APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1994, p. 2384–2388
0099-2240/94/$04.00+0

Vol. 60, No. 7

Inheritance of Strain Instability (Sectoring) in the Commercial Button Mushroom, *Agaricus bisporus*

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Received 28 February 1994/Accepted 26 April 1994

The button mushroom, *Agaricus bisporus*, is a commercially important cultivated filamentous fungus. During the last decade, the button mushroom industry has depended mainly on two strains (or derivatives of these two strains). Using one of these highly successful strains (strain U1) we examined the phenomenon of strain instability, specifically, the production of irreversible sectors. Three "stromatal" and three "fluffy" sectors were compared with a healthy type U1 strain and with a wild-collected isolate. Compost colonization and fruit body morphology were examined. The main objective of this study, however, was to examine the meiotic stability of the sectored phenotype. Single basidiospores were isolated and subjected to a grain bioassay in which the ability to produce sectors was measured. Our results were as follows: (i) basidiospore cultures obtained from a wild-collected isolate showed no tendency to produce sectors; (ii) approximately 5% of the basidiospore cultures obtained from healthy type U1 strains produced irreversible sectors in the grain bioassay; (iii) the five primary sectors examined produced basidiospore cultures, half of which produced normal-looking growth in the grain bioassay and half of which produced some degree of sectoring; and (iv) the one sectored isolate that represented the F2 generation gave ratios similar to the 1:1 ratio observed for the F1 cultures.

The existence and phenotypic expression of strain instability in fungal isolates that are repeatedly subcultured in the laboratory are a reccurring theme in the industrial microbiological literature (11, 15, 20, 21, 29, 32, 37, 38). Numerous studies have shown that filamentous fungi grown in nutritionally rich laboratory media exhibit extremely high levels of morphological and physiological variation (11, 15, 20, 21, 38), and a high frequency of instability is often encountered in the absence of any external mutagenic agent. Fungal instability has been observed as morphological variations, including differences in sporulation (11), variations in the formation of aerial mycelia (36), and variations in the pigmentation of hyphae (38). In addition, workers have observed physiological variations, including changes in virulence (5, 35, 36) and changes in the production of secondary metabolites (26, 34). In a number of filamentous fungi, morphological variants are often observed as sectors that appear on radially growing fungal colonies on solid laboratory media (20).

The button mushroom, *Agaricus bisporus*, is one of the world's most economically important vegetable crops (13). Mushroom growers inoculate compost beds with spawn, which is grain colonized with a specific commercial strain. Mushroom spawn is generally rye grain or millet that has been heat sterilized and inoculated with mycelium from an axenic culture of a particular commercial strain (30, 33). Strain stability is of great importance to both spawn producers and mushroom growers. Mushroom strains are usually propagated vegetatively on nutritionally rich substrates, and abnormal growth and poor yield have been recorded occasionally (21, 22, 31). Of particular concern to the spawn industry is the phenomenon of sectoring of the mycelium colonizing the grain.

When *A. bisporus* produces sectors on grain or on compost, the sectors usually appear as fluffy patches of rapidly growing mycelium or as thick, rubbery areas of matted growth (2). These distinctive morphologies can be observed on agar plates, grain, compost, and the casing material during fruiting (2). When sectored mycelia complete the growth cycle, it has been observed that they give poor yields and produce poor-quality mushrooms (2, 21). Severe sectors may not produce any mushrooms at all (2, 21). Although the appearance of sectors has been observed for many years in the mushroom industry (21), the biological mechanisms that are associated with this growth form are not well understood. The fact that sectoring may appear during the production of spawn or during the colonization of compost on mushroom farms suggests that commercial mushroom strains may possess an inherent ability to spontaneously produce sectors.

Many sectors produced by commercial mushroom strains have the ability to go through the growth cycle and produce mushrooms. In this study we examined the meiotic stability of cultures derived from single basidiospores recovered from fruit bodies of normal and sectored isolates of a commercial strain (strain U1). We also compared the data which we obtained with data obtained from germinated basidiospores of a wild-collected isolate of *A. bisporus*. In addition, we examined the sporophore morphology of normal U1 mushrooms and mushrooms derived from sectored U1 isolates. We also observed compost and casing colonization by the sectored U1 isolates.

