Immunodetection and Quantification of *Botrytis cinerea* on Harvested Wine Grapes

Robert W. Ricker, James J. Marois, Jeffery W. Dlott, Richard M. Bostock, and Janice C. Morrison

First four authors: Department of Plant Pathology, University of California, Davis 95616; and fifth author: Department of Viticulture and Enology, University of California, Davis 95616.

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ABSTRACT

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Water-soluble antigens produced by *Botrytis cinerea* were detected in spiked and naturally infected grape juice by using an enzyme immunoassay with an indirect format of antibody horseradish-peroxidase conjugates bound to polyclonal rabbit antibodies directed against *B. cinerea* (anti-Bc IgG). Protein A purified gamma globulin from an early-bled antiserum (803-7), which reacted primarily with low molecular weight compounds present only in extracts of *B. cinerea*, was used to specifically detect *B. cinerea* and quantify levels of infection in juice from infected grape berries. Late-bled, higher titer antiserum (803-19), which cross-reacted with proteins and carbohydrates present in extracts from species of *Botrytis, Aspergillus, Penicillium,* and *Uncinula*, was used to quantify levels of rot caused by the presence of multiple fungi. Minimum detectable levels of infection, based on mixtures of clean and infected juice, were 0.25–0.5% with 803-7 IgG, and 0.02% with 803-19 IgG. Affinity purification of 803-19 IgG by using antigens from *Aspergillus niger* coupled to Sepharose beads improved specificity of anti-Bc IgG to *B. cinerea* but decreased detection sensitivity to approximately 0.5% infection. Cross-reactivity of all anti-Bc IgG collections was consistently low with juice extracted from uninfected grape berries. In contrast, cross-reactivity of anti-Bc IgG with water-soluble antigens extracted from sterile and reproductive structures of several fungi was negligible in early-bled antiserum and increased in subsequent collections. The increase in cross-reactivity in late-bled antisera corresponded with an increase in the overall serum titers for anti-Bc IgG to antigens from *B. cinerea*. Nonspecific binding of 803-19 IgG was high with extracts from *A. niger* and an unidentified species of *Penicillium*, suggesting numerous epitopes common to antigens from these fungi.

Additional keywords: EIA, mold detection, *Uncinula necator*, *Vitis vinifera*.

Botrytis cinerea Pers.:Fr. is a widespread pathogen commercially important on wine and table grapes (2). Although sometimes desirable and known as "noble rot" when present on varieties of grapes used for sweet, late-harvest wines, *B. cinerea* generally is undesirable. Known as "bunch rot" or gray mold, *B. cinerea* is detrimental to the quality of grape berries. Mold analysis is an important step in determining grape berry quality, and as such partly determines the price paid for grapes and, in some

cases, whether or not the grapes are accepted or rejected at wineries. Visual inspection of grape clusters and berries is currently the most widely used method for detecting and quantifying the fungus. This procedure, however, is labor-intensive, time-consuming, and highly dependent on the skills of the inspector. It also cannot be applied to accurately measure levels of fungus in machine-harvested grapes. Glycerol, which has been related to mold growth, was proposed as a marker compound for quantifying levels of fungal infection in grape juice. Ravji et al (20), however, demonstrated a poor correlation between glycerol production and mycelial mass, as well as interspecific differences in rates of glycerol production dependent on grape variety, the method of juice preparation, and incubation conditions.

Savage and Sall (21,22), using polyclonal rabbit antibodies, indicated that immunosorbent-type antibody assays could provide a reliable technique for detecting low amounts of *B. cinerea* in grape juice. Although these authors demonstrated the use of an immunosorbent assay for mold detection, their protocol was time-consuming (5–12 hr) and required radioactive-labeled antibodies. During the past eight years, numerous improvements in immunoassay formats and revised protocols have resulted in assay times as short as 5 min (1,27). Shortened assay times, in addition to the use of nonradioactive probes (3,4,28), have made immunoassays safe, viable, and attractive detection methods for use in field conditions.

Our objectives in the present study were to produce polyclonal rabbit antibodies to crude extracts of *B. cinerea* (anti-Bc IgG); to characterize anti-Bc IgG specificities by using extracts from *B. cinerea*, *Aspergillus niger* Tiegh (the causal agent of black-mold rot), *Penicillium* sp. (a causal agent of blue-mold rot), *Uncinula necator* (Schwein.) Burrill (the causal agent of vine powdery mildew), and assorted varieties of *Vitis vinifera* L.; and to determine immunoassay sensitivities for detecting *B. cinerea* in juice from harvested grape berries by using indirect noncompetitive and competitive enzyme immunoassay (EIA) formats with microtiter plates.

MATERIALS AND METHODS

Fungal cultures. *B. cinerea, A. niger, Penicillium* sp., and *U. necator* were isolated from grape berries or grape vine tissues from plants in California and Chile. Cultures of *B. cinerea* were started with inoculum derived from several grape varieties including Chardonnay (*cu* isolates; Napa, CA), Red Emperor (*del* isolates, Delano, CA; *chil* isolates, Chile), Ribier (*R*78-72-3 and *R*81-71 isolates, Delano, CA), and Thompson Seedless (*tgs* isolates, grocery store). *A. niger* and *Penicillium* sp. were isolated from several varieties of grapes collected near Davis and Napa. All fungi, except *U. necator*, were initially isolated on potato-dextrose agar (PDA) plates and grown at room temperature. Single-spore isolates were transferred to agar plates prepared with a glucose-modified version of Czapek's defined media supplemented with asparagine (CzA). After several transfers on CzA, conidial suspensions were harvested and mixed with activated silica gel crystals without indicating dye (Fisher Scientific Co., Pittsburgh, PA; product with Davison chemical code 0400808237, mesh size 6–12). Dried, spore-coated crystals, which required no maintenance and provided long-term storage of cultures at 4 C (19,29), were used to inoculate both agar plate or broth cultures.

Conidia and hyphae from *U. necator* were washed off the surfaces of infected leaves and stems from small vines inoculated with conidia and grown under laboratory conditions with a variable light cycle for 3–5 wk in 20-L plastic bottles covered with multiple layers of cheesecloth. Fungal tissue was stored frozen at −80 C in a minimal amount of wash solution, which consisted of 1.5% NaCl and 0.01% Tween-20 in sterile deionized water.

Growth media. Undefined media used in the present studies included potato-dextrose agar (Difco Laboratories, Inc., Detroit, MI), tomato juice agar (prepared by mixing V-8 juice with Bacto-agar [Difco; 15 g/L]), and grape juice agar (prepared by mixing filtered [Whatman No. 1] juice extracts from Chardonnay, Chenin blanc, Cabernet Sauvignon, and Rubired berries with Bacto-agar [15 g/L]). Defined medium was CzA, which was prepared either as a broth or solidified with agar.

