Viruslike Particles Transmitted by and Detected in Spawn of the Cultivated Mushroom, Agaricus bisporus

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ABSTRACT

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Viruslike particles (VLPs) were detected in healthy appearing spawn of *Agaricus bisporus* by serology and electron microscopy. These VLPs also were detected in mycelial cultures and sporophores produced from the indexed spawn. Serologic indexing of spawn was a reliable method for detecting mushroom VLPs.

In the 1950s mushroom growers in several countries suffered heavy losses from a serious disease that caused malformed mushrooms (12,20). Various names were proposed including La France disease, Brown disease, Watery Stipe, X disease, and die-back (13).

Hollings et al (11) were the first (1962) to show that this disease was associated with viruses. Mushroom viruses are spread by spores (5,9,18,19) or by hyphal anastomosis (3,4,7,8,10,14). Virus transmission from a cell-free suspension into a sporophore by nibbing on the sporophore surface or by injection generally has been inconsistent (9,10). Attempts to transmit these viruses to several higher plants have failed (9,10). Transmission of the viruses of *Agaricus bisporus* to other fungi has not been demonstrated. Similarly, viruses affecting other fungal species do not infect *A. bisporus* (2,14).

A question of major importance is whether viruses can be transmitted by and detected in healthy appearing spawn. Hollings and Stone (12) stated: "Spawn is unlikely to be a major source of infection, for spawn made from virus-infected cultures fails to colonize the compost." But they recognized that virus infection at low concentrations might not produce symptoms. Dieleman-van Zaayen (6) scraped mycelium off spawn grains but could not detect virus in the small amount of material available. She also ground grains and mycelium together but the amount of debris made visualization of virus particles difficult by electron microscopy. She suggested that: "...to answer the question whether spawn can be virus contaminated or not, detection methods for virus in spawn need improvement." In 1975 Moyer and Smith (15,16) purified and prepared antisera against the three-virus particles associated with La France disease as it occurs in Pennsylvania: two polyhedral particles measuring 25 and 34 nm in diameter and a bacilliform-shaped particle 19 × 50 nm. These particles are referred to as viruslike particles (VLPs) as transmission was obtained only by hyphal anastomosis between diseased and healthy mushroom isolates. Moyer and Smith (16) also determined that the VLPs were more reliably and consistently detected by use of antiserum than electron microscopy.

For this reason, the next step and the objective of this study was to determine whether VLPs could be detected serologically in healthy appearing spawn and in mycelial cultures or sporophores produced from this spawn.

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MATERIALS AND METHODS

Five strains of healthy appearing *A. bisporus* (Lang.) Imbach designated as P_1, P_2, P_3, P_4, and P_5 were tested. Antisera against the three VLPs (25 nm, 34 nm, and 19 × 50 nm) were the same as that used in previous studies (15,16). Absorbed antisera were obtained by incubating antiserum with an equal volume of healthy sporophore extract.

Serologic indexing of spawn. Spawn medium (50 g of rye grain, 1 g of $CaCO_3$ and 75 ml of water) was mixed in 250-ml Erlenmeyer flasks, sterilzied by autoclaving at 1 atm for 30 min, and cooled to room temperature. Spawn was produced by inoculating spawn media with a few spawned grains of the desired isolate and allowing 3–4 wk for colonization at room temperature (22–23 C). Twelve flasks were used for each strain.

A previously described purification procedure (19,20) was modified to obtain a virus preparation for serologic indexing. Fifty grams of spawn was homogenized in 3 volumes (150 ml) of 0.05 M potasium phosphate buffer, pH 7.2, containing 0.05% 2-mercaptoethanol for 2 min in a Waring Blendor. This buffer was used throughout the extraction; the extract was maintained at 4 C or in an ice bath. The homogenate was centrifuged (9,400 *g*, 20 min; SS-34 Sorvall rotor was used for all centrifugations unless otherwise designated), and the resulting supernatant solution was collected. A second homogenization and centrifugation (9,400 *g*, 20 min) of the pellet followed, and the combined supernatant solutions were adjusted to 10% polyethylene glycol (PEG) 6,000 MW and 0.6 M NaCl. This preparation was stirred slowly for 2.5 hr and the precipitate was collected by centrifugation (20,000 *g*, 20 min), resuspended in buffer (1 ml/5 g of original host material), and stored overnight at 4 C. The resuspended pellet was stirred slowly for 15 min; the supernatant was collected by a low speed centrifugation (10,000 *g*, 15 min) and adjusted to 10% PEG and 0.6 M NaCl. After 2.5 hr of slow stirring, the precipitate consisting of host material and the three kinds of VLPs was collected by centrifugation (20,000 *g*, 20 min), resuspended in buffer (1 ml/50 g of original host material), and stored overnight at 4 C. The precipitate was stirred for 15 min and then centrifuged at 10,000 *g* for 10 min. This preparation containing partially purified VLPs was stored at 4 C and used for serologic indexing.

