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Electrophoretic and Immunological Comparisons of Developmentally Regulated Proteins in Members of the *Sclerotiniaceae* and Other Sclerotial Fungi

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The fungal stroma is a distinct developmental stage, a compact mass of hyphal cells enveloped by a melanized layer of rind cells which is produced from vegetative mycelium. Two types of stromata that are characteristic of members of the *Sclerotiniaceae* but are also produced in a wide range of other fungi, i.e., the determinate tuberlike sclerotium and the indeterminate platelike substratal stroma, were compared in these studies. Developmental proteins found in determinate sclerotial and indeterminate substratal stromata, but not in mycelia, were characterized and compared in 52 isolates of fungi, both ascomycetes (including 18 species in the *Sclerotiniaceae* and 5 species of *Aspergillus*) and the basidiomycete *Sclerotium rolfsii*. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of mycelial, stromatal initial, and stromatal extracts demonstrated that all members of the *Sclerotiniaceae* produced proteins unique to stromatal extracts within a molecular weight range of 31,000 to 39,500 which composed 13 to 58% of the total protein in stromata. Proteins unique to the sclerotial stage were also produced in *Sclerotium rolfsii* and the *Aspergillus* species but within a generally lower-molecular-weight range of 11,000 to 30,000. The proteins were then characterized by two-dimensional electrophoresis to determine the number and isoelectric point of polypeptides composing each protein. Polyclonal antibodies were raised to the major 36-kDa sclerotial protein of *Sclerotinia sclerotiorum* (Ssp). Immunoblots demonstrated that all sclerotial proteins from species in the *Sclerotiniaceae* cross-reacted with anti-Ssp antibodies, while no cross-reaction was observed with proteins from substratal stromatal species in the *Sclerotiniaceae*, sclerotial species of *Aspergillus*, or *Sclerotium rolfsii*. Results of discriminant analysis of the data from competitive inhibition enzyme-linked immunosorbent assays were consistent with the results of immunoblotting. Three groupings, sclerotial species in the *Sclerotiniaceae*, substratal stromatal species in the family, and sclerotial species outside the family, were delimited on the basis of relative decreasing ability to compete for anti-Ssp antibody. These data demonstrate that stromatal proteins differ among different taxonomic groups of fungi and suggest that the *Sclerotiniaceae* may include two distinct lineages of genera.

The fungal stroma, a compact mass of hyphal cells enveloped by a melanized layer of rind cells or extracellular material, is an ecologically significant and developmentally distinct stage in the life cycle of many soil-borne fungi. Produced from vegetative mycelium, the stroma can function as a resistant propagule, capable of surviving freezing and desiccation. After physiological conditioning, it is capable of germinating to produce a mycelium, a conidial anamorph, or a sexual teleomorph (fruiting body). Stromata appear to have evolved convergently among ascomycetes and basidiomycetes, including ectomycorrhizal and aflatoxin-producing fungi, as well as a large group of soil-borne plant pathogens. All members of the ascomycete family, the *Sclerotiniaceae*, produce one of two basic types of stroma (37). The first type is the determinate tuberlike sclerotium (Fig. 1A), detaching from the plant host usually to infest soil and plant debris and associated with those genera accommodating necrotrophic, plant-pathogenic species. The second type is the indeterminate platelike substratal stroma (Fig. 1B), penetrating and enveloping the substrate on which it develops and associated with those genera including species presumed to be saprophytes, with the notable exception of *Sclerotinia homoeocarpa*, the causal agent of dollar spot of turf (14). Like other stromatal fungi, many of these fungi produce only vegetative mycelium and stromata when isolated from nature in pure culture. Production of the sexual fruiting structures necessary for conclusive classification or identification can be problematic.

Developmentally regulated proteins produced abundantly in stromata, but generally not in other vegetative or reproductive stages of the life cycle, have been reported in ascomycetes such as *Aspergillus* spp. (24) and several members of the *Sclerotiniaceae* (11, 23, 26, 29) and in some basidiomycetes such as *Typhula*, *Coprinus*, *Sclerotium rolfsii* (11, 18), and *Hygrophoropsis* (1). In some species, these proteins have been immunochemically localized in cytoplasmic protein bodies within the stroma (19, 30). The proteins, composing 15 to 60% of the total protein in stromata, may be ubiquitous among stromatal fungi and probably serve as reserves for germination (2, 4, 8, 14, 38). Given the similarity in structure and function of fungal stromata, it is possible that the developmental stromatal proteins, at least in some domains, are conserved among stromatal fungi. It is also likely, however, that the proteins differ among different taxonomic groups of fungi, perhaps reflecting the phylogeny of stromatal fungi. If the proteins differ, at what taxonomic rank do they differ? Do they differ at the same taxonomic rank in different groups of fungi?

Data from previous studies based on an immunological comparison of stromatal proteins suggest that stromatal proteins differ among different fungi, although few such studies have been performed. Among species in the genus *Sclerotinia*, the most abundant stromatal proteins have been

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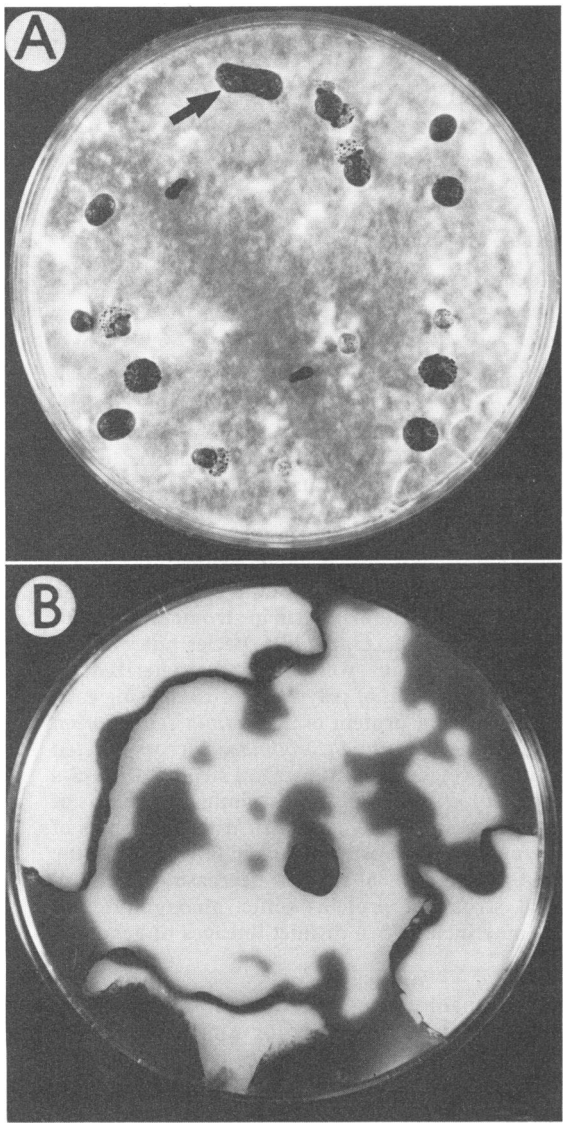