Fungal extracts. Various isolates of the same species were grown in defined media for 3–6 wk and harvested by pouring the culture broth containing fungal hyphae and conidia into a Buchner funnel covered with miracloth (Calbiochem-Behring, LaJolla, CA). All isolates were pooled to avoid isolate-specific properties. The total fungal mass was washed with 500 ml of 17.5 mM sodium phosphate buffer with 145 mM NaCl, pH 7.4 (PBS; [6]) and either frozen and lyophilized or resuspended (v/v) in one-tenth volume of 10× PBS with additives (see below) for immediate extraction. Large amounts of fungal tissue suspended in extraction buffer were chilled in an ice bath and homogenized with either a Waring blender or a Polytron homogenizer (Brinkman Instruments Co., Westbury, NY) for three 30-sec bursts separated by 1-min intervals. Small amounts of fungal tissue were homogenized manually by grinding in 400–1,000 μl of buffer with micropestles and 1.5-ml microfuge tubes on ice. Three to five percent (w/v) polyvinylpolypyrrolidone (PVPP; Sigma Chemical Co., St. Louis, MO), acid-washed and neutralized (13), was routinely added to samples immediately after tissue homogenization and before centrifugation. Large volume samples were centrifuged at 4 C at 10,000 *g* for 15 min. Small volume (<1.5 ml) samples were centrifuged at room temperature at 16,000 *g* for 5 min. Cell fragments were pelleted and discarded, and supernatants with soluble antigens received phenylmethyl sulfonyl fluoride (PMSF) to a final concentration of 1.0 mM. Extracts were either quickly frozen in liquid nitrogen and stored at −20 or −80 C or stored at 4 C for antigen analysis soon after extraction. Extracts stored at 4 C contained 0.05% NaN_3.

Extraction buffers contained either a nonionic detergent (PBS at pH 7.4, a reducing agent [either 30 mM ascorbic acid or 5–10 mM β-mercaptoethanol], 1.0 mM EDTA, and 0.02% [v/v] Tween-20) or an ionic detergent (62.5 mM Tris-HCl at pH 6.8, 145 mM NaCl, 5.0 mM DL-dithiothreitol, 1.0 mM EDTA, and 2.0% sodium dodecyl sulfate [SDS]).

Grape juice samples. Thompson Seedless grape berries were used for negative control juice samples and inoculation with fungal conidia. Berries visibly free of mold were selected and surface-sterilized for 30 sec by immersion in 0.5% sodium hypochlorite followed by three washes with sterile water. Each berry was injected with 50–100 μl of conidial suspension (12.5×10^4 conidia per milliliter of sterile water) and stored at 20–25 C until covered by mycelium-bearing conidia. Completely infected berries were extracted and processed as above in a PBS-Tween buffer. Clarified buffered juice, considered to be 100% infected with *B. cinerea*, was quickly frozen in liquid nitrogen and stored at −20 or −80 C.

Several varieties of grape berries, harvested either by machine or by hand, were collected from truck gondolas at grape inspection stations. Samples of Cabernet Sauvignon and Chardonnay with no visible mold were extracted for negative control juice. All juice from inspection stations was obtained by using a commercial crusher (a press with closely spaced rollers) and initially was partially clarified by low-speed centrifugation (<1,000 *g*) or coarse filtration. Juice was mixed with either 10× (phosphate) or 5× (borate) concentrated liquid buffer stock, resulting in a juice dilution of 90 or 80% of the original concentration, respectively. Phosphate-buffered samples had a pH of 6.5–7.3 and contained: 100 mM Na_2HPO_4/NaH_2PO_4, 145 mM NaCl, 1.25 mM EDTA, 1.0 mM PMSF, 0.02% (v/v) Tween-20, 0.02% thimerosal, and 3% PVPP. Borate-buffered samples had a pH of 3.5–4.0 and contained: 50 mM $Na_2B_4O_7$, 145 mM NaCl, 1.25 mM EDTA, 1.0 mM PMSF, 0.025% (v/v) Triton X-100, and 6% (w/v) of a mixture containing equal amounts of PVPP and Amberlite XAD-4 (Rohm & Haas Co., Philadelphia, PA). After approximately 6–12 hr storage on ice, samples were filtered through porous plastic disks (Porex X-4900, Fine PE = 20 μm limit; Porex Technologies, Fairburn, GA) to remove resins and suspended particulates. Aliquots of processed juice were quickly frozen in liquid nitrogen and stored at −80 C.

Berries from clusters harvested by hand were sorted according to infection levels of *B. cinerea*. Collections included berries from Malvasia, Sauvignon blanc, and Sémillon grapes. Unfiltered juice from each infection class was extracted and mixed with lyophilisate from freeze-dried 2.0 M phosphate buffer mixture prepared at pH 7.2, which resulted in juice dilution to 92% of its original concentration and a rise in pH to 6.5–7.0. Approximate final concentrations in processed samples were: 400 mM Na_2HPO_4/NaH_2PO_4, 145 mM NaCl, 1.0 mM EDTA, 5.0 mM cysteine, 1.0 mM PMSF, 0.02% (v/v) Triton X-100, and 20% (w/v) of wet PVPP. Resins mixed with juice were acid-washed and neutralized before use (13) and stored at room temperature as a thick slurry in 1.0 M borate buffer at pH 8.0.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blots. Soluble components from extracts of fungal tissues homogenized in Tris-SDS buffer, and Amersham Rainbow molecular weight markers (Amersham Corp., Arlington Heights, IL), were separated by SDS-PAGE (10) by using a 12%, 0.75-mm thick gel for 45 min with a constant potential of 200–205 V. Immediately after electrophoresis, gels were washed and equilibrated in two changes of transfer buffer, pH 8.3 (20 mM Tris, 150 mM glycine, 20% methanol) for a total of 15 min at 20–25 C. Separated components were transferred from gels onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH; BA-83) with a transfer apparatus (Bio-Rad mini trans-blot electrophoretic transfer cell; Bio-Rad Laboratories, Richmond, CA) in an ice bath with constant potential (100 V for the first 30 min, decreased to 50 V for the next 5.5 hr). Nitrocellulose membranes containing blotted compounds (western blots; [25]) were sequentially incubated in blocking buffer (5% [w/v] nonfat milk powder in PBS), primary antibody (either 803-7 IgG or 803-19 IgG at 5 μg/ml in EIA dilution buffer [PBS with 0.5% bovine serum albumin, 0.02% Tween-20, and 0.01% thimerosal]), goat-anti-rabbit IgG conjugated to horseradish peroxidase (GAR-HRP; Boehringer Mannheim Biochemicals, Indianapolis, IN; diluted [1:1,000] in EIA dilution buffer), and substrate (0.006% 4-chloro-1-naphthol [Sigma] and 0.003% H_2O_2 in PBS). All incubations were at 20–25 C for 2 hr, except the final step with substrate, which was 30 min. Membranes were washed three times with EIA wash solution (145 mM NaCl and 0.05% Tween-20) between incubation steps.

