. Plant Disease 72:664-667.

A qualitative, highly sensitive dot immunobinding assay for detection of the pathogenic fungus *Sirococcus strobilinus* on spruce seed is described. This assay employs *S. strobilinus*-specific monoclonal antibodies to detect fungal material (antigens) in spruce (*Picea* spp.) seed extracts applied in small volumes (0.5–1 μl) to nitrocellulose paper. The dot immunoassay allows detection of as little as 1 ng of *S. strobilinus* antigen in fungal extracts making it 5–25 times more sensitive than the enzyme-linked immunosorbent assay (ELISA) previously described. This assay is relatively easy to perform, requires minimal laboratory equipment, and is potentially applicable for use in seed testing laboratories.

In a previous paper (5), an indirect enzyme-linked immunosorbent assay (ELISA) for detection of *Sirococcus strobilinus* Preuss constituents (antigens) in spruce (*Picea* spp.) seed extracts was described. Although the ELISA is quantitative, highly specific, and sensitive relative to conventional (detection-by-plating) tests for this conifer pathogen, special equipment and some technical expertise are required to perform the assay. This paper describes the development of a relatively facile and ultrasensitive dot immunobinding assay. This qualitative assay, in which *S. strobilinus*-specific monoclonal antibodies are used to detect their specific antigens in seed extracts applied as small (1 μl) dots onto nitrocellulose paper, requires minimal laboratory equipment and is potentially applicable for use in seed testing laboratories.

MATERIALS AND METHODS

***Sirococcus strobilinus*-soluble antigens.** Fungal soluble antigens (crude mycelial extracts) were prepared from axenic cultures of *S. strobilinus* isolated, cultured, and extracted as previously described (4,5). Protein concentrations were determined by the method of Lowry et al (2). *Sirococcus strobilinus*-soluble antigen standards ranging from 1 to 1,000 μg/ml were prepared by diluting mycelial extracts in distilled water.

Seed extracts. Nine seedlots collected in August and September of 1982 from Engelmann spruce (*Picea engelmanni* Parry ex Engelm.) and hybrid spruce (*Picea* spp.) from adjacent seed zones (elevation: 1.2–1.6 km) in the British Columbia (B.C.) interior and three seedlots collected in 1984 from disease-free seed trees at the B.C. Forest Service (B.C.F.S.) Seed Orchard, Duncan, B.C., were obtained from the B.C.F.S. seed repository. Each seedlot was tested for *S. strobilinus* by plating 250 surface-sterilized (8) seeds on nutrient agar and identifying the emerging fungi after 10–21 days' culture at alternating 12-hr cycles of 21 C and 16 C with an 8-hr photoperiod. Five hundred seeds from each seedlot were surface-sterilized and suspended at 100 seeds/ml in 0.05 M NH_4HCO_3 containing 10 mM NaN_3. Ten microliters of the proteolysis inhibitors, phenylmethylsulfonyl fluoride (1 M)/Pepstatin (5 mg/ml) in 95% ethanol, were added and the seeds were extracted as previously described (5).

Monoclonal antibodies. Monoclonal antibodies (McAbs) directed to mycelial or secreted (culture filtrate) antigens of *S. strobilinus* were prepared as previously described (4). Monoclonal antibodies used were: SCA 1/217.24 and SCA 2/108.5 (directed to secreted antigens) and SDM 1/441.8.20, SDM 1/434.28.3, SDM 1/475.6.2, SDM 1/171.27.13, SDM 2/392.10.8 (all directed to mycelial antigens of *S. strobilinus*). All McAbs were specific for *S. strobilinus* and showed no reactivity with saprobes commonly associated with *Sirococcus* in seeds (4,5).

