Electrophoretic Analysis of Double-Stranded RNA in Stocks of Cultivated Mushroom (*Agaricus brunnescens*)

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ABSTRACT

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Because an earlier detailed comparison by electron microscopy indicated that the detection of double-stranded RNA (dsRNA) by polyacrylamide gel electrophoresis (PAGE) was a very reliable diagnostic technique for virus infection in mushrooms, we initiated a study to determine if the PAGE method could be used to detect dsRNA in stock strains used to generate commercial spawns (seed). Sporophores were derived directly from test spawns by covering the spawn with a layer of casing material. Sporophores were grown in environmentally controlled isolation chambers. Based on an extensive evaluation analysis of spawns from eight separate national and international stock programs, 41% of the strains tested contained dsRNA. None of the isolates showed aberrant growth, but several strains had abnormal growth rates. Although fruiting initiation by several of the strains was difficult, there was no correlation between fruiting ability and dsRNA content.

Additional key words: fungal virus, mushroom disease

La France disease is one of the most important diseases of *Agaricus brunnescens* known today. It spreads rapidly during commercial mushroom culture, and if unchecked, the disease can reach epidemic proportions with substantial economic losses.

Since the first report of virus(es) in cultivated mushrooms (19), there has been disagreement regarding the presence or extent of contamination in the spawn stocks. Initial reports indicated that virus particles were not present in spawn (7,8); however, Hollings (6) found viruslike particles (VLPs) in two of 19 strains tested. Passmore and Frost (14) determined that VLPs were present in all eight of their strains. The discrepancy in results may be attributed to incomplete knowledge regarding virus infection or the type of tissue tested and/or the methods of analysis.

Different types of mushroom tissue may be used for virus detection, i.e., sporophores produced from compost under commercial or pilot plant mushroom house conditions (18,20), mycelia grown aseptically on nutrient agar (9,20), and sporophores produced on grain under controlled (pest-free) conditions (15).

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A variety of techniques exist for detecting VLPs, i.e., electron microscopy (5,20), serology (13), and immunoelectrophoresis (4). A sensitive and specific assay method was described by Marino et al (11) with polyacrylamide gel electrophoresis (PAGE). Six molecular weight forms of double-stranded RNA (dsRNA) were associated with VLPs in *A. brunnescens*. The dsRNA detected is thought to be the viral genomic nucleic acid. Generally dsRNA may be present in the VLPs of spherical morphology (10–12). None of the bands has been directly correlated with specific morphology or size of the VLPs, however. Marino et al (11) detected three additional dsRNA species associated with virus-infected mushroom by PAGE and ethidium bromide staining.

Few data are available on the occurrence of virus in the numerous commercial stock cultures used in the mushroom industry. A program was initiated at Beltsville to systematically examine cultures used by the mushroom industry and in six research programs. The Beltsville collection, preserved by storage in the vapor phase of liquid nitrogen (17), provided a reliable source of cultures. This report presents the results of screening 43 strains of *A. brunnescens* by electrophoretic analyses of dsRNA. Preliminary results from this study have been published (2).

MATERIALS AND METHODS

Spawn culture. *A. brunnescens* stocks were maintained in the vapor phase of liquid nitrogen (−160 to −196 C) (16,17). Sporophores were produced on rye grain by a modified standard procedure (15). Rye grain medium (175 g of rye grain, 3.5 g of $CaCO_3$, and 220 ml of distilled water) was placed in a 1-L Erlenmeyer flask fitted with a cotton plug and sterilized in a steam sterilizer by "precooking" at 100 C for 90 min, then immediately heating for 90 min at 121 C and 1 kg/cm pressure. The cooled substrate was inoculated with a 1-cm^2 agar block of mycelium from a culture of *A. brunnescens* grown on potato-dextrose agar. The spawn grain culture was incubated at 24 C and shaken at 3-day intervals to distribute mushroom mycelium throughout the grain medium. When mushroom mycelium completely colonized the grain (12–14 days), the grain was mixed thoroughly by shaking and poured into a pasteurized plastic tray 14 cm long × 23 cm wide × 6 cm high. The leveled grain was layered with 2 cm of pasteurized casing soil that was wetted with distilled water to field capacity. Cased grain spawn was incubated for 40–60 days under conditions conducive to formation of mushroom primordia and rapid growth of mushrooms (15). Fresh laboratory air entering the incubator (Forma Scientific, Marietta, OH; model 3188 with special blower) was filtered through several layers of fiberglass. Each mushroom was picked and weighed when the veil began to stretch but before gills were exposed. Mushrooms were observed for symptoms (18) of virus infection. Student's *t* test was used to test the comparison between infection and production efficiency; probability values of 0.05 or less were considered significant.