Antiserum production. Four female New Zealand white rabbits were given subcutaneous injections, each consisting of 1 ml of immunogen (equal parts of antigen and adjuvant) distributed between three to four sites along the sides of the body. Initial injections were biweekly, but subsequent immunizations were less frequent (2- to 8-wk intervals over 12 mo). Immunogens were administered either as water-in-oil emulsions, with an oil-based adjuvant, or as a suspension of fungal antigens in physiological saline or PBS. The oil-based mixtures included either Freund's complete adjuvant, Freund's incomplete adjuvant, or gliding bacterial adjuvant (GBA; [26]). Antigens, obtained by extracting hyphae and conidia of *B. cinerea* in PBS plus 0.02% Tween-20, consisted of crude mixtures of soluble components separated from cell-wall debris and other particulate matter by centrifugation at 4 C, 10,000 *g*, for 30 min. Doses were determined as wet weight of fungus per milliliter of extraction buffer, with primary injections of 1 mg/ml. Subsequent injections consisted of 0.5 mg/ml until collection of 803-7 IgG, after which rabbits were injected with 2.5 mg/ml. An intramuscular injection of 0.5 ml of pertussis mixture (diphtheria and tetanus toxoids and pertussis vaccine adsorbed USP, Connaught NDC 49281-280-84; Connaught Laboratories Inc., Ontario, Canada) per rabbit was given in the upper thighs (0.25 ml/leg) to enhance the immune response (23,24) (Fig. 1), 188 and 358 days after the primary injection.

Whole blood was collected from central arteries of rabbit ears by using hubless 22-gauge needles (8). Sera were separated from compacted clots by centrifugation at 4 C, 2,000 *g*, for 5 min, and further clarified by an additional spin at 4 C, 5,000 *g*, for 10 min. One- to two-milliliter aliquots of whole serum were quickly frozen in liquid nitrogen and stored at −80 C. The remaining serum from each collection was stored at 4 C as a slurry of 40% ammonium sulfate, prepared by mixing saturated ammonium sulfate (SAS) solution adjusted to pH 7.4 with serum (9).

Gamma-globulin processing. Amounts of gamma immunoglobulin (IgG) in crude sera were measured by radial immunodiffusion following the manufacturer's instructions in RID kits for determination of rabbit IgG (ICN Biomedicals, Costa Mesa, CA). IgG was separated from whole sera by Protein A-Sepharose affinity chromatography (Pharmacia Inc., Piscataway, NJ; CL-4B) by using an abbreviated version of a protocol by Oi and Herzenberg (18) in which bound IgG was directly eluted with 100 mM glycine-HCl at pH 3.0. Fractions were immediately neutralized with 50 μl of 2.0 M Tris-HCl at pH 8.0 per milliliter of eluant, and IgG concentrated to 5–10 mg/ml using Centricon-30 devices (Amicon Corp., Danvers, MA) or a stir cell with an Omega series membrane (30,000 NMW limit; Filtron Corp., Clinton, MA). Aliquots of 200 μl were quickly frozen in liquid nitrogen and stored at −80 C.

Undesired, cross-reacting antibodies in 803-19 IgG were removed by affinity chromatography with grape and fungal components covalently bound to Sepharose columns. Chenin blanc berries visibly free of fungal infection were quickly frozen and crushed to a fine powder in liquid nitrogen by using a mortar and pestle. The frozen powder was extracted with cold acetone (−20 C), and the precipitated proteins resuspended in PBS at pH 7.4. Borate buffer was added, and protein concentration (determined using Bradford dye-binding procedure [Bio-Rad micro-assay]) was adjusted to 0.5 mg/ml in borate-buffered saline (BBS, pH 8.8; [6]). Soluble crude extract (0.6 ml) from *A. niger* (10 mg of lyophilized mycelium and conidia homogenized in 1 ml of Tris-SDS buffer), which contained an undetermined amount of protein, was mixed with 3.4 ml of 100 mM BBS. Components in each extract were covalently bound to Sepharose according to the manufacturer's suggested protocol, with 4 ml of antigen extract mixed with approximately 1 ml of Reacti-gel 6× (Pierce Chemical Co., Rockford, IL) that had been washed in 10 ml of BBS immediately before combining with antigen extract.

EIA protocol. Fungal and grape antigens were adsorbed onto charge-modified surfaces of Immulon 2 polystyrene microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) and detected in noncompetitive and competitive EIAs. Indirect formats with second (goat-anti-rabbit) antibodies conjugated to

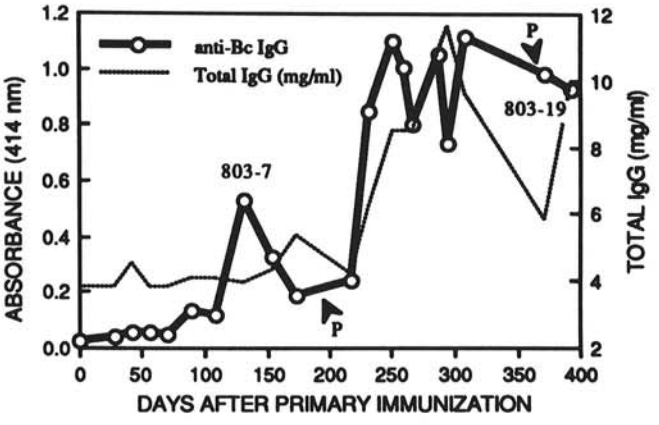

Fig. 1. Levels of total IgG in sera from rabbit 803 (dotted line) and capacity of sera to react with antigens in a crude extract of *Botrytis cinerea* (solid line). Total IgG concentrations (mg/ml of serum) were determined by radial immunodiffusion. Capacity of sera to react with antigens of *B. cinerea*, reflecting levels of antibodies to *B. cinerea* (anti-Bc IgG) in sera, was determined by noncompetitive indirect EIA. Fungal extract was diluted (1:200) in coating buffer and adsorbed onto microwell surfaces; antisera was diluted (1:7,290) in EIA dilution buffer. Increased absorbance (A_{414nm}) corresponded to increased amounts of anti-Bc antibodies. Antisera of 803-7 and 803-19 were collected 131 and 393 days after primary immunization, respectively. An intramuscular injection of 0.5 ml of pertussis/DPT (P) was given in the thighs, 188 and 358 days after primary immunization.

horseradish peroxidase were used for both types of assays. Plates coated with samples containing either known or unknown amounts of fungal and grape compounds diluted in carbonate buffer or grape juice were used for noncompetitive assays. Plates coated with crude extracts from *B. cinerea* grown in CzA broth were used for competitive assays. Samples containing either diluted crude extract from *B. cinerea*, clean grape juice, or juice from berries infected with *B. cinerea* were added to precoated microwells and incubated simultaneously with primary antibody solutions. Variation in adsorption of antigens onto microwell surfaces and interplate differences in the binding of conjugates to primary antibodies were monitored by coating 11 wells on each plate with a (1:2) dilution series of Protein A purified rabbit IgG ranging from 4,000 to 4 ng of IgG per milliliter of 50 mM carbonate buffer at pH 9.6.