Dot immunoassay. The dot immunoassay used to detect *S. strobilinus* antigens in spruce seed extracts was similar to that described by Bennett and Yeoman (1). Nitrocellulose paper (NCP) (BA 85 0.45 μ nitrocellulose membrane from Schleicher and Schuell, Keene, NH, or Transblot transfer medium from Biorad Laboratories, Richmond, CA) was cut into 8 × 12.5 cm rectangles. The NCP was positioned over a 96-well polyvinyl chloride (PVC) microassay plate (Falcon no. 3911, Becton-Dickinson, Mississauga, Ontario), fastened with pins, and placed on a light table. With a microcapillary pipet, fungal-soluble antigen standards or undiluted seed extracts were applied in 1-μl dots to the NCP at each well position, using the PVC plate as a template. The antigen-coated NCP was allowed to dry at 37 C for 15 min. Sites not coated with fungal antigens or seed extracts were blocked by immersing the NCP for 1.5 hr at 37 C in 50 ml of 2.5% (w/v) gelatin in 10 mM phosphate (pH 7.4) containing 3 mM KCl, 140 mM NaCl, and 0.01% (w/v) NaN_3 (phosphate-buffered saline [PBS]). The NCP was then rinsed briefly in PBS containing 0.05% (v/v) Tween 20 and 0.5% (w/v) gelatin (PBST-gelatin) and blotted gently with filter paper. A 96-well polystyrene microculture plate (Cell Wells no. 25860, Corning, Palo Alto, CA) was outfitted with a Parafilm gasket, then 200 μl of specific McAbs (undiluted hybridoma culture fluids) or negative control supernatants (undiluted culture fluids from SP2/0-Ag 14 cells, the myeloma partner used in the derivation of the *S. strobilinus*-specific McAbs) were pipetted into each microplate well.

Accepted for publication 19 February 1988.

The antigen-treated and blocked NCP was inverted onto the microculture plate and overlaid with a second Parafilm strip, a Scotch Brite pad (both cut to fit the microplate), and finally, the microplate lid. The whole assembly (Fig. 1) was clamped together with bulldog clamps, inverted to expose the antigen dots to the culture supernatants, and incubated for 18 hr at 4 C. The NCP was then removed and washed three times (10–15 min each on a horizontal shaker at 70–90 cycles/min) in 100–150 ml of PBST-gelatin, then incubated for 2 hr at room temperature in 35–50 ml of enzyme-conjugated second antibody (affinity-purified alkaline phosphatase-labelled goat anti-murine IgF[ab']$_2$ obtained from Helix Biotech, Vancouver, B.C., or horseradish peroxidase-conjugated goat anti-murine IgG [H + L], or glucose oxidase-conjugated goat anti-murine Igs [H + L], the latter two obtained from HyClone Laboratories, Logan, UT) diluted to 1:500 in 1% (w/v) bovine serum albumin (Fraction V, Sigma, St. Louis, MO) in PBS (pH 7.4). After this incubation, the NCP was washed three times by shaking in PBST-gelatin as described above and incubated in 100 ml of substrate solution (with shaking) until maximum color development occurred at sites of specific antigen-antibody interactions. When alkaline phosphatase-labelled second antibody was used, the substrate solution consisted of 300 mg of Fast Blue BB in 50 ml of 0.2 M Tris HCl (pH 8.2) and 20 mg of α-naphthyl acid phosphate dissolved in 50 ml of distilled water. The two solutions were mixed 1:1 just before use. The substrate solution for glucose oxidase conjugates consisted of 750 mg of β-D-glucose, 50 mg of *p* nitro blue tetrazolium, and 10 mg of phenazine methosulfate dissolved in 100 ml of 0.1 M phosphate (pH 6.9) just before use. When glucose oxidase-labelled second antibodies were used, Tween 20 was deleted from the washing buffer.