MATERIALS AND METHODS

Fungal isolates and culture conditions. The *A. bisporus* isolates used in this study were obtained from commercial type U1 strains generously supplied by the major North American spawn producers. A wild *A. bisporus* isolate (RWK1420) was kindly provided by R. Kerrigan, Sylvan Spawn Laboratories, Inc., Kittanning, Pa. Sr1 was a primary sector isolated from commercial type U1 spawn that produced a stroma-like morphology. Sr1-2 was a single-basidiospore isolate obtained from a deformed fruit body from an Sr1 primary culture. Sr3 was a type U1 isolate obtained from a commercial bed in The

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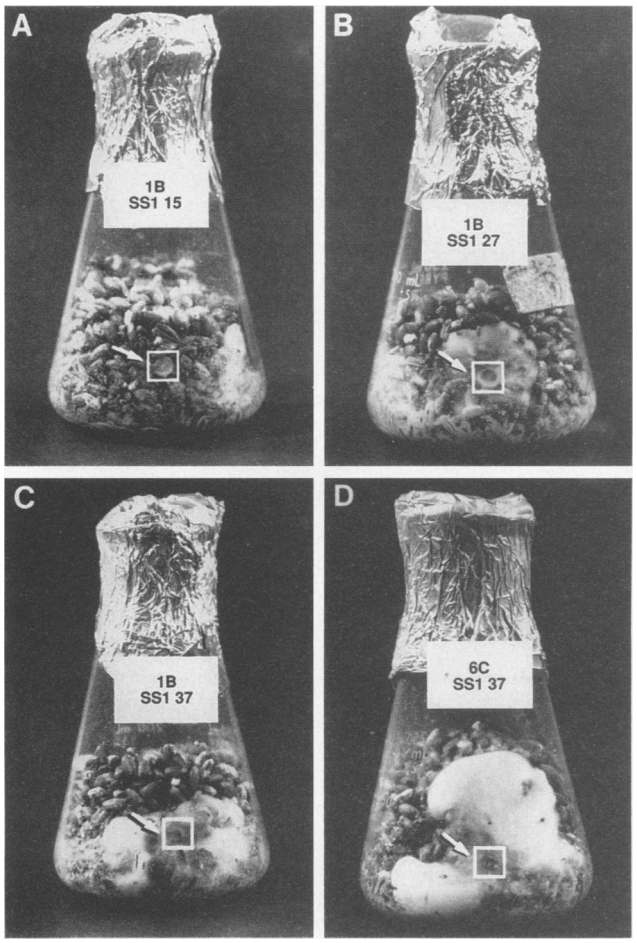

FIG. 1. Grain bioassay of single-basidiospore cultures. Each rye grain medium was prepared and inoculated as described in the text with a seed plug (square). (A) Morphological appearance of a culture exhibiting normal growth. (B) Morphological appearance of a culture exhibiting + sectoring. (C) Morphological appearance of a culture exhibiting +++ sectoring. (D) Morphological appearance of a culture exhibiting ++++ sectoring.

Netherlands that produced a stroma-like sector. Fl1, Fl2, and Fl3 were primary sectors isolated from commercial type U1 spawn that produced a fluffy morphology. All of the sectored isolates produced sectored phenotypes that were irreversible after extensive subculturing on a number of different media. All cultures were maintained either on complete yeast medium (CYM) (27) or on potato dextrose yeast agar (2). To produce fruit bodies, spawn was inoculated into compost which was prepared as described by Begin and Spear (2).

Basidiospore isolation and germination. Spore prints on Whatman no. 1 filter paper were prepared by using fruit bodies of both normal and sectored commercial isolates. Basidiospores were germinated as described previously (14). Single-basidiospore isolates were cultured on CYM or potato dextrose yeast agar.