Initial coating of plates was similar for both types of assay formats. After 60 min of incubation with extracts, plates were washed three times with EIA wash solution and incubated another 30 min with EIA dilution buffer to block free sites remaining on plate surfaces. Plates could be dried and stored at −80 C for future analysis or analyzed immediately. The general EIA protocol included: incubating antigen-coated plates for 30 min with solutions containing 3–5 μg of anti-Bc rabbit antibodies per milliliter of dilution buffer (150 μl/well); washing three times with wash solution; probing for 30 min with GAR-HRP diluted (1:15,000) in dilution buffer (150 μl/well); washing four times with wash solution; and incubating for 30 min with a substrate solution (150 μl/well). In initial experiments, we used a buffered substrate mixture consisting of: 250 μg/ml of 2,2′-azinobis (3-ethylbenz-thiazoline sulfonic acid) (ABTS; Sigma) and 0.003% H_2O_2 in 100 mM sodium phosphate/citric acid buffer at pH 4.0, with absorbance measured at 414 nm. In most experiments, however, we used a buffered substrate mixture consisting of: 0.005% 3,3′,5,5′ tetramethylbenzidine (TMB; Sigma) and 0.003% H_2O_2 in 100 mM sodium acetate/citric acid buffer at pH 6.0 (7). In the latter instance, color development was stopped by the addition of 75 μl of 2.0 M H_2SO_4 to each well that contained 150 μl of buffered substrate. Absorbance, represented only as A_{450nm}, was measured at two wavelengths: 450 nm, at which absorbance of the oxidized substrate (TMB) is highest, and 650 nm, at which absorbance is lowest. Subtraction of A_{650nm} values from A_{450nm} values corrected for any well-to-well differences inherent in the plates. Absorbance measurements of substrate solutions were obtained by using a Vmax microplate reader (Molecular Devices, Palo Alto, CA), which has a dynamic range to 3,000 (−4,000) mOD units.

EIA detection of *B. cinerea* in grape juice. EIA A_{450nm} values from spiked juice standards were used to determine amounts of infection in unknowns. Multiple levels of infection in juice were simulated by a dilution series prepared from juice mixtures (v/v) of visibly uninfected berries with either juice from berries considered to be 100% infected with *B. cinerea* (determined by visible presence of the fungus on berry surfaces) or extracts of *B. cinerea* grown in CzA broth. Five juice samples, representing five grape clusters of Sauvignon blanc, were diluted (1:100) in coating buffer, adsorbed onto surfaces of microwells, and probed with IgG at 5 μg/ml of EIA dilution buffer. Each juice sample represented a specific (w/w) ratio of visibly infected and uninfected berries. Additional juice samples were compared to standard curves (prepared v/v) to examine degrees of correlation between infection level and A_{450nm} value depending on the method that infection was calculated, either as a mixture of infected and uninfected juice volumes (Sauvignon blanc, v/v), berry weights (Malvasia, w/w), or berry numbers (Sémillon, b/b).

RESULTS

B. cinerea, A. niger, and *Penicillium* sp. grew vigorously in undefined culture media prepared from extracts of grape berries, potato tubers, and tomato fruit, but also grew well in CzA agar and broth media. Changes in culture conditions (including light intensity, media composition, temperature, and culture age) greatly affected the degree of sporulation and amount of fungal mass produced. Under all culture conditions, however, *B. cinerea* produced antigenic substances recognized by anti-Bc antibodies (data not shown).

All four rabbits injected with crude extracts of *B. cinerea* responded similarly in producing anti-Bc antibodies and showing overall increases in levels of serum IgG (Fig. 1), although only rabbit 803 was used to produce the results presented in this paper. Early-bled antiserum (803-7), which had a titer of 36,450, contained anti-Bc antibodies that reacted only with antigens present in extracts of *B. cinerea*. Late-bled antiserum (803-19), which had a titer of 109,350, contained anti-Bc antibodies that cross-reacted strongly with extracts of *B. cinerea*, *A. niger*, and *Penicillium* sp. (Fig. 2), and moderately with extracts of *U. necator* (data not shown). Titer represents the reciprocal of the greatest dilution of antiserum causing a color change greater than five times above background.

Western blot analyses revealed high levels of cross-reactivity between 803-19 IgG and soluble components extracted and separated by SDS-PAGE from all fungi studied (Fig. 3A). Blots probed with 803-7 IgG revealed reactivity with compounds present only in extracts of *B. cinerea*. Antibodies reacted primarily with

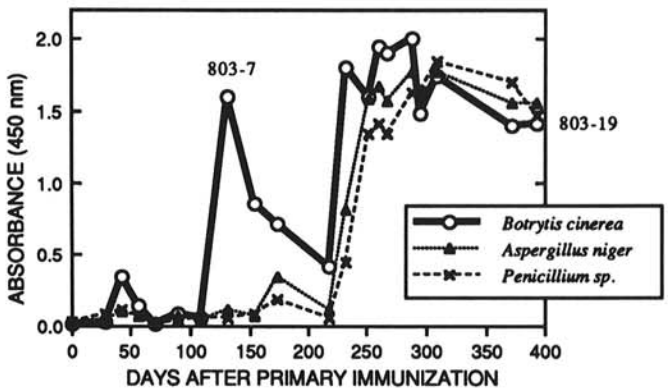

Fig. 2. Levels of cross-reactivity between antibodies to *Botrytis cinerea* (anti-Bc IgG) and crude extracts from *Aspergillus niger*, *B. cinerea*, and *Penicillium* sp. were determined by using noncompetitive indirect EIA format. Soluble fungal components (homogenized, 40 mg of lyophilized fungal tissue per milliliter of Tris-SDS buffer) diluted (1:1,000) in coating buffer and adsorbed onto microwell surfaces. Protein A purified IgG from individual sera was adjusted to 5 μg/ml of EIA dilution buffer. Increased absorbance (A_{450nm}) corresponded to increased binding of anti-Bc antibodies to antigens coated onto microwell surfaces.

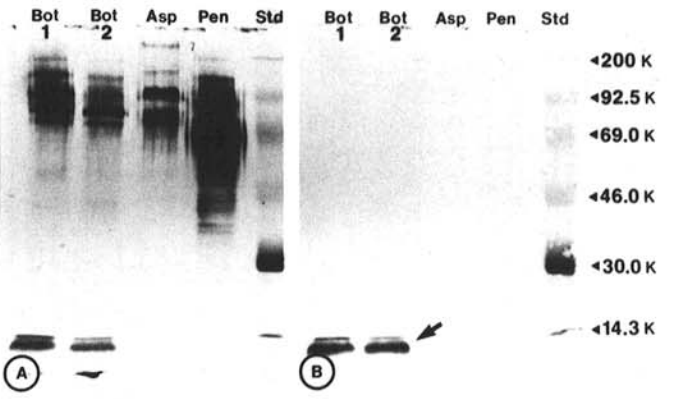

Fig. 3. Western blot analysis of fungal extracts using Protein A purified 803-19 IgG and 803-7 IgG (5 μg/ml of EIA dilution buffer). Components were extracted from mixtures of conidia and mycelia from *Botrytis cinerea* (Bot 1 and 2; two separate extracts), *Aspergillus niger* (Asp), and *Penicillium* sp. (Pen); separated by SDS-PAGE (12% gel); blotted onto nitrocellulose membranes; and probed with 803-19 IgG (**A**) and 803-7 IgG (**B**). Amersham Rainbow markers were used for molecular weight standards (Std).

low molecular weight ($M_r < 2{,}000$; data not shown) compound(s) that migrated at or ahead of the bromophenol blue dye front and were not resolved on 12% SDS-PAGE gels. There was an additional low level of binding with two proteins ($M_r = 80$ and 200 kDa) (Fig. 3B). Neither 803-7 nor 803-19 IgG reacted with blotted compounds from tissues of grape berries (data not shown).