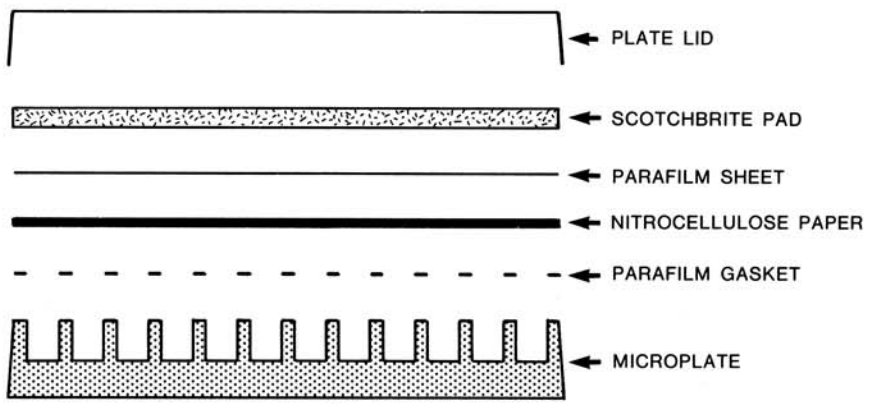

Fig. 1. Microplate assembly for dot immunoassay. See text for details.

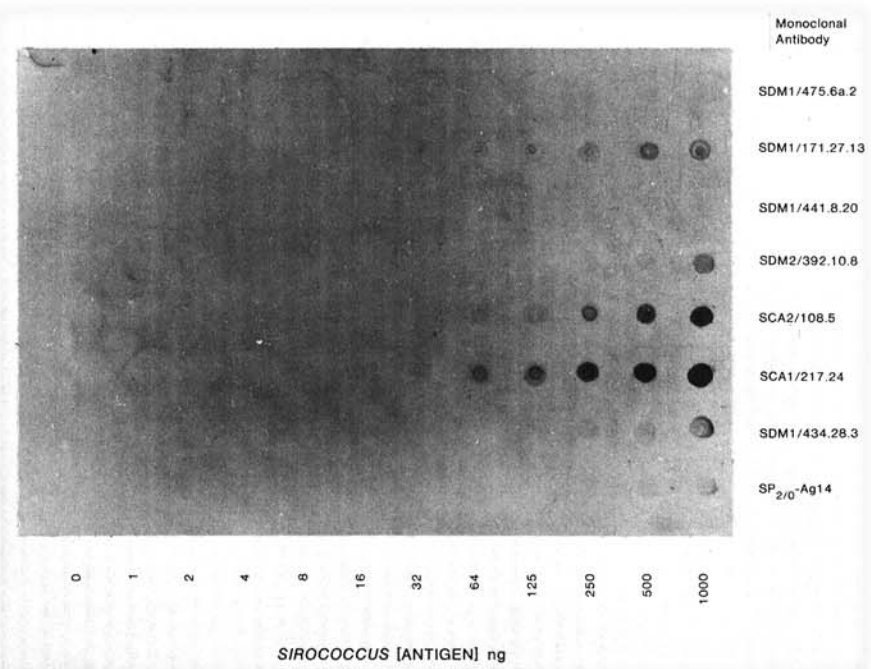

Fig. 2. Dot immunoassay: *Sirococcus strobilinus*-soluble antigens. Fungal-soluble antigen standards (1 μl) were spotted onto nitrocellulose and incubated in succession with hybridoma culture fluids (1° antibody) containing McAbs directed to mycelial (SDM) or secreted (SCA) antigens of *S. strobilinus*, alkaline phosphatase-labelled second antibody, and substrate as described in Materials and Methods. Negative control: SP2/0-Ag 14 myeloma culture fluids. Grey-black precipitates at the sites of antigen dots indicate a positive reaction.

RESULTS AND DISCUSSION

Development of dot immunoassays for *S. strobilinus*. To establish optimal conditions for dot immunoassays for detecting *S. strobilinus* antigens several elements of the assay were examined including nitrocellulose membrane type and porosity, blocking conditions, and second antibody systems. Different nitrocellulose membranes were tested, including Gelman GA-9 Metricel (0.10 μm), Gelman GA-8 Metricel (0.20 μm), Amicon 041255 (0.45 μm), Millipore GSWP 02500 (0.22 μm), Schleicher and Schuell BA85 (0.45 μm), and Biorad Transblot transfer medium (0.45 μm). Nylon transfer medium (Biorad Zeta-probe, 0.45 μm) also was tested. The best results (low background staining, high sensitivity) were obtained with Amicon 041255 (0.45 μm) or Schleicher and Schuell BA85 (0.45 μm) membranes. Lower porosity nitrocellulose (less than 0.45 μm) tended to absorb antigen poorly, resulting in low sensitivity. High background staining was observed when Zetaprobe was used.