Nucleic acid extraction. Nucleic acids were extracted as described previously (21) with several modifications. Frozen sporophore tissue (2–20 g) was ground to a paste in liquid nitrogen and stirred for 30 min in an extraction buffer (30 ml) consisting of 2 ml of 5% sodium dodecyl sulfate (SDS), purified bentonite (27 mg/ml), 2 ml of 0.1 M Na_2EDTA_2 (pH 7.0), 4 ml of 0.1 M Tris-HCl (pH 8.9), 200 µl of 2-mercaptoethanol, and 20 ml of water-saturated phenol per 10 g of tissue. The mixture was centrifuged at 5,000 *g* for 15 min. The aqueous phase was decanted, and an additional one-half volume of the extraction buffer was stirred into the phenol phase. After a second centrifugation, the two aqueous

phases were combined and two volumes of absolute ethanol were added and the mixture stored at 4 C for 2 hr to precipitate the nucleic acids. The precipitate was collected by centrifugation and suspended in a small volume of 0.1X SSC (1X SSC = 0.15 M NaCl in 0.015 M sodium citrate). Ribosomal RNA (rRNA) was precipitated by adding an equal volume of 4 M LiCl in 2X SSC. Most of the rRNA precipitated under these conditions and was removed by centrifugation (7,000 *g*). Nucleic acids remaining in the supernatant solution were ETOH-precipitated, collected by centrifugation (5,000 *g*) for 10 min, and resuspended in 0.5 ml of 0.01 M Tris-hydrochloride (pH 7.4) and 0.5 ml of 0.05 M $MgCl_2$ to remove deoxyribonucleic acid (DNA). This buffer had been equilibrated with 0.1% bentonite that was removed by two successive centrifugations at 7,500 *g*. The nucleic acids were incubated with 100 g of DNase for 30 min at 35 C. This solution was dialyzed for 24 hr in electrophoresis buffer (see below) after centrifugation.

Gel electrophoresis. The dsRNA samples were electrophoresed in 5% polyacrylamide−0.125% *N,N*-methylene-bis-acrylamide gels. Gels were cast in 7-mm (i.d.) glass tubes. Gels were preelectrophoresed without samples at 4 m*A*/gel to remove the ammonium persulfate. Electrophoresis of dsRNA was performed at 100V, 6 m*A* per gel in 0.04 M Tris, 0.02 M Na acetate (pH 7.5) containing 0.002 M Na_2EDTA. Samples (0.1–0.3 ml) were applied with one to three drops of saturated sucrose and electrophoresed for 4 hr at room temperature. Nucleic acid zones were visualized by staining for at least 30 min in ethidium bromide, 1 μg/ml, and photographed under shortwave UV light (Chromato-transilluminator model C-61; Ultraviolet Products Inc., San Gabriel, CA) with Polaroid Type 55 film with a Wratten G yellow filter.

To confirm the double-stranded nature of the nucleic acid in electrophoresis gels, nucleic acid preparations were tested for sensitivity to ribonuclease (RNase) and deoxyribonuclease (DNase). Samples were incubated for 30 min at 37 C with 1 μg/ml RNase (bovine pancreatic ribonuclease A, Sigma Chemical Co., St. Louis, MO) in buffer consisting of 0.01 M Tris-HCl and either 0.03 or 0.01 M NaCl, pH 7.2. Other samples were similarly treated with 20 μg/ml DNase (RNase-free deoxyribonuclease treated with 20 μg/ml DNase; RNase-free deoxyribonuclease, Worthington Biochemical Corp., Freehold, NJ) in 0.01 M Tris-HCl, 5 mM $MgCl_2$ buffer, pH 7.4. Nucleases were removed by adding 50 mg/ml bentonite and centrifuging at 12,000 *g* for 10 min. Samples were then precipitated with ethanol in an ice bath for 1.5 hr and centrifuged at low speed (5,000 *g* for 15 min). The pellets were dissolved in buffer and electrophoresed. Comparisons of the number of dsRNA bands from various isolates and samples were made by mixing and then coelectrophoresing with the dsRNA from appropriate representative profiles and markers. Fungal virus-containing strains of *Penicillium chrysogenum* Thom (ATCC 9480) and *P. stoloniferum* Thom (ATCC 14586) were used as standards in dsRNA extractions and molecular weight estimations.