FIG. 1. Two types of stromata in cultures grown on potato-dextrose agar. (A) *Sclerotinia sclerotiorum*; arrow indicates determinate, sclerotium; (B) *S. homoeocarpa*; colony-reverse, indeterminate, substratal stroma.

reported to be closely related (32), while within other genera, such as the genus *Typhula*, species were reported to be antigenically different (19). In studies associated with those reported here, we found that a sclerotial protein was closely related among three species in the *Flavi* section of the genus *Aspergillus* but unrelated to sclerotial proteins of two species from another section of the genus (24). These studies suggest that stromatal proteins are antigenically different and that these differences resolve at different taxonomic levels (genus, species, and species-aggregate, respectively) among different groups of the fungi that have been studied.

In the *Sclerotiniaceae*, on the basis of histochemical and ultrastructural evidence, cytoplasmic protein storage differs in the two types of stroma, the sclerotium and the substratal stroma. In sclerotia, protein is sequestered in membrane-bound cytoplasmic protein bodies from the time the sclerotia are fully developed through dormancy; on germination, protein reserves are almost completely depleted (2, 4, 6–8, 11, 14, 15, 31, 38). In mature, substratal stromata, lipid is an important cytoplasmic storage product and protein bodies are relatively infrequent, suggesting that protein is not as important a long-term storage product in substratal stromata as in sclerotia (14, 15). On the basis of electrophoretic studies, developmental proteins within a narrow molecular size range (32 to 38 kDa) and composing up to 60% of the total protein have been reported in both sclerotial (11, 26, 29) and substratal stromatal (23) members of the family. Developmental sclerotial proteins, with molecular weights of 35,500 and 37,000, have also been reported in the stromatal anamorph, *Sclerotium cepivorum* (23), the causal agent of soft rot of onions, which was accommodated in the family (37) but lacks a sexual fruiting body and therefore cannot be placed taxonomically among the sexually reproducing fungi. *Sclerotium cepivorum*, in addition to producing major stromatal proteins in the same molecular weight range as members of the *Sclerotiniaceae*, produces sclerotia that are anatomically and histochemically characteristic of sclerotial members of the family and produces a *Myrioconium*-like microconidial state (probably spermatial) typical of the family (14).

The primary objective of this study was to determine, within a family of stromatal fungi, i.e., the *Sclerotiniaceae*, whether developmental stromatal proteins differ and whether differences reflect hypotheses about groups within the family, especially differences between the plant-pathogenic sclerotial and the mostly saprophytic substratal stromatal groups of genera. Proteins from 18 species in the *Sclerotiniaceae*, including 31 sclerotial and 17 substratal stromatal isolates, were examined. In addition, five isolates representing two sections of the genus *Aspergillus* were included as an ascomycetous outgroup as well as three isolates of the basidiomycete *Sclerotium rolfsii*. Stromatal proteins were characterized by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional electrophoresis and then were compared by Western blot (immunoblot) and competitive enzyme-linked immunosorbent assays (ELISAs) to determine affinities on the basis of simple cross-reactivity and relative competitive ability to bind with specific antibodies, respectively.

MATERIALS AND METHODS

Cultures. Isolates used in this study are listed in Table 1. Cultures were grown in potato-dextrose broth (33) at room temperature (20 to 23°C) in the dark. Mycelia were harvested after 2 to 4 days, stromatal initials were harvested as white tufts of mycelium after 4 to 7 days, and sclerotia and substratal stromata were harvested after 4 to 6 weeks by filtration. All fungal material was freeze-dried for 2 to 4 days prior to protein extraction.

Protein extraction. Samples were ground to a fine powder in liquid nitrogen by using a mortar and pestle. The powder was suspended in cold extraction buffer (11, 20) and homogenized for 1 to 2 min with a Polytron homogenizer at 4°C, and the homogenates were centrifuged at 38,000 rpm (Beckman SW40 rotor) at 4°C for 3 to 6 h. The resulting supernatants were stored at −20°C until required. Total protein was determined by the method of Lowry et al. (17) by using bovine serum albumin (BSA) as a standard.

One- and two-dimensional electrophoresis. One-dimensional SDS-PAGE was carried out on all isolates by using the methods of Laemmli (16). Denaturing buffer (23) was added

