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Physiological and Genetic Variability of Commercial Isolates of Culinary–Medicinal Mushroom *Agaricus brasiliensis* S. Wasser et al. (Agaricomycetideae) Cultivated in Brazil

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ABSTRACT: *Agaricus brasiliensis* S. Wasser et al. ("Cogumelo medicinal"), a culinary–medicinal mushroom native to Brazil, is cultivated mostly for exports. Strain characteristics influence productivity, but little is known about ecological and genetic variation among commercial strains. We examined six strains of *A. brasiliensis* to determine the characteristics of colony growth and genetic variability. Two colony growth studies were conducted: (1) on ordinary laboratory media, malt extract agar, and potato dextrose agar at several incubation temperatures (20°, 25°, 28°, 30°C); (2) on minimal media supplemented with glucose) at three pH (6.0, 7.0, 8.0). We also evaluated linear growth on "race tubes" filled with commercial compost (at 28°C). Generally, strain 22, commonly considered by producers to have low productivity, tended to grow the slowest on both media at all temperatures, with significant differences in rates found between it and all other strains on both media at 30°C. At pH 6.0, strain 22 grew more slowly than strains 21, 23, and 24 and presented similar rates to strains 33 and 34. At pH 8.0, no isolate was able to grow. Strain 22 also grew the slowest in race tubes. PCR-RFLPs of the ITS region identified all strains as *A. brasiliensis*. RAPD patterns identified two groupings: strain 22 and all other strains. Even though we found low genetic variability, important strain characteristics (e.g., low colonization rate) considered related to productivity were detected for strain 22. We conclude that five of the strains may belong to the same ecological/genetic biotype. Low variability among Brazilian commercial strains might suggest a common origin of a single isolate recently brought into cultivation.

KEY WORDS: *Agaricus brasiliensis*, intraspecific variability, mycelium growth rate, genetic variability, culinary–medicinal mushrooms

ABBREVIATIONS

ITS: intergenic spacer; **MEPA:** malt extract enriched with peptone agar; **MMG:** minimal media supplemented with glucose; **PCR:** polymerase chain reaction; **RFLP:** restriction fragments length polymorphism; **RAPD:** random amplified polymorphic DNA; **PDA:** potato dextrose agar

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INTRODUCTION

Agaricus brasiliensis S. Wasser et al., also known as *Agaricus blazei* Murrill ss. Heinem., is a culinary–medicinal mushroom native to Brazil, naturally occurring in the mountainous areas of the Atlantic forest, in the states of São Paulo (Eira, 1997) and Paraná (Wasser et al., 2002). Cultivation was pioneered in the 1960s by Japanese immigrants, first outdoors and later under controlled conditions after Brazilian isolates were sent to Japan and reintroduced (Chen, 2001).

Areas of cultivation have expanded in the country, particularly in the last decade, as mushroom production generated a good source of income for small farmers. The state of São Paulo, in the Southeast region, is responsible for most production, but other regions in Brazil, particularly the states of Minas Gerais (Southeast region), Paraná, and Santa Catarina (South region), recently have been involved in cultivation (Fig. 1). This interest is due to the commercial value of the mushroom in the international market, particularly in Japan. The mushroom has the potential for use in both food and the pharmaceutical industries. Most production is for export as a nutriceutical with antitumor, immune-modulation, and antimicrobial medicinal properties (Fujimiya et al., 1999; Mizuno 1999; Stamets, 2000).

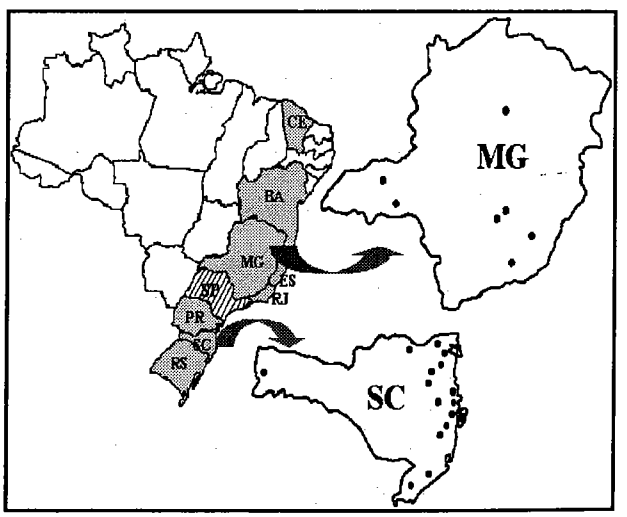

FIGURE 1. States in Brazil in which *Agaricus brasiliensis* is cultivated. SP is evidenced as the state in which most production occurs. SC and MG show different geographic centers of production.