Addition of ionic (Fig. 4) and nonionic detergents to buffers enhanced extraction of antigenic compounds from grape and fungal tissues. The increased extraction and solubilization of antigens caused by SDS permitted samples to be serially diluted 20–30 times more than samples lacking detergent without diminishing A_{450nm} levels. However, SDS concentrations greater than 0.04% (1:50 dilution of sample containing 2% SDS) reduced adsorption of antigens to polystyrene microwells (Fig. 5). SDS concentrations between 0.005% and 0.01% caused the least interference with adsorption of antigenic substances to microwell surfaces (data not shown).

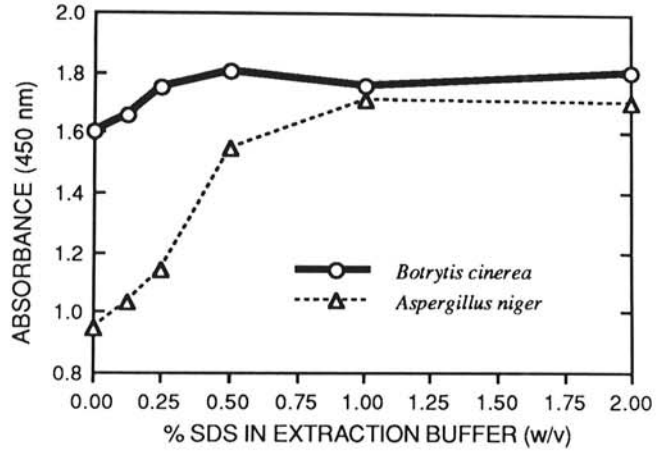

Fig. 4. Effects of sodium dodecyl sulfate (SDS) concentrations on extraction of antigenic substances from *Botrytis cinerea* (solid line) and *Aspergillus niger* (dashed line) were determined by using noncompetitive indirect EIA format. Extracts (40 mg of lyophilized fungal tissue per milliliter of extraction buffer containing discrete amounts of SDS) were diluted (1:1,350) in coating buffer and adsorbed onto microwell surfaces. Protein A purified 803-19 IgG was adjusted to 5 μg/ml of EIA dilution buffer. Increased absorbance (A_{450nm}) corresponded to increased binding of anti-Bc antibodies to antigens coated onto microwell surfaces.

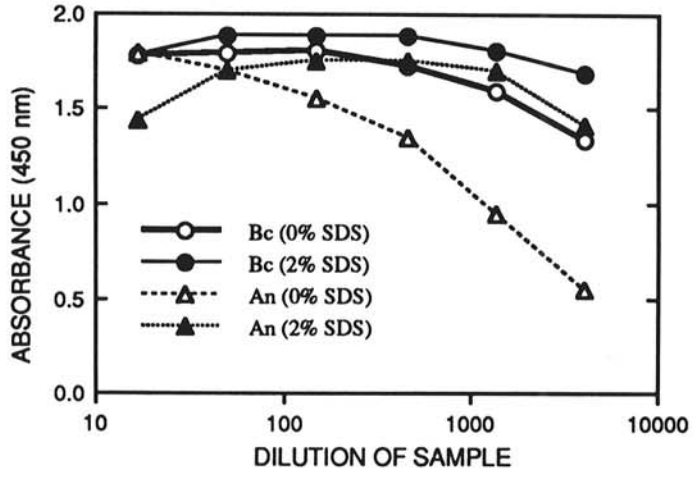

Fig. 5. Effects of sodium dodecyl sulfate (SDS) concentrations on antigen solubilization and adsorption onto microwell surfaces were determined by using noncompetitive indirect EIA format. Dilution of extracts from *Botrytis cinerea* (Bc) and *Aspergillus niger* (An) (40 mg of lyophilized tissue per milliliter of extraction buffer containing either 0 or 2% SDS) decreased concentrations of antigens and SDS. Diluted samples were adsorbed onto microwell surfaces. Protein A purified 803-19 IgG was adjusted to 5 μg/ml of EIA dilution buffer. Increased absorbance (A_{450nm}) corresponded to increased binding of anti-Bc antibodies to antigens coated onto microwell surfaces.

Affinity purification of 803-19 IgG with grape proteins coupled to Sepharose did not reduce levels of antibody binding to either fungal or grape antigens (data not shown). Affinity purification of 803-19 IgG with antigens from *A. niger* coupled to Sepharose or incubation of 803-19 IgG with a crude extract from *A. niger* before EIA analysis greatly reduced cross-reactivity of anti-Bc IgG with extracts of *A. niger*, *Penicillium* sp. (data not shown), *U. necator*, and Cabernet Sauvignon grape berries (Fig. 6). Cross-absorption of 803-19 IgG also significantly reduced its specific activity but still allowed detection of *B. cinerea* well below 0.5% infection in juice from visibly uninfected Cabernet Sauvignon berries spiked (v/v) with 100% infected juice (Fig. 7). Unabsorbed

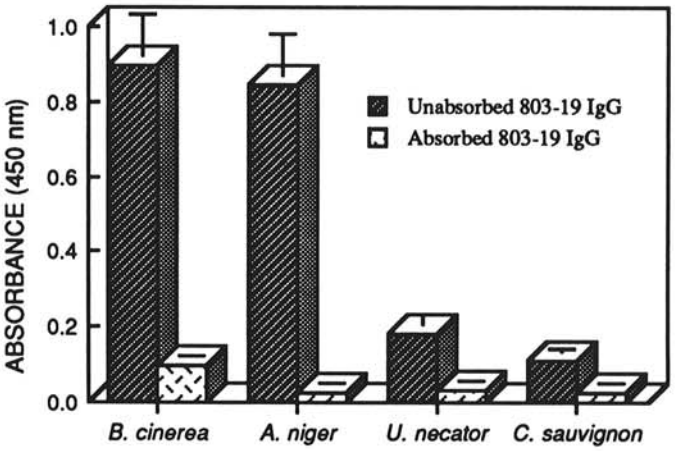

Fig. 6. Cross-reactivity of unabsorbed and preabsorbed (with a crude extract from *Aspergillus niger*) 803-19 antibodies (anti-Bc IgG) with water-soluble extracts from *Botrytis cinerea*, *A. niger*, *Uncinula necator*, and Cabernet Sauvignon berries was determined by using noncompetitive indirect EIA format. Extracts were diluted (1:1,000) in coating buffer and adsorbed onto surfaces of microwells. IgG was adjusted to 3.4 μg/ml of EIA dilution buffer. Increased absorbance (A_{450nm}) corresponded to increased binding of anti-Bc antibodies to antigens coated onto microwell surfaces. Bars indicate standard deviations for means of three replicates.