Grain bioassay. To 250-ml Erlenmeyer flasks we added 50 g of rye grain, 1 g of calcium carbonate, and 66 ml of distilled water. The rye grain flasks were then autoclaved at 121°C for 30 min and stored at room temperature (22 ± 2°C). Although the experiments described below were performed with rye grain, similar results with respect to sectored morphology were observed if the rye grain was replaced by millet. Sterilized rye grain was inoculated with 5 g of normal commercial spawn to provide the "background" inoculum (2). A 5-mm agar plug ("seed plug") from each basidiospore culture was placed into each inoculated rye grain flask. Each seed plug was placed below the surface of the grain and against the inner glass wall of the flask for easy observation (Fig. 1). The flask was incubated without shaking at room temperature for 14 days, during which time the growth and appearance of both the background inoculum and the seed plug were monitored daily. The morphological appearance of each seed plug was arbitrarily defined as follows: 0 indicated that the seed plug exhibited normal growth and therefore was indistinguishable from the background growth (Fig. 1A); + to ++++ indicated that the seed plug exhibited abnormal growth ranging from fluffy to stroma-like (Fig. 1B through D). For each bioassay, at least 100 basidiospore-derived cultures were examined. The data in Table 1 represent the averages of the data obtained from two or three separate experiments. To verify that the morphology was irreversible, a number of transfers were made from recovered seed plugs on CYM, and another grain bioassay was performed.

TABLE 1. Morphological appearance of single-basidiospore cultures in the grain bioassay

Fungal isolate	% of cultures that exhibit[a]:				
	0 Growth	+ Growth	++ Growth	+++ Growth	++++ Growth
Wild isolate	100.0 ± 0				
Normal U1	92.5 ± 3.5	3.0 ± 1.4	4.5 ± 2.1		
Sr1	48.5 ± 6.4	20.0 ± 7.1	19.5 ± 3.5	5.5 ± 0.7	6.0 ± 4.2
Sr1-2	42.0 ± 4.2	24.0 ± 4.2	16.5 ± 3.5	7.5 ± 0.7	9.5 ± 6.3
Sr3	32.0 ± 5.3	10.7 ± 3.1	14.0 ± 5.3	14.0 ± 5.3	28.7 ± 7.1
Fl1	44.0 ± 4.0	13.3 ± 3.1	24.0 ± 2.0	16.0 ± 3.5	4.0 ± 2.0
Fl2	50.5 ± 2.1	33.0 ± 7.1	9.5 ± 0.7	6.0 ± 2.8	1.0 ± 1.4
Fl3	51.5 ± 4.9	23.5 ± 2.1	16.5 ± 2.1	7.5 ± 3.5	1.0 ± 1.4

[a] The morphological appearance of isolates was defined as follows: 0, normal (characterized by growth identical to the growth of the inoculated spawn); + to ++++, sectored (characterized by colonies that range from very mildly fluffy [+] to stroma-like [++++]). The values are the means ± standard deviations calculated from the results of two or three separate experiments for each isolate; a total of 150 to 200 spores were used for each isolate.

Fruit body production. Mycelia isolated from normal and sectored type U1 strains were fruited in compost substrate at the research facility of Sylvan Spawn Laboratories. Photographs of fruit bodies were taken with Kodak TMAX-100 black and white film.

DNA isolation and Southern hybridization. The procedures used for DNA isolation and DNA-DNA hybridization have been described previously (6).

RESULTS

In fruiting trials, the normal type U1 strain exhibited slow and uniform colonization of the compost. In general, the sectored U1 strains, especially the stromatal sectors (Sr1-2 and Sr3), exhibited much faster and sometimes less uniform colonization of the compost (Fig. 2). Furthermore, Sr3 produced large patches of matted mycelium on the surface of the cased compost (Fig. 2C). The level of pin formation was significantly lower in the sectored strains than in the normal U1 strain. All of the sectored isolates examined (Sr1, Sr1-2, Sr3, Fl1, Fl2, and Fl3) produced fruit bodies. In an attempt to obtain some information that related sectoring to mushroom quality (morphology), we compared mushrooms derived from normal strain U1 with mushrooms derived from both stromatal and