TABLE 1. Origin of isolates

LMK no. and species	Host	Locality	Source[a]
Sclerotial stromatal species			
2 *Sclerotinia sclerotiorum*	Lettuce	LaSalle, Ontario	Jarvis #128
34 *S. sclerotiorum*	Cabbage	Australia	ATCC 34325
79 *S. sclerotiorum*	Carrot	Ontario	Kohn
81 *S. sclerotiorum*	Soybean	Australia	Steadman #147
83 *S. sclerotiorum*	Snapbean	New York	Steadman #155
87 *S. sclerotiorum*	Canada thistle	Montana	Steadman #176
91 *S. sclerotiorum*	Alfalfa	Washington	Gilbert, U.S. Department of Agriculture
92 *S. sclerotiorum*	Soybean	Kansas	Jardine
94 *S. sclerotiorum*	White bean	Ontario	Kohn and Grenville
121 *S. sclerotiorum*	Rapeseed	Grang Xi, China	Wong
122 *S. sclerotiorum*	Soybean	China	Wong
3 *Sclerotinia minor*	Lettuce	LaSalle, Ontario	Jarvis #129
35 *S. minor*	Potato	Australia	ATCC 34324
4 *Sclerotinia trifoliorum*	Alfalfa	Virginia	Srvcek
103 *S. trifoliorum*	Milk vetch		ATCC 52595
12 *Monilinia fructicola*	Peach (Red Haven)	Ontario	Biggs
17 *Myriosclerotinia dennisii*	*Eriophorum angustifolum*	Oppland, Norway	Kohn and Schumacher
18 *Botrytis cinerea*	Onion	Ontario	Jarvis #11
19 *Botrytis porri*	Garlic	Oregon	Jarvis #130
33 *Ciboria acerina*			ATCC 14978
50 *Dumontinia tuberosa*	Bloodroot	Ontario	Kohn and Malloch
51 *D. tuberosa*	Bloodroot	Ontario	Kohn and Malloch
52 *Ciborinia erythronii*	*Erythronium* sp.	Ontario	Kohn and Malloch
53 *C. erythronii*	*Erythronium* sp.	Ontario	Kohn and Malloch
1 *Sclerotium cepivorum*	Onion	Netherlands	Gams #83-11
70 *S. cepivorum*		Nevada	Adams, Sc-US-60B
71 *S. cepivorum*	Onion (soil)	Elmer, N.J.	Adams, Sc-103
72 *S. cepivorum*	Onion (soil)	New Jersey	Adams, Sc-NJ-3
73 *S. cepivorum*		Brazil	Adams, Sc-W-10
74 *S. cepivorum*	Onion	New Jersey	Adams, Sc-102
126 *S. cepivorum*	Green onion	Ontario	ATCC 66452
164 *Sclerotium rolfsii*	Carrot	North Carolina	Punja #153
165 *S. rolfsii*	Carrot	North Carolina	Punja #169
166 *S. rolfsii*	Goosegrass	North Carolina	Punja #177
127 *Aspergillus alliaceus*	Blister beetle		Wicklow, NRRL315
128 *Aspergillus auricomus*			Wicklow, NRRL391
135 *Aspergillus parasiticus*	Corn	North Carolina	NRRL6433
136 *Aspergillus flavus*	Corn	North Carolina	NRRL6541
137 *Aspergillus nomius*	Moldy wheat	United States	NRRL13137
Substratal stromatal species			
8 *Sclerotinia homoeocarpa*	*Agrostis palustris*	Pennsylvania	Burpee, S84
9 *S. homoeocarpa*	*A. palustris*	Pennsylvania	Burpee, S38
10 *S. homoeocarpa*	*A. palustris*	Cambridge, Ontario	Burpee, S037
11 *S. homoeocarpa*	*A. palustris*	Cambridge, Ontario	Burpee, S001
37 *S. homoeocarpa*	*A. palustris* cv. Pemcross		ATCC 56039
78 *S. homoeocarpa*	*A. palustris*	Pennsylvania	Burpee, S9
5 *Lambertella subrenispora*	*Aster ageratoides* var. *ovata*	Honshu, Japan	Kohn, CUP-JA3663
399 *Lambertella langei*	*Andromeda polyfolia*	Hedmark, Norway	Jensen, S-681.1
102 *Rutstroemia bulgarioides*	Spruce cones	Palgrave, Ontario	Thorne
394 *Poculum henningsianum*	*Carex lasiocarpa*	Buskerud, Norway	Jensen, S-540.1
395 *P. henningsianum*	*Carex juncella*	Oslo, Norway	Jensen, S-601.1
402 *Poculum petiolarum*	*Quercus* or *Fagus* sp.	Oestfold, Norway	Jensen, S-756.1
403 *Poculum sydowianum*	*Fagus sylvatica*	Zealand, Denmark	Jensen, S-775.1

[a] ATCC, American Type Culture Collection; CUP, Cornell University Plant Pathology Herbarium; NRRL, Northern Regional Research Laboratories, Peoria, Ill.

to samples to give a final concentration of 1.0 to 1.5 μg of protein per μl, and the samples were denatured by boiling for 5 min. Samples (60 μg of protein per well) were separated on 8 to 20% (wt/vol) SDS-polyacrylamide linear gradient gels at a constant voltage, 1 V/cm, for 7 h at 4°C. Low-molecular-weight markers (Bio-Rad) were used as standards. Gels were stained with Coomassie blue (Sigma), destained, and photographed as described by Novak and Kohn (23). Gels of separated stromatal proteins of *Sclerotinia sclerotiorum*, *Sclerotinia homoeocarpa*, *Sclerotium cepivorum*, and *Lambertella subrenispora* were also stained with periodic acid-Schiff reagent (13) to determine whether the proteins were glycosylated. Gels were dried onto cellulose membranes (Bio-Rad) to facilitate densitometric scanning (23).

Two-dimensional electrophoretic analysis was carried out on one isolate each of *Sclerotinia sclerotiorum* (LMK2),

Sclerotinia homoeocarpa (LMK11), *Sclerotium cepivorum* (LMK1), and *L. subrenispora* (LMK5) by the methods of O'Farrell (25). Samples (2 to 5 μg of protein per μl) were focused on tube gels containing 5% (vol/vol) pH 5 to 7 and pH 3.5 to 10 ampholytes (1:4) (LKB) overnight for 2 h at 200 V, 15 h at 400 V, and 1 h at 800 V (7,200 V · h). Tube gels were overlaid onto 8 to 20% (wt/vol) SDS-polyacrylamide linear gradient gels and electrophoresed for 18 h at 0.625 V/cm at 4°C. Two-dimensional gels were visualized after Coomassie blue staining (23).

Protein purification. Crude sclerotial protein extracts of *Sclerotinia sclerotiorum* (LMK2) were focused as previously described but by using tube gels which contained 5% (vol/vol) pH 5.0 to 6.5 and pH 4 to 6 ampholytes (2:1). After being focused, tube gels were incubated in reducing buffer (25) at 4°C after which the protein band corresponding to the major polypeptide (pH 6.0) of the 36-kDa sclerotial protein of LMK2 (Ssp) was excised. The gel pieces were chopped into 1-mm cubes, transferred into the large cups of an electro-eluter, and eluted at 200 V for 2 h at 4°C. The eluting buffer was the same as that described for one-dimensional SDS-PAGE. Concentrated protein (300 μl) was removed from the small cups of the electro-eluter and stored at −20°C until required. This purified, SDS-denatured protein was designated denatured Ssp (denSsp). Purity of the protein was determined by densitometric scanning of SDS-polyacrylamide gels (23).

Native Ssp (natSsp) was purified by separating crude sclerotial extracts of LMK2 on one-dimensional 10% (wt/vol) polyacrylamide gels. An 8-mm strip was excised from each side of the gel and stained in Coomassie blue for 30 min. After destaining, these strips were aligned with the unstained portion of the gel, and the area corresponding to the major polypeptide (pH 6.0) of the 36-kDa sclerotial protein band of LMK2 was excised. The gel strip was chopped into 1-mm cubes and electro-eluted as described above except that the eluting buffer did not contain SDS. Concentrated protein (300 μl) was stored, and its purity was determined as described above for denSsp.