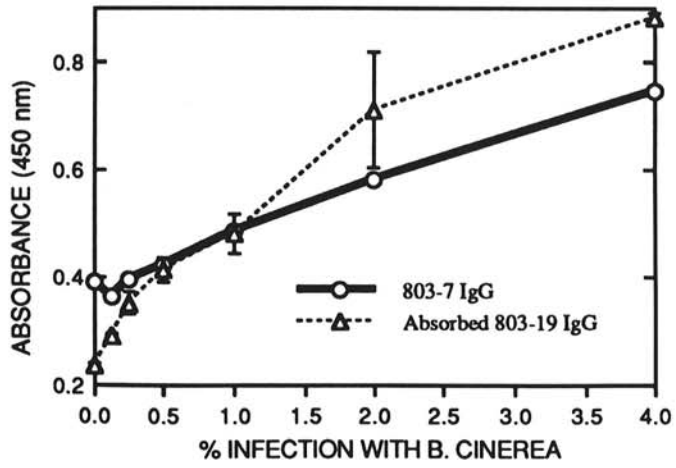

Fig. 7. Detection of *Botrytis cinerea* in spiked grape juice by using noncompetitive indirect EIA format. Gradient of infection levels was prepared by mixing juice (v/v) from visually infected (considered to represent 100% infection) and visually uninfected (considered to represent 0% infection) berries. Juice samples were diluted (1:5) in coating buffer and adsorbed onto surfaces of microwells. Cabernet Sauvignon berry juice (dashed line) was probed with 3.4 μg of 803-19 IgG (preabsorbed with extract from *Aspergillus niger*) per milliliter of EIA dilution buffer. Chardonnay berry juice (solid line) was probed with 5 μg of 803-7 IgG per milliliter of EIA dilution buffer. Increased absorbance (A_{450nm}) corresponded to increased levels of infection by *B. cinerea*. Bars indicate standard deviations for means of three replicates. Standard deviations for 803-7 IgG were less than the diameter of plot symbols.

803-7 IgG also allowed *B. cinerea* to be detected below 0.5% infection in similarly spiked juice from visibly uninfected Chardonnay berries (Fig. 7).

Noncompetitive indirect EIA using 803-7 IgG revealed a strong correlation ($r^2 = 0.924$) between A_{450nm} values, representing levels of fungal antigens in juice extracted from clusters of Sauvignon blanc berries naturally infected with *B. cinerea*, and percentage of infection as determined by a (w/w) ratio of visibly infected berries to uninfected berries (Fig. 8). Generally, correlations between antigen level and percentage of infection were best when

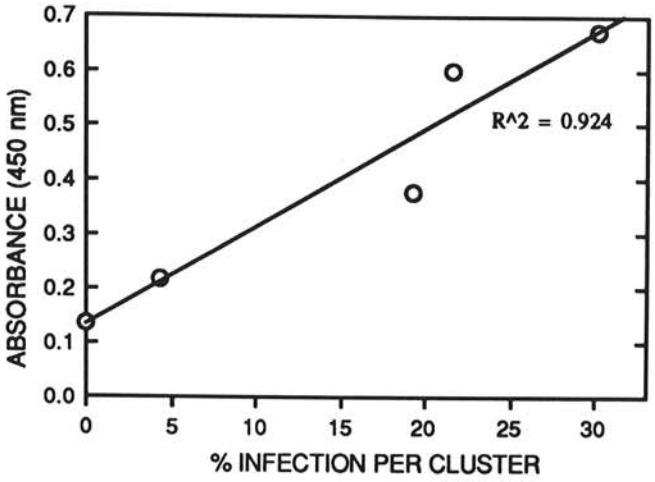

Fig. 8. Detection of *Botrytis cinerea* in naturally infected Sauvignon blanc berry juice by using noncompetitive indirect EIA format. Berries from five clusters were sorted according to presence or absence of visible fungus, and (w/w) ratios of infected and uninfected fruit were used to determine the percentage of infection for each cluster. Juice (from entire cluster) was diluted (1:10) in coating buffer and adsorbed onto surfaces of microwells. The 803-7 IgG was adjusted to 5 μg/ml of EIA dilution buffer. Increased absorbance (A_{450nm}) corresponded to increased levels of infection by *B. cinerea*. Data points represent averages of three replicates. Simple linear regression analysis revealed $Y = 0.13044 + 1.7926^{-2x}$; $r^2 = 0.924$.

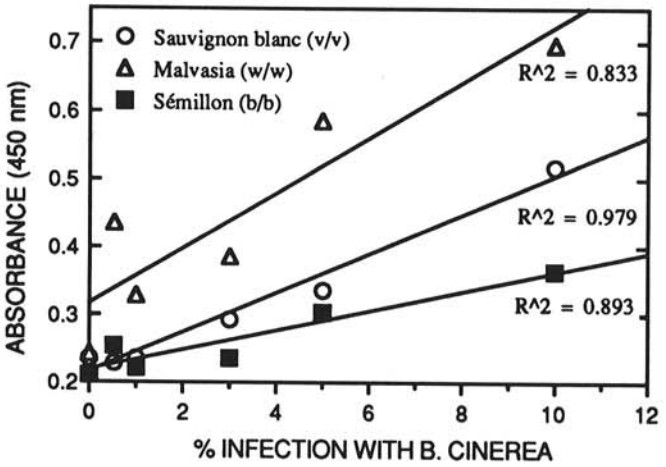

Fig. 9. Determination of the correlation between quantity of fungal antigen in grape juice and percentage of visual infection with *Botrytis cinerea* by using noncompetitive indirect EIA format. Samples were prepared by mixing juice volumes (Sauvignon blanc, v/v), berry weights (Malvasia, w/w), or berry numbers (Sémillon, b/b) from infected and uninfected fruit. Juice was diluted (1:50) in coating buffer and adsorbed onto microwell surfaces. The 803-7 IgG was adjusted to 5 μg/ml of EIA dilution buffer. Increased absorbance (A_{450nm}) corresponded to increased amounts of cross-reactive antigens attached to microwells. The quantity of antigen and percentage of infection correlated best in (v/v) mixtures of infected and uninfected juice from berries of Sauvignon blanc ($r^2 = 0.979$). Simple linear regression analysis revealed: $Y = 0.21345 + 2.9144^{-2x}$ (Sauvignon blanc); $Y = 0.31446 + 4.0473^{-2x}$ (Malvasia); $Y = 0.21822 + 1.4393^{-2x}$ (Sémillon).

standards were prepared from specific (v/v) ratios of juice from infected and uninfected berries (Fig. 9; Sauvignon blanc with $r^2 = 0.979$). Competitive indirect EIAs using 803-7 IgG also showed strong correlations between assay signal and concentration of antigenic compounds diluted with clean grape juice. Dilutions ranging from 1:20 to 1:320 (equivalent to sample concentrations of 5.0–0.3%) of crude extracts from *B. cinerea* grown in CzA broth showed $r^2 = 0.902$. Dilutions ranging from 1:20 to 1:160 (equivalent to sample concentrations of 5.0–0.6%) of juice extracted from Sémillon berries infected with *B. cinerea* showed $r^2 = 0.874$. Juice from uninfected Sémillon berries, at any concentration, did not significantly reduce A_{450nm} values (Fig. 10).

DISCUSSION

Levels of infection in grape berries caused by *B. cinerea* were measured by immunoanalysis of grape juice by using early-bled (803-7) polyclonal antibodies from rabbits immunized with crude extracts of *B. cinerea*. The range of detection in EIAs extended below 1% fungal infection in grape berries, which currently is the lower detection limit desired by members of the wine-grape industry for quantifying amounts of rot-causing organisms. Quantifying levels of fungal infections in berries by measuring amounts of certain fungal components soluble in grape juice represents a major advantage over the existing visual detection methods. Aside from providing speed, sensitivity, and highly reproducible results, an EIA permits accurate measurement of rot levels in machine-harvested fruit, which until now could not be critically assessed by visual inspection because of the decreased amounts of intact berries and the abundance of juice and skins.