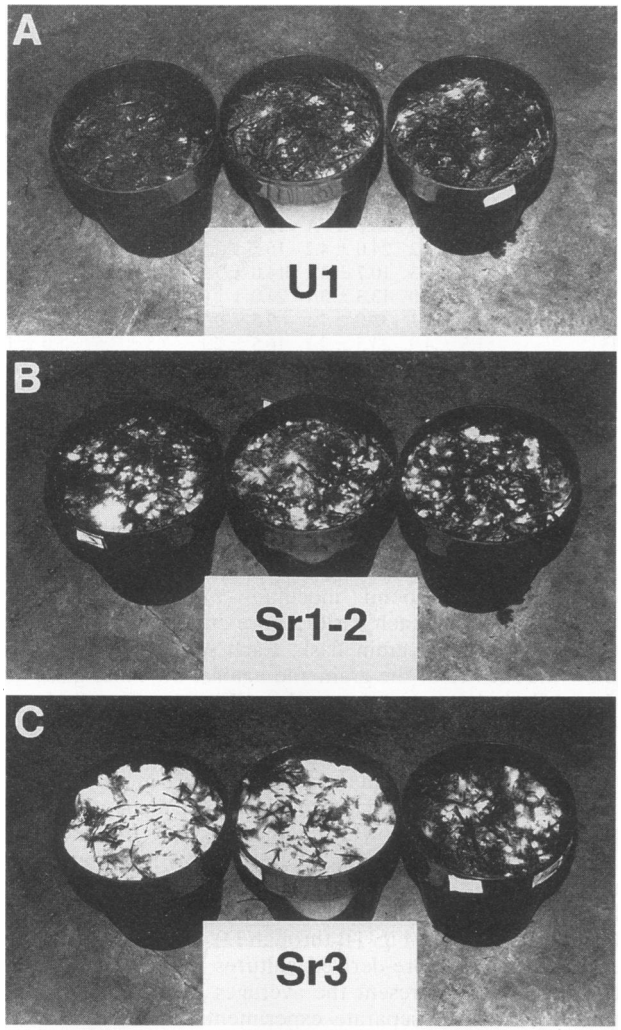

FIG. 2. Commercial compost medium inoculated with a normal type U1 strain (A), Sr1-2 (B), and Sr3 (C). Compared with the normal strain, Sr1-2 and Sr3 grew faster and produced significantly more fluffy and matted mycelia on the surface of the compost substrate. The conditions used for the three isolates were identical.

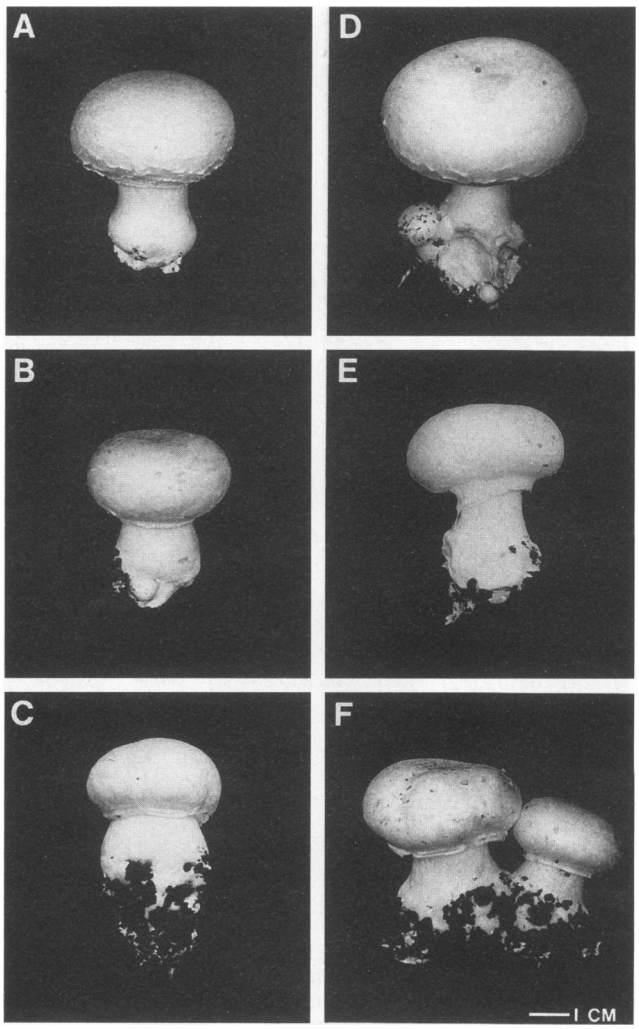

FIG. 3. Mushrooms produced by both normal and sectored commercial strains. (A) Mushroom from a normal type U1 strain. (B) Mushroom from isolate Sr1, showing the swollen stipe. (C) Mushroom from Sr3, showing the severely swollen stipe. (D) Mushroom from isolate Fl1 showing the flat cap and central depression. (E) Mushroom from Fl2. (F) Mushroom from isolate Fl3. The frequency of deformed mushrooms in healthy beds of U1 is extremely low (<0.1%). On the basis of a very limited sample size (20 to 30 mushrooms), the frequencies of abnormal mushrooms were approximately 10% for Fl1, Fl2, and Fl3, 25 to 30% for Sr1, and nearly 100% for Sr3.