Among Brazilian farmers, colloquial names are generally used, and several different strains or even species of *Agaricus* (such as *A. sylvaticus*) may have been in cultivation in different regions under the name of Cogumelo Medicinal. In South Brazil Cogumelo de Piedade and Cogumelo Blazei are other examples. Farmers have no precise taxonomic identification of their produced mushrooms.

Despite its economic importance, cultivation of *A. brasiliensis* in the country is based upon low-level technologies. As a consequence, yield is usually low (Braga, 1999). *A. brasiliensis* is mostly a seasonal crop, with production occurring in the warmest months, from October to April. The mushroom is cultivated under uncontrolled or low-tech cultivation conditions. This lack of environmental control is one of the factors affecting productivity, which makes the cultivation of *A. brasiliensis* under the present conditions highly risky. Furthermore, production techniques (compost formulations and production) have been adapted from *A. brunnescens*, which mostly has been cultivated under temperate conditions. In contrast, *A. brasiliensis* is a tropical mushroom cultivated in composted substrates based on straw, and sugar cane bagasse with supplements and correctives in different formulations, most of which have not yet been experimentally evaluated. Colonized compost is usually commercialized by private enterprises, with spawn being produced by specialized companies or a few major producers who concentrate on production of compost, spawn, mushrooms, and processed products.

Although strain attribute is one of the most important factors responsible for productivity, the intraspecific variabilities of *A. brasiliensis* have not been studied. Mushroom strains are known to differ substantially in their growth rate at all stages of the mushroom cycle under different environmental conditions, such as incubation temperatures and substrate pH. For instance, oyster mushroom strains under the same conditions typically take 5–10 days to colonize the culture media in standard size Petri plates. All conditions being the same, strains which take longer than 3 weeks to colonize culture media,

grain, or bulk substrates have a higher susceptibility to contamination (Stamets, 2000). Mycelial growth rates *in vitro* and in compost were used to differentiate strains of *Agaricus subfloccosus* originated from different regions (Noble et al., 1995). Similarly, growth rates in different substrates were used to characterize strains of another cultivated mushroom, *Volvariella volvaceae* (Reyes et al., 1998). Of particular interest is the report that optimal growth temperatures were shown to vary among strains of *Agaricus brunnescens* (Horna and Royse, 1983), which is closely related to *A. brasiliensis*.

Molecular biology has become a very important tool in the evaluation of intraspecific genetic variability of microorganisms. The establishment of improvement programs for the development of superior cultivars requires a previous evaluation of the level of isolate variability, which can be performed using molecular biology techniques. The PCR (polymerase chain reaction) techniques and protocols for DNA analysis, such as the RAPD (random amplified polymorphic DNA) (Williams et al., 1990) and the RFLP have been used to study the genetic variability of isolates of other mushrooms (Buynard et al., 1996). DNA sequences have been found to vary within genera and even among isolates of a particular species (O'Donnell, 1992; Muthumeenakshi et al., 1994; Kusaba and Tsuge, 1995). A wide genetic diversity among isolates allows a greater genetic base for the development and selection of new lines. On the contrary, if a low diversity crop is found, for example in *A. brunnescens*, where only five genotypic classes have been found among 34 commercial isolates examined (Royse and May, 1982), then variation among lines should be increased.

Recently, Fukuda et al. (2003), who have studied genetic variation among seven cultivated Japanese strains and one Brazilian strain of *A. brasiliensis* (=*A blazei* ss. Heinem.), found that genetic composition of the Brazilian strain was different from that of the Japanese strains. They also suggested that variation between the two groups of strains might be low and that strains may have been selected from the same wild-type population of *A. brasiliensis*. They have concluded that intraspecific variation of *A. brasiliensis* was originally low in comparison with other cultivated mushrooms. As a conclusion, they suggested the development of studies of genetic divergence among strains integrated to several other aspects, such as physiology and productivity, in order to have a precise and consistent strain analysis and efficient development of superior strains for cultivation.

Knowledge of intraspecific variability of mushrooms is important for the improvement of commercial production in order to select isolates best suited to specific environmental conditions. The understanding of the trophic requirements of each isolate is instrumental to obtaining the physiological basis for mushroom improvement. Furthermore, the knowledge of optimal ecological parameters is important for efficient control during each phase of mushroom production for yield improvement. Finally, the understanding of physiological variability among isolates may identify which suboptimal cultural practices have been used in production systems or which strains may be required for optimal adaptation to different environmental conditions, such as those occurring in different regions in the country.

The aim of this work was to establish the link between growth rate and genetic variation among six commercial strains of *A. brasiliensis*. Physiological variation was evaluated by using mycelial growth on different substrates, incubation temperatures, and substrate pH. Genetic variation was evaluated by using PCR- and RFLP-based genotyping. For the objective of genetic variability, molecular markers based on the PCR were used.

Prior to physiological and genetic investigations, taxonomic identification of strains obtained as mycelial cultures were made by using traditional techniques of producing fruiting bodies for studies.