Antibody preparation. Chickens were selected for production of polyclonal antibodies to both denSsp and natSsp because they produce abundant antibodies (immunoglobulin Y) that are easily extracted from egg yolk in a relatively pure form (12). Three White Leghorn hens for each antigen (denSsp and natSsp) were allowed to acclimatize to their new environment for 2 to 6 weeks prior to injections, during which time eggs were collected for extraction of preimmune antibodies. The first injection consisted of 100 μg of denSsp or natSsp in complete Freund adjuvant (Sigma) (1:1), with the total volume not exceeding 1.0 ml. The next four injections consisted of 150 μg of denSsp or natSsp, but in Freund incomplete adjuvant (1:1), with the total volume not exceeding 0.5 ml. Each dose of antigen was given as two intramuscular injections in different areas of the breast muscle. Hens were injected weekly for 5 weeks. Eggs from which specific (anti-denSsp or anti-natSsp) antibodies were extracted were collected 4 to 8 weeks after the first injection.

Antibodies were extracted from egg yolks by precipitation with dextran sulfate (12), further purified by reprecipitation with 14% (wt/vol) sodium sulfate, and dialyzed against Tris-buffered saline (TBS) (12) for 2 days. Purity of the antibodies was determined by SDS-PAGE. Antibodies were stored at 4°C until required.

Western blot. Mycelial, stromatal initial, and sclerotial or substratal stromatal extracts from all of the isolates listed in Table 1 were separated by one-dimensional SDS-PAGE as described above. Proteins were electro-transferred to 0.2-μm-pore-size nitrocellulose membranes (Schleicher & Schuell) by the methods of Towbin et al. (35) and were either stained for total protein with the colloidal gold protein detection kit (Bio-Rad) by the methods of Rohringer and Holden (27) or immunoassayed by the procedure of Tijssen (34) except that BSA was used instead of gelatin as a blocking agent. Alkaline phosphatase-conjugated anti-chicken rabbit antibodies (Sigma) were used as secondary antibodies to label anti-denSsp and anti-natSsp chicken antibodies.

Several controls suggested by Bendayan (5) were used as follows. (i) Immunoblots of sclerotial and substratal stromatal proteins were treated with preimmune antibodies. (ii) Immunoblots of mycelial and stromatal initial proteins were treated with anti-denSsp and anti-natSsp antibodies. (iii) Immunoblots of sclerotial and substratal stromatal proteins were treated with secondary antibodies only to detect nonspecific binding. (iv) Immunoblots of sclerotial and substratal stromatal proteins were treated with anti-denSsp or anti-natSsp antibodies that had been adsorbed with an excess of Ssp for 12 h at 4°C to verify the specificity of the antigen-antibody reaction. These controls were also used for ELISAs.

ELISAs. The indirect noncompetitive methods of Voller et al. (36) and Tijssen (34) were used to establish a standard curve for anti-denSsp and anti-natSsp antibodies from which the amount of antibody bound to Ssp could be determined. Nunc polystyrene microwell plates were coated with several dilutions of crude Ssp in coating buffer (36) overnight at 4°C. Wells were washed with TBS-Tween (0.01 M Tris–1.37 M NaCl–0.05% [vol/vol] Tween-20 [pH 7.4]) prior to adding 3% (wt/vol) BSA in TBS and incubating at 37°C for 30 min. This step, necessary to block nonspecific binding sites on the solid phase, was repeated once. All subsequent incubations were done at 37°C, and each step after incubation with primary antibody was separated by five washes with TBS-Tween. Microwells were treated with several dilutions of anti-denSsp or anti-natSsp chicken antibodies in TBS for 1 h, followed by treatment with alkaline phosphatase-conjugated anti-chicken rabbit antibodies in TBS for 1 h. The substrate (*p*-nitrophenyl phosphate) was added in modified dimethylformamide buffer, pH 9.8 (36), and incubated for 1 h. The reaction was stopped with 2.5 N NaOH, and the plates were allowed to stand at room temp for 30 min prior to reading absorbances (optical density at 405 nm) with an enzyme immunoassay reader (Mandel). These assays were repeated three times for both anti-denSsp and anti-natSsp antibodies, with eight replicates of Ssp per assay. The weighted four-parameter logistic curve-fitting program developed by Davis et al. in 1980 (9) was used to generate standard (concentration of antibody [units per milliliter] versus optical density at 405 nm) curves for anti-denSsp and anti-natSsp antibodies.

After working dilutions of Ssp and anti-denSsp and anti-natSsp antibodies were established (34), the modified indirect inhibition enzyme immunoassay of Voller et al. (36) was used to compare the binding abilities of other sclerotial and substratal stromatal proteins (i.e., inhibitors) for anti-denSsp and anti-natSsp antibodies. The following representative isolates from each of the species listed in Table 1 were designated inhibitors: LMK1, 2, 3, 5, 11, 12, 17, 18, 19, 33, 50, 52, 102, 103, 127, 128, 135, 136, 137, 164, 394, 399, and 403. Microwells were coated with Ssp as described above. In competitive assays with anti-denSsp antibodies, several dilutions of the inhibitor were boiled for 5 min prior to being mixed with a known constant concentration of antibodies. In