A variety of blocking times, temperatures, and media were investigated, including 1–3% bovine serum albumin in PBS (pH 7.4), 10% horse or fetal bovine serum in PBS (pH 7.4), and 2.5% gelatin in PBS (pH 7.4) for 1–3 hr at room temperature or at 37 C. No differences were observed in the results obtained with the various blocking conditions.

Three different second antibody systems for detecting specific antigen-McAb complexes were tested, including horseradish peroxidase-labelled goat anti-murine IgG (H + L), glucose oxidase-labelled goat anti-murine Igs (H + L), and alkaline phosphatase-labelled goat anti-murine IgF(ab')$_2$. Although excellent results were obtained with all three second antibodies when mycelial extracts (*S. strobilinus* soluble antigens) were tested, high background staining was observed with peroxidase conjugates when seed extracts were tested (see below). This may have been due to endogenous peroxidase activity in the seed extracts, but was not inhibited by heating the seed extracts (56 C for 30 min) or by adding NaN_3 or H_2O_2 to the seed extract before applying it to the nitrocellulose paper. This precluded the use of peroxidase conjugates in immunodetection of *S. strobilinus* in seeds.

Detection of *S. strobilinus* antigens in

dot immunoassays. The dot immunoassay was more sensitive than the previously described enzyme-linked immunosorbent assay (ELISA) (5) in detecting *S. strobilinus* antigens. When *S. strobilinus*-soluble antigen standards (dilutions of crude mycelial extracts) were applied in 1-μl dots onto NCP, as little as 8–16 ng of antigen could be detected with some of the McAbs (Fig. 2). When equal volume mixtures of the above *S. strobilinus*-specific McAbs were employed (not shown), sensitivities of 1–5 ng of fungal antigen could often be achieved, whereas the lower limit of *S. strobilinus* antigen detection was 25 ng with the ELISA under the same conditions. In dot immunoassays with soluble *S. strobilinus* antigens, no background staining was observed in the absence of specific antibody (SP2/0 negative control) with the exception of some nonspecific binding of enzyme-labelled second antibody observed at higher antigen concentrations (500–1,000 ng).

When seed extracts were tested (Fig. 3), positive reactions (formation of a grey-black precipitate) using the alkaline phosphatase detection system were observed with seed extracts from seedlots (8178 to 8189) shown to be infested with *S. strobilinus* by the plate test or to contain *S. strobilinus* antigens by the ELISA (Table 1). Some variability in *S. strobilinus* antigen detection was observed with different McAbs on different seed extracts (Fig. 3). By definition, each McAb is directed to a single antigenic site on a given antigen (3). Hence, this phenomenon may relate to variability in antigen expression in *S. strobilinus*, as was previously observed in different isolates of the fungus (4). This problem may be alleviated by using a mixture of McAbs (each directed to a different antigenic site) to optimize antigen detection. The lower reactivity of some McAbs may also, in part, reflect differences in affinity or avidity for antigens. No precipitates were observed when extracts of seeds from seedlots 6153, 4388, and 4405, obtained from disease-free seed orchard trees, were reacted with any of the McAbs (Fig. 3), indicating absence of *S. strobilinus* antigens. These seedlots were also negative for *S. strobilinus* in the plate tests and ELISA (Table 1). Background staining was observed occasionally when some extracts from infested seeds were tested in the dot immunoassay. This may have been due to seed alkaline phosphatase as has been observed in *Calascypha fulgens*-infected spruce seeds (7), as background staining was not observed in tests with noninfested seeds. Selective inhibition of seed alkaline phosphatase was attempted by adding Levamisole (0.25–2 mM) to the substrate buffer (6). However, this did not reduce background staining. Nevertheless, after 30–45 min of incubation of the NCP in the substrate solution, positive reactions (grey-black precipitates) were clearly distinguishable from background (red-orange coloration) that appeared within 2 min after substrate addition and did not alter appreciably during the course of the incubation. Formation of precipitates after substrate addition has also been observed occasionally in ELISAs performed with extracts from *S. strobilinus*-infested seeds (5). Similar background staining problems occurred intermittently with the glucose oxidase detection system. The nature of these chemical reactions is unknown. However, these spurious background problems evoke important considerations in dealing with plant material in immunoassays in which enzyme-labelled antibodies are used. Careful attention should be given to selection of appropriate enzyme labels and controls for endogenous enzyme activity and/or other interfering substances. As noted earlier, it was