Serologic tests were performed by a constant-feed micro double diffusion technique (1), using 0.75% agarose and 0.14 M NaCl in 0.02 M potassium phosphate buffer, pH 7.2, containing 0.02% sodium azide.

Serologic indexing of mycelium. Potato dextrose yeast (PDY) broth (10 g of dextrose, 1.5 g of yeast extract in 1:1 distilled water containing the extract of 250 g of potatoes) was poured into 250-ml Erlenmeyer flasks in 100-ml aliquots, sterilized by autoclaving at 1 atm pressure for 30 min, and cooled to room temperature. Mycelium was obtained by inoculating PDY broth with the desired strain and allowing 4–5 wk for colonization at room temperature 22–23 C. Inoculum consisted of a few grains of spawn from the same samples tested for spawn indexing. Twelve flasks were used for each strain.

The colonized media were filtered through four layers of cheesecloth and the drained mycelium was extracted for use in serologic indexing.

The purification procedure described for serologic indexing of spawn was used, with the following modifications: the mycelium was homogenized in an equal volume of 0.05 M potassium phosphate buffer, pH 7.2 (w/v 1:1), containing 0.1% 2-mercaptoethanol. A centrifugation (12,000 *g*, 15 min) was used to obtain the final, partially purified preparation.

Extracts from mycelial cultures were tested in a micro double diffusion test as described for spawn indexing.

Serologic indexing of sporophores. Sporophores, derived from the spawn used for spawn and mycelium indexing, were grown in the Mushroom Research Center of The Pennsylvania State University. Young sporophores, of the early flushes of the cropping cycle, were collected, frozen in liquid nitrogen, and stored at −20 C.

Extracts were prepared as described for serologic indexing of spawn except for the use of 0.05 M potassium phosphate buffer, pH 7.2, containing 0.1% 2-mercaptoethanol instead of 0.05% to avoid oxidation as indicated by pellet discoloration. Serologic indexing was as described previously.

In the absence of spawn strains not containing VLPs, extracts of sporophores of a strain that did not contain detectable VLPs and did not show symptoms and of a strain containing VLPs and