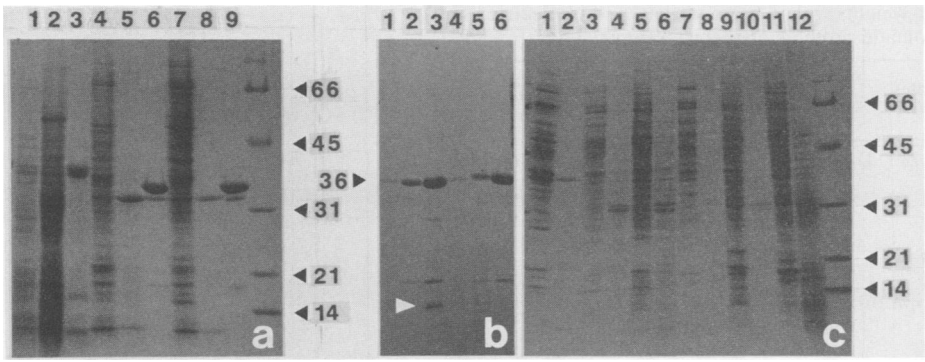

FIG. 2. One-dimensional 8 to 20% (wt/vol) SDS-PAGE of sclerotial and substratal stromatal species in the *Sclerotiniaceae*. (A) Mycelial, stromatal initial, and sclerotial extracts of *Botrytis porri* (lanes 1 to 3) and *Dumontinia tuberosa* LMK50 (lanes 4 to 6) and LMK51 (lanes 7 to 9), respectively. (B) Mycelial, stromatal initial, and sclerotial extracts of *Ciborinia erythronii* LMK52 (lanes 1 to 3) and LMK53 (lanes 4 to 6), respectively. Arrow denotes sclerotial protein (molecular weight, 11,000) produced in LMK52 (lane 3) but absent in sclerotial extract of LMK53 (lane 6). (C) Mycelial and substratal stromatal extracts of *Rutstroemia bulgarioides* (lanes 1 and 2), *Poculum henningsianum* LMK394 (lanes 3 and 4) and LMK395 (lanes 5 and 6), *Lambertella langei* (lanes 7 and 8), *P. petiolarum* (lanes 9 and 10), and *P. sydowianum* (lanes 11 and 12). Molecular weight standards are phosphorylase *b* (92,000), BSA (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,000), and α-lactoalbumin (14,000).

competitive assays with anti-natSsp antibodies, several dilutions of the inhibitor were mixed with a known constant concentration of antibodies without prior boiling. The inhibitor plus anti-Ssp-antibody mixtures were incubated overnight at 37°C with vigorous shaking (150 rpm). Microwells were blocked as described above. Inhibitor plus anti-Ssp-antibody mixtures were centrifuged at high speed (Beckman Microfuge) at room temperature for 15 min to pellet immune complexes, the supernatants were added to microwells, and the plates were incubated at 37°C for 1 h. The supernatants of inhibitor plus anti-Ssp antibody served as the primary antibodies. The procedure after incubation with primary antibodies was as described above. These assays were repeated twice for both anti-denSsp and anti-natSsp antibodies, with eight replicates for each inhibitor concentration per assay. The resulting absorbances were used to obtain corresponding antibody concentrations (units per milliliter) from the standard (antibody concentration [units per milliliter] versus optical density at 405 nm) curves generated from the indirect noncompetitive assays. Linear regressions of antibody concentration (units per milliliter) to $\log_{10}$ inhibitor concentration (micrograms per milliliter) were generated for both sets of inhibition assays. These curves were used to obtain the inhibitor concentration required to reduce the concentration of anti-denSsp or anti-natSsp by 50% and to establish a ranking of inhibitors based on this property. Discriminant analysis, developed by using the SPSS-X statistics program (21, 22), was performed on the ranked inhibitors to determine whether isolates could be classified into groups based on their ability to compete for specific antibody.

RESULTS

One- and two-dimensional analysis. Examples of one-dimensional SDS-PAGE of total protein extracted from mycelia, stromatal initials, and sclerotia or substratal stromata are shown in Fig. 2. Stromatal protein production was first detected in maturing stromatal initials. In all species examined, except *Ciborinia erythronii*, one to three protein bands present in sclerotial or substratal stromatal extracts were not observed in mycelial or stromatal initial extracts. In *C. erythronii*, protein bands with molecular weights corresponding to the intensely staining 35.5-kDa sclerotial protein (Fig. 2B, lanes 3 and 6), were observed in both mycelial and stromatal initial extracts (Fig. 2B, lanes 1, 2, 4 and 5) but in lesser amounts. In general, there was no intraspecific variation in the molecular weights of the major stromatal proteins, but in *C. erythronii*, one isolate (LMK52) consistently produced an additional, low-molecular-weight sclerotial protein band (molecular weight, 11,000) (Fig. 2B, lane 3) which the other isolate (LMK53) did not.

All of the isolates from members of the *Sclerotiniaceae* produced sclerotial or substratal stromatal proteins within a narrow molecular weight range, 31,000 to 39,500 (Table 2). Substratal stromatal extracts of *Sclerotinia homoeocarpa* and *L. subrenispora* were each found to contain one protein in addition to the proteins previously reported (23) because of the enhanced resolution achieved by separating proteins on linear gradient gels. Some isolates also produced stromatal proteins of lower molecular weight, 11,000 to 20,000, but in all cases the major proportion of sclerotial or substratal stromatal protein was of higher molecular weight (Table 2). In the basidiomycetous isolate *Sclerotium rolfsii*, a major protein with a molecular weight of 44,000 as well as some minor sclerotial proteins (Table 2) were observed in addition to protein bands previously reported (11). Two groups of sclerotial proteins were observed in the five *Aspergillus* isolates examined: a narrow range of low-molecular-weight (12,000 to 22,000) proteins which generally composed 24 to 48% of the total protein and a wide range of higher-molecular-weight (29,000 to 79,000) proteins which generally composed only 1 to 8% of the total protein in sclerotia (24). A sclerotial or substratal stromatal protein was designated major if it constituted more than 13% of the total protein in stromatal extracts as measured by densitometric scans of one-dimensional SDS-gels. Stromatal proteins did not stain with periodic acid-Schiff reagent, suggesting that they are not glycosylated (data not shown), although not all fungal glucans are periodic acid-Schiff positive.

A representative two-dimensional separation is shown in Fig. 3. Two-dimensional stromatal protein banding patterns were unique for the four isolates examined (Table 2). The isoelectric points of the sclerotial proteins of *Sclerotinia sclerotiorum* and *Sclerotium cepivorum* were slightly more

TABLE 2. Molecular weights, relative proportion, and isoelectric points of stromatal proteins of species examined