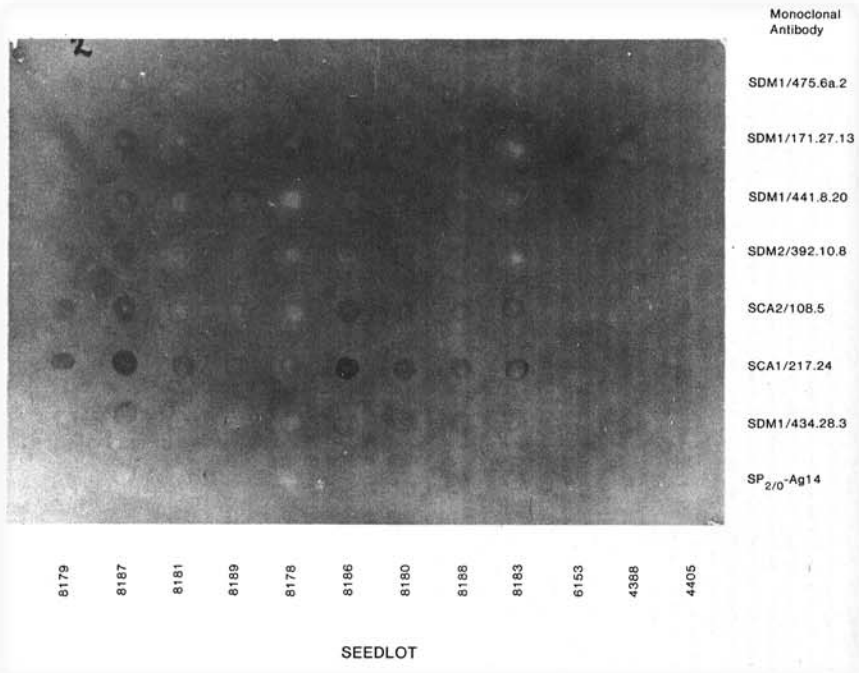

Fig. 3. Dot immunoassay: detection of *Sirococcus strobilinus* antigens in spruce seed extracts. Extracts from infected (8178–8189) and noninfected (6153, 4388, and 4405) seedlots were applied as 1-μl dots to nitrocellulose paper and incubated in succession with McAbs (1° antibody), alkaline phosphatase-labelled second antibody, and substrate as described in Materials and Methods. Formation of a grey-black precipitate indicates the presence of *S. strobilinus* antigen in the seed extract. Negative control: SP2/0-Ag 14 myeloma culture fluids.

Table 1. Results of plate tests, ELISAs, and dot immunoassays to detect *Sirococcus strobilinus* in spruce seedlots

Seedlot[a]	Plate test[b]	ELISA[c]	Dot immunoassay[d]
8178	1.8	0.26 ± 0.03	+
8179	0.0	0.32 ± 0.05	+
8180	0.04	0.36 ± 0.05	+
8181	0.08	0.35 ± 0.006	+
8183	2.4	0.37 ± 0.06	+
8186	1.2	0.40 ± 0.04	+
8187	0.08	0.39 ± 0.07	+
8188	0.08	0.32 ± 0.02	+
8189	0.0	0.15 ± 0.04	+
6153	0.0	0.04 ± 0.04	0
4388	0.0	0.04 ± 0.03	0
4405	0.0	0.03 ± 0.03	0