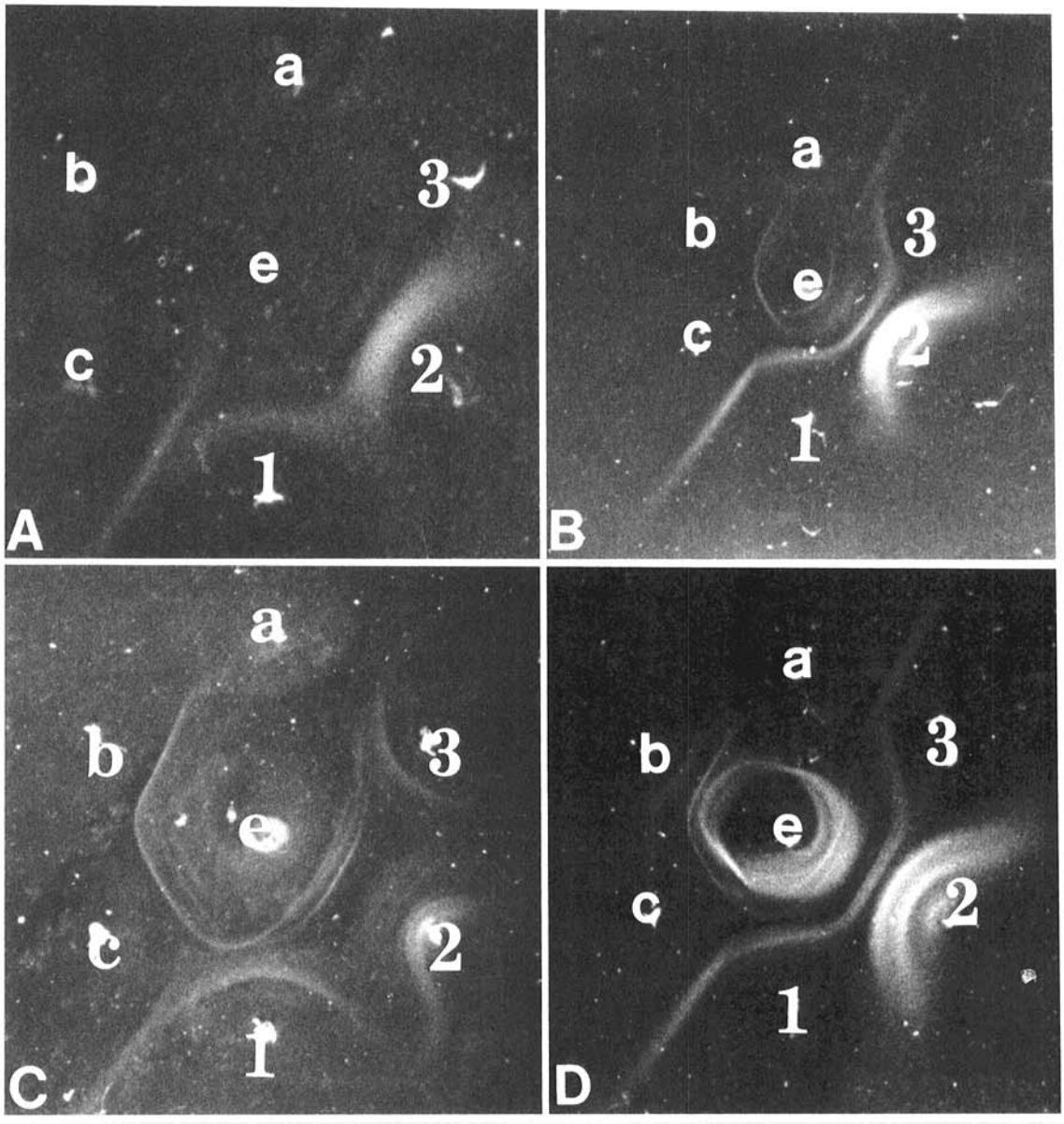

Fig. 1-(A to D). Reactions of unabsorbed and absorbed antisera to viruslike particles (VLPs) with concentrated mushroom extracts in constant feed microdouble diffusion tests. The final extracts usually were opalescent and slightly turbid and retained their characteristics during the incubation period. Unabsorbed antisera were applied at positions 1 (undiluted antiserum to the 19 × 50 VLP), **2)** (antiserum to the 25 nm and 34 nm VLPs), and **3)** (antiserum to the 19 × 50 nm VLP diluted 1:2). Absorbed antisera were applied at positions a (antiserum to the 19 × 50 nm VLP), b (antiserum to the 34 nm VLP), and c (antiserum to the 25 nm VLP). Position e contained concentrated extracts of: **A)** sporophores of a strain not containing detectable VLPs; **B)** sporophores of strain P_1; **C)**mycelial culture of P_2; and **D)** spawn of P_3. Plexiglass templates with funnel-shaped wells, spaced 0.5 cm apart, were placed on a 0.1 cm layer of agar formed on a microscope slide. The slides were placed in a moist chamber and incubated at room temperature. Three replications of each extract resulted in reproducible patterns.

exhibiting La France disease symptoms also were prepared and used as negative and positive controls respectively. In addition to serology, electron microscopy was used to determine the presence or absence of VLPs. Each preparation was examined on grids coated with 0.3% Formvar. Negative staining with phosphotungstic acid, pH 7.0 (0.2%, w/v), and octadecanol (0.015%, w/v) as wetting agent gave satisfactory results. Staining time was approximately 45 sec. About 0.5 μl of a solution of octadecanol in hexane was delivered to the surface of a stain droplet before removing the excess stain. Eight to 10 grids per negative control were examined; no VLPs were observed.

RESULTS

The antisera were prepared with the sucrose gradient centrifugation fractions that contained the 25, 34, and 19 × 50 nm VLPs, respectively (15,16). However, the antisera to the 34 and the 19 × 50 nm VLPs also contained antibodies to the 25 nm VLP. Thus, these tests were used to indicate the presence or absence of VLPs based on the presence or absence of a reaction against the antisera (Table 1, Fig. 1).