Table 1. Detection of double-stranded RNA (dsRNA) by polyacrylamide gel electrophoresis (PAGE) in tissue extracts from sporophores of *Agaricus*

Strain[a]	ATCC accession number	Source[b]	Color by cap	Average production efficiency on rye grain[c]	Presence of dsRNA
7	56137	United States (5)	White	727	−
11, 52[d]	56141, 56159	United States (1)	Intermediate	658[e], 535	−
62	56165	England (5)	White	916	−
94	56318	United States (5)	Brown	351	+
111, 128	56358, 56375	Netherlands (3)	Intermediate	949, 824	−, +
130, 136	56377, 56383	England (5)	White, intermediate	762, 933	+, −
146	56392	Australia (5)	White	1,110	−
172	10892	United States (4)	White	701	+
183	56415	United States (5)	Intermediate	89	+
187	···	France (5)	Intermediate	942	−
222	···	United States (1)	Intermediate	590	−
254, 261	52977, 52978	Czechoslovakia (2)	Brown	392, 50	−, +
289	32675	Taiwan (4)	White	318	−
374	34803	Scotland (6)	Intermediate	339	−
397	34732	United States (6)	Intermediate	239	+
439	···	United States (1)	Intermediate	687	−
446	···	United States (1)	White	549	+
463, 464	52991, 52979	United States (2)	White, intermediate	254, 52	−
465, 496	56055, 56056	United States (2)	White	650, 198	+, −
499, 500	24558, 24559	United States (1)	White	814, 653	+
501	···	United States (1)	White	1,035	−
502, 503	60210, 60211	United States (5)	Brown, white	468, 717	+
504, 536	60212, 56056	United States (5)	Intermediate	331, 717	+, −
537	56057	United States (5)	Brown	944	−
538	56058	PI (7)	White	177	+
540, 541	52980, 52981	United States (2)	Intermediate	406, 50	−
549	···	United States (5)	White	550	+
550	···	Germany (8)	White	690	+
551, 552	52980, 52981	Germany (8)	Brown	245, 364	−
555	···	United States (1)	Intermediate	430	−
556	···	United States (5)	White	387	+
566	···	United States (5)	White	550	−

[a] Number of culture in Beltsville cultivated mushroom collection stored in liquid nitrogen.
[b] Culture sources: 1 = isolated from sporophore collected from the wild by second author; 2 = Central Bureau voor Schimmelcultures-Barn, Netherlands; 3 = American Type Culture Collection, Rockville, MD; 4 = C. Raper, Wellesley College, Wellesley, MA; 5 = T. Quimo, Los Baños College, Laguna, Philippines; 6 = G. Eger, Marburg, West Germany; 7 = commercial spawnmaker; and 8 = isolated from sporophore produced by commercially available spawn.
[c] Efficiency = g mushroom (mushroom cut at surface of substratum)/kg (dry wt) of rye grain at inoculation.
[d] Grouped because of similarities.
[e] First and second numbers refer to respective strain numbers in first column.

RESULTS AND DISCUSSION

Forty-two percent or 18 of 43 strains of *A. brunnescens* examined were found to contain dsRNA (Table 1). dsRNA-positive and dsRNA-negative strains were found in three commercial mushroom cultivars of *A. brunnescens*, i.e., white, intermediate, and brown cap colors. dsRNA was present in two of eight strains (128, source 2; 172, source 3) received from established culture collections and in two of eight cultures (261 and 397) obtained from the wild in the United States and Czechoslovakia. We failed to detect dsRNA in the other 25 strains tested in this study. It is possible, however, that the amount of dsRNA was below the level of detection in these strains.