fluffy sectors. The sectored isolates that we examined all produced abnormal-looking fruit bodies (Fig. 3). Strain Sr1 produced mushrooms with flat tops and fat stipes (Fig. 3B). Sr3 produced mushrooms with small caps and extremely fat stipes (Fig. 3C). The mushroom caps produced by Fl1 appeared stretched when they were compared with normal U1 mushroom caps at the same stage in development (Fig. 3D). Fl2 produced mushrooms with flat tops and fat stipes (Fig. 3E). Fl3 produced mushroom caps with large depressions (Fig. 3F) and exhibited a tendency to produce mushrooms with open veils. The percentages of abnormal fruit bodies ranged from 10% for the fluffy sectors to 35 to 100% for the stromatal sectors.

Basidiospores were collected from fruit bodies of the wild-collected isolate (RWK1420), normal strain U1, and sectors Sr1, Sr1-2, Sr3, Fl1, Fl2, and Fl3. The basidiospores were germinated on solid CYM and allowed to grow for 10 to 12 days. Mycelia from the germinated basidiospores were used in the grain bioassay. The grain bioassay which we used was a modification of a previously described bioassay in which 1-liter grain flasks were used (2). We developed a scoring system that rated the degree of sectoring from 0 to ++++; 0 indicated that there was no sectoring (normal growth), and ++++ suggested that severe stromatal sectoring occurred (Fig. 1). Our results indicate that normal commercial strain U1 has a natural tendency to produce sectors in the F1 generation (Table 1). All of the basidiospore-derived cultures obtained from a wild-collected isolate that we studied produced normal background mycelial growth in the grain bioassay. The + and ++ seed plugs in the U1 grain bioassay were subcultured four or five times on CYM and then reinoculated as seed plugs into other grain bioassay preparations. A total of 80% of the subcultures retained the sectored morphology in the second grain bioassay, whereas 20% of the cultures reverted to the control 0 or background growth.

The ratios of normal morphology to sectored morphology

for basidiospore cultures obtained from sector Sr-1, Sr-3, Fl1, Fl2, and Fl3 preparations were approximately 1:1 (Table 1). A single spore was isolated from a fruit body of Sr1 and was allowed to grow and produce a mushroom, from which single spores were isolated. These spores represented the F2 generation (Sr1-2). When these spores were germinated and subjected to the grain bioassay, the ratio of normal growth to sectored growth (42:58) was similar to the ratio observed for the F1 generation (1:1) (Table 1).

One possible explanation for the difference in growth characteristics between the normal and sectored isolates may be related to the natural heterokaryotic nature of the mycelium and the possibility of deheterokaryotization. One of the two homokaryotic nuclei in a fertile mycelium may not undergo mitotic division or may undergo mitosis at a rate much lower than the mitotic rate of the other nuclear type. Informative restriction fragment length polymorphic probes can distinguish nuclear types and have been utilized to identify the homokaryotic state during protoplast regeneration or basidiospore germination (6, 14, 20). DNAs were isolated from the type U1 strains examined in this study, the wild-collected isolate, and all of the primary sectors shown in Table 1. In addition, DNAs were isolated from approximately 30 randomly selected basidiospore cultures that exhibited + to ++++ growth. These DNAs were digested with *Eco*RI, electrophoresed into agarose gels, Southern blotted onto GeneScreen Plus, and hybridized with restriction fragment length polymorphism probe p33n10 (6, 13, 19). The autoradiograms obtained all suggested that the heterokaryotic state was present, and there was no evidence of any variation in the ratio of the two nuclear types (data not shown).

DISCUSSION

Strain instability in filamentous fungi implies that there are permanent changes over time. In the commercial mushroom *A. bisporus*, the phenotypic expression of changes may frequently be manifested as morphological variations in mycelial cultures that have been termed sectors (2, 10, 22). In this study we examined a number of permanent and irreversible sectors and compared these sectors with the normal commercial strain from which they were derived. We consider this type of permanent and irreversible sector one form of mushroom strain degeneration. As a reference point, we made a number of observations with a wild-collected *A. bisporus* strain which had never been subjected to the environmental conditions associated with mushroom cultivation. We made a number of comparative measurements associated with compost colonization and fruit body morphology. Restriction fragment length polymorphism data suggested that all of the primary sectors were heterokaryotic and that the apparent ratio of the two nuclear types was 1:1. The results of serial transfers of the primary sectors examined in this study suggested that the changes observed were mitotically stable. This characteristic clearly distinguishes this type of sector from sectors that may revert to the normal phenotype after subculturing (2).