Species (no. of isolates examined)	Mol wt of protein[a] (10^3)	Relative % of total protein	pI of protein[b]
Sclerotinia sclerotiorum (11)	36	42.0–45.0	5.8, 6.0, 6.2
Sclerotinia minor (2)	35	48.0–48.5	
Sclerotinia trifoliorum (2)	36	33.8–34.0	
Sclerotinia homoeocarpa (6)	31.5	13.5–14.5	6.6–6.7
	32.5	16.0–18.0	6.6–6.8
	34.5	32.0–34.0	6.8–6.9
Sclerotium cepivorum (7)	35.5	13.0–13.5	5.8, 6.1
	37.5	26.0–30.0	6.0, 6.2
Sclerotium rolfsii (3)	44	24.6–25.0	
	38, 30, 27	6.0–9.0	
	17	49.0	
Myriosclerotinia dennisii (1)	35.5	57.0–57.9	
	19	9.0–9.4	
Botrytis cinerea (1)	39.5	25.0–25.2	
	18.5	9.5–9.8	
Botrytis porri (1)	38.5	13.0–13.5	
	37.5	13.0–13.4	
	18	5.0–5.3	
Monilinia fructicola (1)	36	16.0	
Dumontinia tuberosa (2)	36	32.5–32.8	
	20	10.0–10.5	
Ciboria acerina (1)	36	34.5	
Ciborinia erythroni (2)	35.5	27.0	
	27	5.0–5.3	
Rutstroemia bulgarioides (1)	39.5	12.9	
Lambertella subrenispora (1)	31	35.0–38.0	6.6–6.7
	33.5	12.0–14.0	6.6–6.8
	35	8.5–9.0	6.8–6.9
Lambertella langei (1)	33	19.0	
Poculum henningsianum (2)	32.5	13.3	
	31.5	10.0–10.2	
Poculum petiolarum (1)	39	15.0–15.2	
	31	9.0–9.3	
Poculum sydowianum (1)	37.5	7.5–7.6	
	34.5	5.0–5.1	

[a] Average based on data from all isolates examined.
[b] Based on data from single isolates examined; see text for details.

acidic (pI 5.8 to 6.2) than those of substratal stromatal proteins from *Sclerotinia homoeocarpa* and *L. subrenispora* (pI 6.6 to 7.1). Also, the sclerotial proteins consisted of two or three polypeptides while the substratal stromatal proteins consisted of single polypeptides.

Purification of Ssp and specific antibodies. The major polypeptide (pI 6.0) of the 36-kDa sclerotial protein of *Sclerotinia sclerotiorum* (Ssp) was purified and recovered from isoelectric-focusing tube gels (denSsp) and one-dimensional polyacrylamide gels (natSsp) at concentrations of 1.0 to 2.0 and 0.5 to 0.7 μg of protein per μl, respectively. SDS-PAGE and densitometric scans of gels of denSsp and natSsp indicated that both preparations consisted of single polypeptides with molecular weights of 36,000 of at least 85% purity.

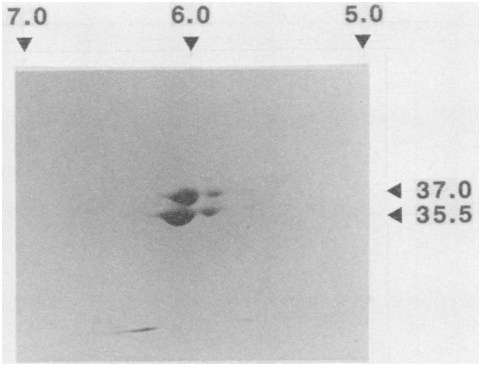

FIG. 3. Two-dimensional separation of a sclerotial extract of *Sclerotium cepivorum* (LMK1). Detail of gel showing 37-kDa protein resolved into two polypeptides (pI 6.2 and 6.0) and 35.5-kDa protein resolved into two polypeptides (pI 6.1 and 5.8).

After 5 weeks of injections with denSsp or natSsp, in each case two of the three hens produced antibodies in a high enough titer for immunoblotting and enzyme immunoassays. SDS-PAGE of anti-denSsp and anti-natSsp antibodies indicated protein bands with molecular weights corresponding to hen immunoglobulin Y (fowl immunoglobulin G equivalent) proteins (28). Reprecipitation with 14% (wt/vol) sodium sulfate eliminated any contaminating egg-yolk proteins from antibody samples.

Western blot. Sclerotial proteins of eight isolates of *Sclerotinia sclerotiorum* electro-transferred to nitrocellulose membranes and probed with anti-denSsp antibodies are shown in Fig. 4A. A cross-reaction was observed not only with the isolate to which the antibodies were raised (lane 1) but also with the other isolates of *Sclerotinia sclerotiorum* (lanes 2 to 6). The same cross-reaction was also observed when these isolates were probed with anti-natSsp antibodies. The cross-reactivity between the low-molecular-weight protein (molecular weight, 17,000) in sclerotial extracts of isolates of *Sclerotinia sclerotiorum* and anti-denSsp (Fig. 4A) or anti-natSsp antibodies was attributed to the presence of preimmune antibodies sensitized to the low-molecular-weight protein. These preimmune antibodies did not interfere with the immunoassays.

Anti-denSsp and anti-natSsp antibodies cross-reacted strongly with the 37-kDa and weakly with the 35.5-kDa proteins of sclerotial extracts of isolates of *Sclerotium cepivorum* (data not shown). Preimmune antibodies did not cross-react with the sclerotial proteins of any of the isolates of *Sclerotium cepivorum*.

The results of immunoblotting of all of the sclerotial and substratal stromatal proteins with anti-denSsp and anti-natSsp antibodies are summarized in Table 3. Sclerotial proteins of all of the isolates of sclerotial species in the *Sclerotiniaceae* cross-reacted when treated with anti-denSsp (Fig. 4B) or anti-natSsp antibodies, but substratal stromatal proteins did not cross-react when treated with either anti-denSsp or anti-natSsp antibodies. The five isolates of the