[a] See text for origin of seedlots.
[b] Incidence (%) of *S. strobilinus*-infected seeds in a random sample of 250 seeds from each seedlot as determined by the plate test (see Materials and Methods).
[c] ELISA absorbance values (A_{410nm}) in tests performed with 25 μl of an extract of 500 seeds per seedlot. Positive A_{410nm} values indicate the presence of *S. strobilinus* antigens in the seed extracts as detected with a mixture of specific McAbs. Values are the $\bar{X} \pm SD$ of three separate ELISAs performed with each seed extract.
[d] Presence or absence of *S. strobilinus* as determined by dot immunoassay using the McAb, SCA 1/217.24.

observed in preliminary investigations in this study that horseradish peroxidase-labelled second antibodies could not be used in immunodetection of *S. strobilinus* in conifer seed because of interference from seed peroxidase.

The dot immunoassay for detecting the fungal pathogen *S. strobilinus* offers several advantages over the ELISA in that it is up to 25 times more sensitive (detecting as little as 1 ng of antigen), requires smaller volumes of seed extract (1 μl as opposed to 25–50 μl), and is relatively easy to perform. However, the ELISA is both qualitative and quantitative, whereas the dot immunoassay is only qualitative (although precipitates at the sites of positive reactions could possibly be measured by densitometry). Although in the initial development of the dot immunoassay fungal antigens or seed extracts applied to nitrocellulose paper were exposed to specific McAbs using a microplate assembly, preliminary investigations suggest that immersion of the antigen-coated nitrocellulose paper in McAb solution may be sufficient. If similar results are obtained without the microplate assembly, a simple kit test consisting of a nitrocellulose card and vials of blocking reagent, McAb, enzyme-labelled second antibody, and substrate may be constructed. Hence, the relative simplicity of the dot immunoassay makes it potentially useful for seed testing laboratories where elaborate equipment may not be available.

ACKNOWLEDGMENTS

The author thanks J. R. Sutherland, R. S. Hunt, and D. J. Morrison for their time and effort in reviewing this manuscript and the British Columbia Ministry of Forests for providing spruce seed samples.

LITERATURE CITED

1. Bennett, F. C., and Yeoman, L. C. 1983. An improved procedure for the "dot immunobinding" analysis of hybridoma supernatants. J. Immunol. Meth. 61:201-207.
2. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
3. Milstein, C., Galfre, G., Secher, D. S., and Springer, T. 1979. Monoclonal antibodies and cell surface antigens. Cell Biol. Int. Rep. 3:1-16.
4. Mitchell, L. A. 1986. Derivation of *Sirococcus strobilinus*-specific monoclonal antibodies. Can. J. For. Res. 16:939-944.
5. Mitchell, L. A., and Sutherland, J. R. 1986. Detection of seed-borne *Sirococcus strobilinus* with monoclonal antibodies in an enzyme-linked immunosorbent assay (ELISA). Can. J. For. Res. 16:945-948.
6. Morris, R. E., and Horowitz, D. A. 1984. Cellular enzyme-linked immunospecific assay (CELISA). IV. Inhibition of endogenous cellular alkaline phosphatase activity. J. Immunol. Meth. 68:11-18.
7. Sutherland, J. R., Rink, U., McMullan, E. E., and Woods, T. A. D. 1981. Isozyme characteristics of *Calascypha fulgens*-infested and pathogen-free spruce seed samples and use of alkaline phosphatase activity for qualitative and quantitative disease incidence assays. Can. J. For. Res. 11:200-205.
8. Sutherland, J. R., Woods, T. A. D., Lock, W., and Gaudet, D. A. 1978. Evaluation of surface sterilants for isolation of the fungus *Geniculodendon pyiforme* from Sitka spruce seeds. Can. For. Serv. Bi-monthly Res. Notes 34:20-21.