The stability and irreversibility of the changes observed in the primary sectors studied suggest that there was some type of genetic change. In an attempt to understand the nature of this change, we examined the stability of the sectored morphology through the sexual reproductive process. *A. bisporus* is a secondarily homothallic fungus that produces basidiospores that are mostly binucleate and self-fertile (8, 13, 23). Therefore, if a single basidiospore is isolated, it is usually heterokaryotic and contains two sexually compatible nuclei. To determine whether the F1 selfed progeny expressed the sectored phenotype, we utilized a grain bioassay in which we essentially mixed a specific inoculum with a background of normal colonized grain. The inocula used (seed plugs) produced growth that either blended in with the healthy colonized grain (0) or produced some degree of sectoring (+ to ++++).

The results of our meiotic stability measurements are shown in Table 1. A number of important and reproducible trends were observed. In the grain bioassay all of the seed plugs obtained from single-spore cultures of the wild-collected isolate exhibited normal (0) growth. Conversely, approximately 5 to 10% of the single basidiospores isolated from the commercial type U1 strains produced a sectored phenotype (Table 1). Since reversible fluffy sectors may appear in normal U1 cultures and may have no effect on strain performance, it was important to determine that the seed plugs obtained from single basidiospores of U1 contained sectors that were indeed irreversible. Subculturing the sectors and performing a second grain bioassay established that 80% of the initial single-spore sectors were irreversible. These data suggest that there is a natural tendency for type U1 strains to produce sectors; the biological mechanisms involved are unknown at this time. The results obtained with the wild-collected isolate (no sectors) certainly support the proposal that wild germ plasm should be introduced into commercial lines. Some wild strains do sector, however, as do their spore offspring (17a).

When the primary sectors examined in this study produced fruit bodies, they all produced abnormal mushrooms (Fig. 3). If single-basidiospore cultures were isolated from these mushrooms and subjected to the grain bioassay, the ratio of normal phenotype (0) to sectored phenotype (+ to ++++) (Table 1) was approximately 1:1. One of the single-spore cultures obtained from a fruit body of Sr1 was allowed to go through the growth cycle, and it produced an F1 mushroom, which released basidiospores which represented the F2 generation (Sr1-2). Basidiospores from Sr1-2 were subjected to the grain bioassay, and the results again suggested that the ratio of normal phenotype to sectored phenotype was 1:1 (Table 1).

Because a mating event need not occur in this secondarily homothallic organism, there is a natural tendency toward inbreeding. Until recently, little was known about the classical organization of the *A. bisporus* genome. Royer et al. (28) described an electrophoretic karyotype for *A. bisporus* which suggested that the haploid chromosome number was 13. In our recent study dealing with meiosis and linkage relationships in *A. bisporus*, we reported that the button mushroom undergoes a conventional meiotic process with both independent assortment and joint segregation of markers and that crossing over is infrequent over most of the genome (18). The biochemical and morphological changes displayed by a number of other filamentous fungi in culture have, in some cases, been attributed to heterokaryosis (7), chromosome instability (9), mobile DNA elements (1, 3, 4), or cytoplasmic inheritance (4, 16, 17). When considering interpretations of the results described in this study, workers must examine both classical Mendelian mechanisms and non-Mendelian alternatives (12, 24, 25).

We provide data that suggest that a primary irreversibly sectored type U1 strain transmits its sectored phenotype to the F1 generation and perhaps the F2 generation. While the mechanistic cause of the altered phenotype and the explanation for the inheritance of the altered phenotype are not known, the consequences of this type of strain degeneration are potentially very serious. In this paper we describe our initial attempt to begin understanding this phenomenon in the cultivated button mushroom, *A. bisporus*.

ACKNOWLEDGMENTS

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

We thank Farid Jalali and Mary Ann Isabella for their technical assistance. We also thank Richard Kerrigan, Alan Castle, and James Anderson for critically reading the manuscript.

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