EIAs and western blot analyses revealed vastly different binding specificities of antisera; early-bled antiserum (803-7 IgG) was specific for detecting *B. cinerea* while late-bled antiserum (803-19 IgG) was less specific. For measuring levels of rot in juice with as low as 0.02% infection caused by a collection of fungi, 803-19 IgG was useful. Antibodies of 803-7 reacted primarily with low molecular weight compounds present only in extracts of *B. cinerea*. These compounds migrated in a narrow band at or ahead of the dye front on 12% SDS-PAGE gels. Low molecular weight lipids and lipid conjugates migrate similarly on comparable gels, whereas simple sugars and some low molecular weight

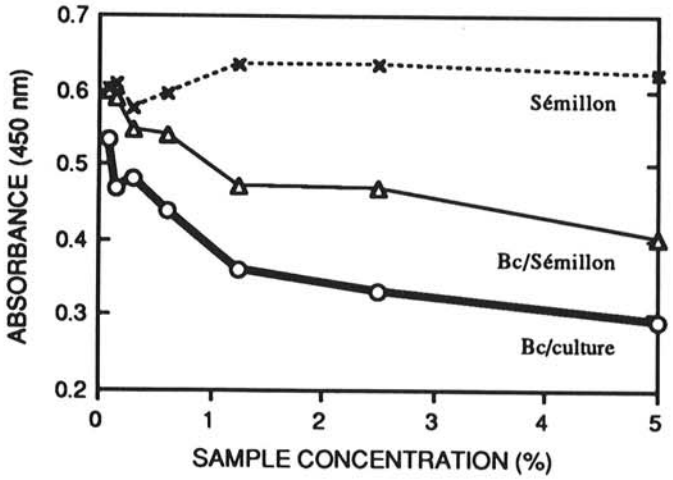

Fig. 10. Detection of fungal antigens from *Botrytis cinerea* by using competitive indirect EIA format. Crude extracts of *B. cinerea* were diluted (1:50) in coating buffer and adsorbed onto surfaces of microwells. The percentage of concentration of competing antigens from extracts of culture-grown *B. cinerea* (Bc/culture) and Sémillon berries naturally infected with *B. cinerea* (Bc/Sémillon), and noncompeting antigens from uninfected berries (Sémillon) were adjusted by diluting samples with clean grape juice. Samples were incubated in microwells simultaneously with 803-7 IgG at 3 μg/ml. Increased absorbance (A_{450nm}) corresponded inversely to increased amounts of antigens from *B. cinerea* in solution.

oligosaccharides often smear and do not migrate in narrow bands. Much of the cross-reactivity (forming smears) observed on blots probed with 803-19 IgG (Fig. 3A) was with simple sugars. Based on preliminary studies with periodate oxidation, 803-7 antibodies react with a carbohydrate epitope on the low molecular weight "lipids". The putative antigenic glycolipid(s) are considered to be immunodominant molecules in crude extracts of *B. cinerea* because of their early stimulation of an immune response.

Late-bled antiserum contained antibodies to compounds that were either less antigenic or less abundant in the immunogen preparations. These antigens had epitopes in common with compounds occurring in other fungal extracts. The high levels of cross-reactivity with extracts of *A. niger* and *Penicillium* sp. prevented specific detection of *B. cinerea* using unabsorbed 803-19 IgG, but permitted measurement of total fungal rot caused by a collection of fungi. Increased specificity of late-bled antiserum for detecting only *B. cinerea* could be achieved by affinity purifying 803-19 IgG with compounds from a crude extract of *A. niger*. Such purification decreased the dynamic range of assay signals but still provided detection sensitivity below 0.5% infection. None of the fungal antigens detected by anti-Bc antibodies appeared to have epitopes in common with compounds present in clean grape juice.

Surfactants, which aid solubilization of lipids, lipid-containing compounds, and various membrane components, can cause desorption in solid-phase immunoassays. Coating proteins onto microtiter plates in the presence of detergents at concentrations equal to or higher than their respective critical micelle concentrations prevents protein adsorption onto the surfaces of polystyrene microwells (5). In the present study, samples were prepared in solutions containing either anionic (Tris-SDS buffer) or nonionic (PBS-Tween buffer) detergents. Concentrations of SDS that exceeded 400 μg per milliliter of coating buffer, or 0.04% (w/v), enhanced antigen extraction from fungal tissues but prevented antigen attachment to well surfaces; whereas, lower concentrations also aided antigen solubilization but did not adversely affect adsorption (Figs. 4 and 5). Buffers of relatively low concentrations (<0.02%, v/v) of nonionic surfactants also effectively solubilized antigenic substances but did not cause desorption of antigens from surfaces of polystyrene microwells. The enhanced solubilization of fungal antigens in buffers containing either anionic or nonionic detergents supports our preliminary identification of the low molecular weight antigenic compounds as lipids with some degree of hydrophobicity.

Levels of analyte in juice samples were represented as percentage of infection, which was based on ratios of either juice volume (v/v), berry weight (w/w), or berry number (b/b) of infected and uninfected berries. The percentage of infected berries by weight is the current standard of measurement used in the wine-grape industry for designating levels of mold on harvested fruit. Depending on the basis for determining percentage of infection ratios, the degree of correlation between antigen level and percentage of infection was high or low. Generally, the highest correlations between visual levels of infection and immunoassay results were obtained from infection gradients prepared with mixtures of juice (v/v) infected with *B. cinerea* and juice not infected. This is attributed to the discrete steps in a dilution obtained by mixing an absolute amount of fungal antigen, as present in a juice sample considered to be 100% infected, with clean juice. Gradients prepared from either b/b or w/w ratios of infected and clean fruit usually had lower correlations because individual samples in a series were not prepared from dilutions of a single infected sample. Each sample was prepared from a separate lot of infected grape berries that, although considered to represent 100% infection, often contained more or fewer fungal antigens than present in other lots also considered to represent 100% infection.

Noncompetitive, indirect EIAs that produced signals directly proportional to the concentrations of measured compounds revealed a high correlation between levels of visual infection and levels of antigenic compounds covering a range from 0 to 30% infection. Competitive, indirect EIAs also revealed a high correlation between levels of visual infection and levels of antigenic compounds, but across a smaller range from 0 to 5% infection. Signals in competitive assays corresponded inversely with amounts of antigenic material in samples because increased levels of antigens in solution decreased antibody binding to microwell surfaces coated with fungal antigens. Both types of assays produced levels of detection that would meet the needs of the grape industry.