The spontaneous precipitation obtained after constant feed micro double diffusion with the highly concentrated, unabsorbed antiserum to the 19 × 50 nm VLP (position 1) was partially alleviated by a 1:2 dilution (position 3) or eliminated by utilizing the absorbed antiserum to the 19 × 50 nm VLP (Fig. 1). The formation of more than one precipitation line in some reactions may be attributed to: i) the presence of more than one type of VLP in the mushroom extract, ii) antibodies to more than one VLP in the antisera, or iii) multiple zones of equivalence of the reactants due to high concentrations.

Serologic indexing of an extract of sporophores that did not contain VLPs as determined by electron microscopy, using absorbed antisera, resulted in no precipitation lines (Fig. 1A). The serologic indexing procedure was performed with extracts of spawn media to test the possibility of nonspecific reactions attributable to ribonucleic acids and proteins in the rye grains. These tests resulted in negative reactions.

A major problem encountered was inability to locate spawn lines not containing VLPs to serve as controls. Mature sporophores contain virus inhibitors (21) that greatly reduce the concentrations of the VLPs. The consistently reduced concentrations of VLPs in the sporophores, compared with spawn, is evidenced in Table 1. The strains reported in Table 1 were selected because sufficient VLPs remained in the sporophores to be detected by serology.

Electron microscopy confirmed the presence of the VLPs in extracts that gave positive serologic reactions. In most instances examination of more than a single grid of any one sample was necessary before the VLPs were observed. Due to difficulties in observing the VLPs, electron microscopy was considered to be less reliable than serology for indexing.

TABLE 1. Results of serologic indexing of spawn, mycelial cultures, and sporophores of five *Agaricus bisporus* strains

Strain	Degree of reaction[a]					
	Spawn		Mycelial cultures		Sporophores	
	Intensity	Time (hr)	Intensity	Time (hr)	Intensity	Time (hr)
P_1	++++	24	++	24	+	48–72
P_2	+++	24	++	48	+	48
P_3	+++	24	++	48	+	48–72
P_4	++++	24	+	48–72	+	48–72
P_5	++++	24	++	48–72	+	48–72

[a]The degree of the serologic reaction was characterized as weak (+), moderate (++), strong (+++), or very strong (++++) according to the density, width (broad or narrow, sharp or diffuse) and time of appearance of the precipitation lines between extracts and absorbed antisera. Observations of the progress of the reactions were recorded at 12-hr intervals.

DISCUSSION

Viruslike particles can be detected in and transmitted by spawn. These VLPs were detected in symptomless spawn cultures and in mycelial cultures and sporophores derived from the original spawn samples. Based on these results, we conclude that symptomless spawn can be virus-infected. This virus-infected spawn can colonize the compost of the trays or beds and give rise to sporophores that appear symptomless.

Based on these results, we also conclude that serologic indexing of spawn is a reliable method of detecting mushroom VLPs. With spawn, the precipitation lines were denser and sharper and became visible more quickly than with serologic indexing of mycelium or sporophores (Table 1).

Viruslike particles were detected by serology and electron microscopy in spawn, mycelium, and sporophores of all five strains of *A. bisporus*. Passmore and Frost (17) examined approximately 550 samples of mushrooms from 13 farms that collectively produce about 35% of the total mushroom crop in the United Kingdom. They detected VLPs in all 550 samples of cultivated mushrooms, whether they appeared to be healthy or diseased. The particles also were found in all eight commercial mushroom spawns that were examined. Hollings and Stone (13) confirmed virus infection on 78 of 117 mushroom farms in Britain. Dieleman-van Zaayen (6) reported that one of three mushroom houses of 1,100 surveyed were affected by die-back disease in The Netherlands in 1967. These reports probably would have indicated even higher percentages of infection if a serologic indexing had also been done. These findings and the results of our study suggest the following questions: First, if virus infection is common in mushrooms, what conditions are necessary to trigger the appearance of the symptoms associated with La France disease? Second, is there any way of obtaining virus-free mushrooms? In regard to the second question, we believe that single-spore cultures might be the answer, if mushroom viruses are not transmitted by all of the basidiospores produced from an infected sporophore.

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