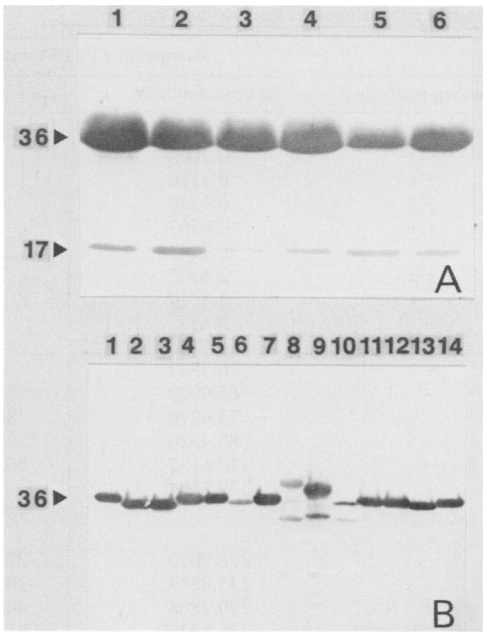

FIG. 4. Western blots of sclerotial extracts probed with anti-denSsp antibodies. (A) Cross-reactions occurred with major sclerotial proteins (molecular weight, 36,000) of all isolates of *Sclerotinia sclerotiorum*: lane 1, LMK2; lane 2, LMK34; lane 3, LMK79; lane 4, LMK81; lane 5, LMK94; lane 6, LMK121. Note weak cross-reactions with a minor protein (molecular weight, 17,000) due to preimmune antibodies. (B) Cross-reactions occurred with major sclerotial proteins of *Sclerotinia sclerotiorum* LMK2 (lane 1), *Sclerotinia minor* LMK3 (lane 2) and LMK35 (lane 3), *Sclerotinia trifoliorum* LMK4 (lane 4) and LMK103 (lane 5), *Monilinia fructicola* LMK12 (lane 6), *Myriosclerotinia dennisii* LMK17 (lane 7), *Botrytis cinerea* LMK18 (lane 8), *B. porri* LMK19 (lane 9), *Ciboria acerina* LMK33 (lane 10), *Dumontinia tuberosa* LMK50 (lane 11) and LMK51 (lane 12), and *Ciborinia erythronii* LMK52 (lane 13) and LMK53 (lane 14).

genus *Aspergillus* and *Sclerotium rolfsii* did not cross-react when treated with either specific antibody.

In control experiments, when mycelial and stromatal initial extracts of sclerotial isolates of species in the *Sclerotiniaceae* were treated with either anti-denSsp or anti-natSsp antibodies, no cross-reactions were observed with mycelial proteins. However, weak cross-reactions were observed with those stromatal initial proteins with molecular weights corresponding to the major sclerotial proteins. No cross-reactivity was observed in the other control experiments.

ELISAs. An example of the standard curves obtained from indirect noncompetitive assays of anti-Ssp antibodies with Ssp is shown in Fig. 5. Working dilutions of both Ssp (1 to 10 μg/ml) and anti-Ssp antibodies (1/250 to 1/500 dilution) were obtained from these curves. The assay limit of detection of Ssp in crude sclerotial extracts of *Sclerotinia sclerotiorum* was 0.25 to 0.30 μg/ml.

Table 3 lists the sclerotial and substratal stromatal protein concentrations (i.e., inhibitor concentrations) of representative isolates derived from indirect competitive inhibition ELISAs. Discriminant analysis of these protein concentrations demonstrated that the representative sclerotial or substratal stromatal isolates were clustered into three groups on the basis of their relative abilities to compete for specific antibodies. The three groups observed were sclerotial isolates representing species in the *Sclerotiniaceae*, substratal stromatal isolates representing species in the *Sclerotiniaceae*, and sclerotial species representing outgroups (Fig. 6A). Although the group of sclerotial species in the *Sclerotiniaceae* (Fig. 6A), which includes species that are generally equivalent in their abilities to compete for specific antibody, is tightly clustered, the relationship of these species can be further resolved by expanding the *x* axis (Fig. 6B).

DISCUSSION

Results of the biochemical and immunological procedures used in this study demonstrate both that stromatal proteins in the *Sclerotiniaceae* are distinct from those of other groups and that differences among the proteins occur within the family. The differences between developmental stromatal proteins produced in sclerotial isolates and those produced in substratal stromatal isolates observed with two-dimensional electrophoresis, Western blot, and ELISA techniques confirm observations based on comparative anatomical and histochemical studies (14, 15) of stromata and suggest that the *Sclerotiniaceae* may include at least two distinct lineages of genera.

Data from one-dimensional electrophoretic analysis in this study, in agreement with data from a previous study (23), showed that the major, stromatal proteins from representatives of the *Sclerotiniaceae* grouped within a relatively narrow molecular weight range (30,000 to 40,000) in comparison with representatives from other groups. With the exception of one species, *C. erythronii*, no intraspecific variation was observed. This was notable in the 11 isolates of *Sclerotinia sclerotiorum*, considering differences in host and geographical distribution among the isolates. Data from two-dimensional electrophoretic analysis on a subset of these isolates revealed differences between sclerotial and substratal stromatal isolates in the *Sclerotiniaceae*. The sclerotial proteins, composed of multiple polypeptides, were more acidic than the substratal stromatal proteins which were composed of single polypeptides. Results from Western blots and ELISA correlated with the differences observed after two-dimensional electrophoretic separation. Sclerotial proteins from species in the *Sclerotiniaceae* were found to be antigenically different from substratal stromatal proteins of species in the family and sclerotial proteins of *Aspergillus* spp. and *Sclerotium rolfsii*. Faint, Coomassie-stained stromatal initial proteins, with molecular weights corresponding to major stromatal proteins, were observed by SDS-PAGE. In addition, weak cross-reactions between these stromatal initial proteins probed with anti-Ssp antibodies suggest that stromatal protein production may begin late in the stromatal initial stage. The relative ranking of sclerotial and substratal stromatal protein affinity for specific antibody, developed from ELISA results, indicated that the *Sclerotiniaceae* comprise at least two groups of genera. The antigenic unrelatedness of *Aspergillus* spp. and *Sclerotium rolfsii* to sclerotial members in the *Sclerotiniaceae* was also demonstrated.

Our studies confirm the affinities of *Sclerotium cepivorum* with members of the *Sclerotiniaceae* suggested by earlier anatomical, histochemical, and electrophoretic studies (14, 15, 23). Although this form-species does not produce a sexual fruiting body, it was accommodated in the family in the genus *Stromatinia* (37), which is characterized by large sclerotia that bear fruit bodies and small sclerotia that reproduce asexually. Some strains of *Sclerotium cepivorum* also produced two sizes of sclerotia (3, 10), but sexual fruiting bodies have not been produced from either type.