Assay format greatly affects assay sensitivity and specificity. Selection of a particular format also can determine speed and simplicity of the assay. Most of the results presented in this paper were obtained from assays that used microplates coated with sample grape juice or fungal extracts. Assay sensitivity with this format typically is less than optimal, being reduced by competition between immunogenic and nonimmunogenic substances binding to the limited number of sites on the microwell surfaces. Double-antibody sandwich assays (DAS), which are used routinely to detect a variety of analytes (11,12,15–17), are attractive because they generally provide increased sensitivity and specificity. DAS formats, however, only detect analytes large enough to have multiple epitopes available to permit simultaneous binding of two or more antibodies. Preliminary experiments in which DAS was used to detect the putative antigenic glycolipid(s) recognized by 803-7 antibodies were nonfunctional, which suggested that the glycolipid(s) could not simultaneously bind two or more antibodies. This binding phenomenon is common to other small molecules such as steroid hormones, which are best detected to a high degree of sensitivity and specificity by using immunoassays with direct competitive formats (14).

This study showed that highly specific and broadly cross-reactive polyclonal antibodies could be produced in rabbits immunized with crude extracts of *B. cinerea*. Depending on the particular IgG sample used, infections caused specifically by *B. cinerea* or general levels of fungal rot on grape berries caused by a collection of fungi could be quantitatively measured. Future work will focus on structural characterization of the putative antigenic glycolipids for development of antigen-labeled conjugates to be used in direct competition assay formats that permit rapid quantification of infections caused specifically by *B. cinerea*.

LITERATURE CITED

1. Anderson, R. R., Lee, T. T., Saewert, D. C., Sowden, K. M., and Valkirs, G. E. 1986. Internally referenced ImmunoConcentration assays. Clin. Chem. 32:1692-1695.
2. Bulit, J., and Dubos, B. 1988. Botrytis bunch rot and blight. Pages 13-15 in: Compendium of Grape Diseases. R. C. Pearson and A. C. Goheen, eds. The American Phytopathological Society, St. Paul, MN.
3. Clark, M. F. 1981. Immunosorbent assays in plant pathology. Pages 83-106 in: Annual Reviews in Phytopathology. Vol. 19. R. G. Grogan, ed. Annual Reviews Inc., Palo Alto, CA.
4. Gaastra, W. 1984. Enzyme-linked immunosorbent assay (ELISA). Pages 349-355 in: Methods in Molecular Biology, Vol. 1. J. M. Walker, ed. Humana Press Inc., Clifton, NJ.
5. Gardas, A., and Lewartowska, A. 1988. Coating of proteins to polystyrene ELISA plates in the presence of detergents. J. Immunol. Methods 106:251-255.
6. Garvey, J. S., Cremer, N. E., and Sussdorf, D. H. 1977. Methods in Immunology. A Laboratory Text for Instruction and Research. 3rd ed. Benjamin-Cummings Publishing Co., Menlo Park, CA. 568 pp.
7. Goka, A. K. J., and Farthing, M. J. G. 1987. The use of 3,3′,5,5′-tetramethylbenzidine as a peroxidase substrate in microplate enzyme-linked immunosorbent assay. J. Immunoassay 8:29-41.
8. Gordon, L. K. 1981. A reliable method for repetitively bleeding rabbits from the central artery of the ear. J. Immunol. Methods 44:241-245.
9. Henry, C. 1980. Storage of antisera. Pages 276-277 in: Selected Methods in Cellular Immunology. B. B. Mishell and S. M. Shiigi, eds. Freeman Publications, San Francisco.
10. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.
11. Lin, H. H., and Cousin, M. A. 1987. Evaluation of enzyme-linked immunosorbent assay for detection of molds in food. J. Food Sci. 52:1089-1094.

12. Lin, H. H., Lister, R. M., and Cousin, M. A. 1986. Enzyme-linked immunosorbent assay for detection of mold in tomato puree. J. Food Sci. 51:180-183, 192.
13. Loomis, W. D. 1974. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods Enzymol. 31A:528-544.
14. Munro, C. J., and Lasley, B. L. 1988. Non-radiometric methods for immunoassay of steroid hormones. Pages 289-329 in: Non-Radiometric Assays: Technology and Application in Polypeptide and Steroid Hormone Detection. B. D. Albertson and F. P. Haseltine, eds. Alan R. Liss, Inc., New York.
15. Notermans, S., and Heuvelman, C. J. 1985. Immunological detection of moulds in food by using the enzyme-linked immunosorbent assay (ELISA); preparation of antigens. Int. J. Food Microbiol. 2:247-258.
16. Notermans, S., Heuvelman, C. J., Beumer, R. R., and Maas, R. 1986. Immunological detection of moulds in food: Relation between antigen production and growth. Int. J. Food Microbiol. 3:253-261.
17. Notermans, S., Heuvelman, C. J., van Egmond, H. P., Paulsch, W. E., and Besling, J. R. 1986. Detection of mold in food by enzyme-linked immunosorbent assay. J. Food Prot. 49:786-791.
18. Oi, V. T., and Herzenberg, L. A. 1980. Immunoglobulin-producing hybrid cell lines. Pages 368-370 in: Selected Methods in Cellular Immunology. B. B. Mishell and S. M. Shiigi, eds. Freeman Publications, San Francisco.
19. Perkins, D. D. 1962. Preservation of Neurospora stock cultures with anhydrous silica gel. Can. J. Microbiol. 8:591-594.
20. Ravji, R. G., Rodriguez, S. B., and Thorton, R. J. 1988. Glycerol production by four common grape molds. Am. J. Enol. Vitic. 39:77-82.
21. Savage, S. D., and Sall, M. A. 1981. Radioimmunosorbent assay for *Botrytis cinerea*. Phytopathology 71:411-415.
22. Savage, S. D., and Sall, M. A. 1982. The use of a radio-immunosorbent assay for *Botrytis cinerea*. EPPO Bull. 12:49-53.
23. Sultzer, B. M., Craig, J. P., and Castagna, R. 1985. The adjuvant effect of Pertussis endotoxin protein in modulating the immune response to cholera toxoid in mice. Pages 225-232 in: Proceedings of the Fourth International Symposium on Pertussis, Joint IABS/WHO Meeting, Geneva, Switzerland, 1984. Vol. 61. C. R. Manclark and W. Hennessen, eds. S. Karger AG, Basel, Switzerland.
24. Sultzer, B. M., Craig, J. P., and Castagna, R. 1987. Immunomodulation by outer membrane proteins associated with the endotoxin of gram-negative bacteria. Pages 113-123 in: Progress in Leukocyte Biology. Vol. 6. J. A. Majde, ed. Alan R. Liss, Inc., New York.
25. Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354.
26. Usinger, W. R., and Mishell, R. I. 1987. Chemical characterization and immunoenhancing activities of purified *Cytophaga* exopolymer. Pages 125-137 in: Progress in Leukocyte Biology. Vol. 6. J. A. Majde, ed. Alan R. Liss, Inc., New York.
27. Valkirs, G. E., and Barton, R. 1985. ImmunoConcentration—A new format for solid-phase immunoassays. Clin. Chem. 31:1427-1431.
28. Walker, J. M. 1987. The enzyme-linked immunosorbent assay (ELISA). Pages 82-97 in: Techniques in Molecular Biology. Vol. 2. J. M. Walker and W. Gaastra, eds. Macmillan Publishing Co., New York.
29. Windels, C. E., Burnes, P. M., and Kommedahl, T. 1988. Five-year preservation of *Fusarium* species on silica gel and soil. Phytopathology 78:107-109.