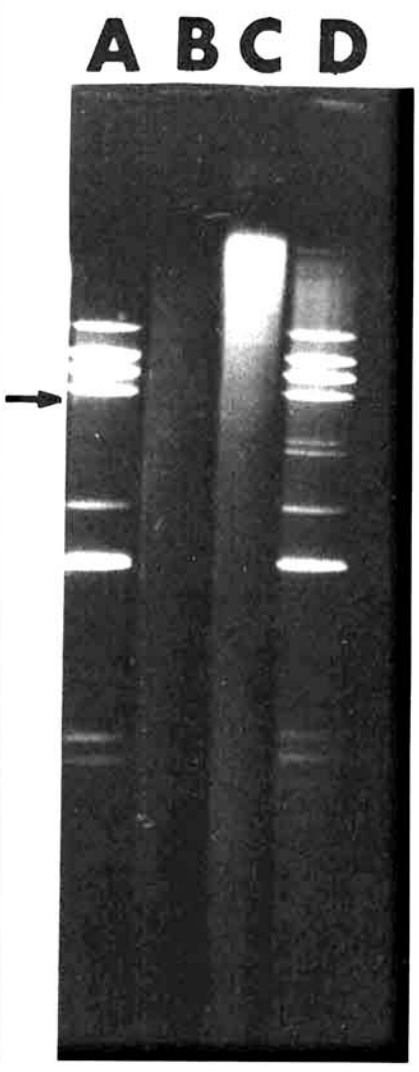

Fig. 1. Representative patterns of dsRNA segments from sporophores of spawn strains of *Agaricus brunnescens*. **(A)** 172 (Table 1) with eight segments; **(B)** 62, no detectable segments; **(C)** 397, one segment; and **(D)** 549, 10 segments. Arrow shows the position on the gel where dsRNA with known molecular weight of 1.6×10^6 daltons from *Penicillium chrysogenum* virus usually migrates on our gels under the same electrophoretic conditions. Separations were made at 6 m*A*/gel for 4 hr at room temperature on 5% polyacrylamide gels and stained with ethidium bromide.

None of the mushrooms produced from the 43 *A. brunnescens* stock cultures showed virus disease symptoms. Earlier descriptions of mushroom virus infection (18) reported a wide variation in symptoms; some infected isolates produced multiple symptoms, whereas others had only normal-appearing sporophores. We have confirmed the existence of dsRNA in normal-appearing mushrooms from field samples (2).

Growth and fruiting patterns varied among the strains; however, these differences were correlated with varietal differences and not with the presence of viral nucleic acid. Some infected strains developed sporophore initials 1 wk after casing, whereas other infected strains failed to fruit for 14 days. This same phenomenon was noted among those strains lacking dsRNA. Usually a characteristic symptom, but again a variable one, is the weak growth of virus-infected mycelium on a culture medium (20). Several workers (7,9,20) have shown that healthy isolates show a vigorous and fluffy mycelial growth pattern, but growth of infected mycelium is sparse, slow, and appressed. We found in this study, however, that some commonly grown strains may harbor the mushroom virus(es) but escape detection by spawnmakers who may rely on cultural symptoms to index for the mushroom virus because some of these strains contained dsRNA segments.

The presence of dsRNA was not associated with a low production efficiency on grain. Although few published reports give tangible figures on the extent of mushroom losses attributable to the mushroom virus, the damage is due mainly to yield reduction (6,9,10,18). In this study, the strains without dsRNA tended to have higher production efficiency ratings, but the differences were not statistically significant.

We have analyzed (3) "virus-infected" sporophores received from P. A. Lemke and from various field-collected mushrooms by this method and have detected the six prominent electrophoretic bands and the three fastest migrating faint bands described previously (10,11).

In contrast to the results obtained with sporophores from commercial beds (3,7,10), the number of dsRNA bands associated with stock cultures varied between one and 10 (Fig. 1). Approximate molecular weight estimates of dsRNA segments ranged from about 0.25 to 2.57 $\times 10^6$ daltons. We do not know if any of these dsRNA segments are associated with the presence of any specific VLP type.

Based on these results, we conclude that electrophoretic analysis of dsRNA extractions from mushroom cultures at the spawnmaking stage may be useful in screening for latent viruses. We have shown that spawn infection occurs and that the grain fruiting approach has potential diagnostic value. The failure of other workers to detect virus in *Agaricus* spawn may be due in part to the fact that negatively stained preparations from aseptically grown mycelial tissue are contaminated with host cellular debris or contain very few virus particles (20). Detection by electron microscopy (EM) may be unreliable under these conditions. Also, mycelium grown in shake or static liquid cultures is extremely slow and is not a good virus source for screening a large number of strains (1,20). Commercial and experimental spawn preparations cannot be examined directly because of the small amount of mycelial tissue (0.1 g) present on the cereal grains used in the product; however, small amounts of sporophore tissue (2–20 g) were sufficient to verify the presence of dsRNA in stock strains.

The presence of virus in certain stock cultures is not perceived as a problem for mushroom growing because virus-infected strains generally grow slower and are eliminated by the spawnmaker. EM studies (20), however, have shown that some virus-infected mycelial cultures have moderate to normal growth rates. Our results agree with these EM studies and suggest that these infected strains may have escaped elimination in routine visual screenings. Last et al (9) proposed that "There is apparently some mechanism that maintains the *status quo* of host and virus in mycelial culture on agar, but not in the growing crops."

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