TABLE 3. Results of immunological analyses by using antibodies raised against denSsp and natSsp

Species	Western blotting data[a]			Competitive ELISA data[b]	
	No. of isolates	denSsp antibody	natSsp antibody	denSsp antibody	natSsp antibody
Sclerotinia sclerotiorum	11	++	++	0.0735	0.3728
Sclerotinia trifoliorum	2	++	++	0.1006	1.2281
Sclerotinia minor	2	++	++	0.1186	1.3198
Myriosclerotinia dennisii	1	++	++	0.1356	1.3932
Sclerotium cepivorum	7	++	++	0.6363	1.8732
Monilinia fructicola	1	+	+	2.2142	2.6534
Ciborinia erythronii	2	++	++	2.6302	2.9151
Dumontinia tuberosa	2	++	++	2.8210	3.1772
Botrytis porri	1	+	+	3.6357	4.2511
Botrytis cinerea	1	+	+	4.0584	4.4917
Ciboria acerina	1	+	+	6.3369	6.4031
Rutstroemia bulgarioides	1	−	−	65.9949	61.9957
Sclerotinia homoeocarpa	6	−	−	73.4276	88.0568
Lambertella subrenispora	1	−	−	88.4520	91.7401
Lambertella langei	1	−	−	110.6132	161.3871
Poculum sydowianum	1	−	−	129.8384	
Poculum henningsianum	2	−	−		184.2668
Poculum petiolarum	1	−	−		
Aspergillus nomius	1	−	−	228.9070	274.6236
Aspergillus flavus	1	−	−	235.0819	301.4232
Aspergillus parasiticus	1	−	−	290.2868[c]	403.1222[c]
Aspergillus alliaceus	1	−	−	326.8535[c]	313.7902[c]
Aspergillus auricomus	1	−	−	336.5483	528.8785
Sclerotium rolfsii	3	−	−	412.3570	1124.4831

[a] ++, Very strong cross-reaction; +, cross-reaction; −, no cross-reaction.
[b] One isolate of each species was used for competitive ELISA analysis; see text for details. Data show concentration of inhibitor required to reduce concentration of specific antibody by 50%.
[c] The disagreement between the antibodies as to the ranking of *Aspergillus parasiticus* and *Aspergillus alliaceus* was not resolved.

While *Sclerotium cepivorum* shows affinities in the *Sclerotiniaceae*, it cannot be classified there without evidence of sexual reproduction. It is also evident that the turf pathogen *Sclerotinia homoeocarpa* shows affinities with the otherwise mainly saprophytic substratal stromatal taxa and not with the sclerotial genera, notably *Sclerotinia*, suggesting that it should be reclassified.

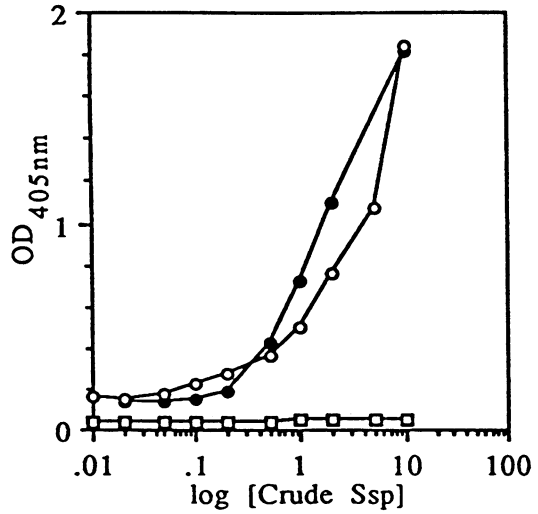

FIG. 5. ELISA titration curve of anti-denSsp (●) and anti-natSsp (○) antibodies to crude sclerotial protein of *Sclerotinia sclerotiorum* (Ssp) including preimmune antibody control (□). Averages of triplicate samples are shown.

Variation among developmental stromatal proteins is a potentially powerful tool in systematic studies, in identification of fungal isolates, and in diagnosis of plant disease. Workers comparing these proteins in taxonomic or phylogenetic studies should be aware that stromatal proteins show variation at different taxonomic levels within different taxa, e.g., at the genus-aggregate level in the *Sclerotiniaceae* and at the species-aggregate level in the genus *Aspergillus* (24). These proteins are especially valuable in identifying affinities of taxa which lack parts of the life cycle important to the identification and classification of the fungus or where the sexual reproductive part of the life cycle is unknown, as is the case with both *Sclerotium cepivorum* and *Sclerotinia homoeocarpa*. Stromatal proteins merit further attention as examples of molecular evolution. Analysis of nucleotide sequences coding the gene(s) of these proteins could proceed along several interesting avenues of research. First, functional characteristics of the protein could be identified as well as constraints on its evolution. Second, at the organismal level, phylogenetic relationships among stromatal fungi may be clarified. Lastly, the abundance of these developmentally regulated proteins provides an ideal system for the study of developmental physiology and regulation in stromatal fungi.

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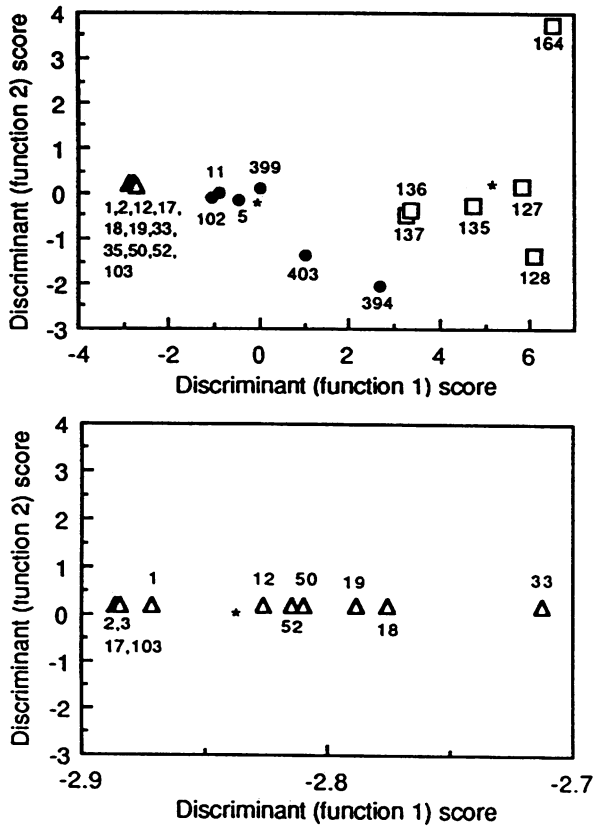

FIG. 6. Scatterplots of discriminant function scores defining membership to groups based on ability of each isolate to compete for specific antibody. (Upper plot) Three groups defined by discriminant analysis of competitive ELISA data are sclerotial isolates in the *Sclerotiniaceae* (group △), substratal stromatal isolates in the *Sclerotiniaceae* (group ●), and nonsclerotiniaceous sclerotial species (group □). (Lower plot) Note that even with modified scale, some members of the originally tightly clustered group remain clustered together (isolates 2, 3, 17, and 103).

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