MATERIALS AND METHODS

Taxonomic Characterization of Strains

Six strains (22, 23, 24, 33, 34, and 35) of *A. brasiliensis* were provided as mycelial cultures, by spawn makers, or isolated from frutifications collected in mushroom farms from different states of Brazil (São

Paulo, Minas Gerais, Santa Catarina, Paraná). Strain 22 was selected because it was cultivated in the state of Santa Catarina and either yield was consistently low or no production was obtained. For all the other strains, no information on productivity was available.

In order to taxonomically identify the strains, basidiomata were obtained using standard cultivation techniques. Macro- and microscopic analyses were done with fresh and dried fruit bodies.

Taxonomic Identification of Cultures Based upon Fruit Body Analysis

A sample of recently collected immature fruit bodies were introduced in a humid chamber to obtain the spore prints and to follow the developmental phases of the mushroom. Macroscopic characteristics of the fruit bodies, such as average size, color and color changes (Munsell, 1975), texture, odor, pileus and lamellae characteristics, veil presence, and spore color, were obtained. Microscopic characteristics and keys for identification were based upon Murrill (1945), Freeman (1979), Moser (1983), and Wasser et al. (2002).

Physiological Variability of Strains

To determine the physiological variability among strains on different culture media, incubation temperatures and pH media were studied in relation to mycelial growth rate. Linear mycelial growth rates were also evaluated on commercial compost substrate.

Mycelial Growth on Different Culture Media and Incubation Temperatures

To compare the *in vitro* mycelial growth of each strain colony diameter on culture media were assessed on malt extract enriched with peptone (30: 3, w/w) agar (MEPA) media and potato dextrose agar (4:20, w/w, potato extract : dextrose), (PDA). One 6 mm-diameter mycelial plug from an actively growing margin of a colony was transferred on to 90 mm-diameter agar plates, each containing media, PDA, and MEPA, and each isolate was incubated at 20±2°, 25±2°, 28±2°, and 30±2°C. Four replicates were used for each media and each temperature level.

Colony diameter (average of two perpendicular diameters) was assessed every 2 days for 10 days for both media.

Mycelial Growth on Different Substrate pH

To evaluate the effect of different substrate pH on the growth of each strain, a minimal media supplemented with 10 g of glucose (MMG) and composed of KH_2PO_4 (1 g); $MgSO_4.7H_2O$ (0.5 g); $CaCl_2$ (0.5 g); $(NH_4)_2SO_4$ (1 g); agar (15 g); and distilled water (1000 mL), as defined by Wenzel and Dias (1999), was prepared, and the pH was adjusted with phosphate buffer to 6.0, 7.0, and 8.0. Media was inoculated with 6 mm plugs of mycelium recovered from an actively growing margin of *A. brasiliensis* colonies on the same media and incubated at 28±2°C. The pH was adjusted before media sterilization and assessed after mycelial growth. Four replicates were used for each pH level and each strain. Mycelial growth was assessed, using the colony diameter (average of two perpendicular diameters) as parameter, and was evaluated every 2 days for 10 days.

Linear Growth Rate of Agaricus brasiliensis Strains in Compost

Portions (ca 30 g) of commercial compost were placed into open-ended glass tubes (25 mm diameter × 185 mm length). Sterile distilled water was added to maintain the compost at 70% (w/w), and under environmentally controlled conditions the tubes were closed with cotton plugs and each column was inoculated at one end with one plug of ca 25 mm diameter of the marginal mycelium growth of a 10-day-old malt extract agar culture of each strain. The columns were incubated at 28°±2C. Average linear growth (4 lines/tube) was measured daily for 10 days. Four replicates were made for each isolate.

Statistical Analysis of the Data

Data on colony diameters (media and incubation temperatures) and linear growth (on compost), average of four repetitions, were used to establish growth curves for each isolate. Subsequently, regression analysis and best fitting lines were determined. Using correlation coefficients (linear and angular) and prediction intervals, it was possible to establish when coefficients were considered statistically different.

Genetic Relatedness Among Strains of *Agaricus brasiliensis*

Isolates and Culture Conditions

The strains were cultivated in 250 mL flasks containing 50 mL of MEPA with no agar media inoculated with 6 mm-diameter mycelial growth disks removed from the margin of 3-week-old colonies grown on MEPA (without agar) medium. Cultures were incubated in stationary conditions at 28°± 2°C for 15 days.

Isolation and Extraction of DNA

Mycelial biomass was harvested onto nylon cloth and washed with distilled water, and DNA extraction was performed as described by Junghans et al. (1998). DNA concentration was estimated through electrophoresis in 1% agarose gels using a lambda phage DNA as a molecular size standard.

Amplification by PCR and RFLP Analysis

The DNA was resuspended in sterile distilled water (SDW) and subsequently used in the PCR. The PCR was used to amplify the ITS region of the rDNA of each isolate. A total of two primers (ITS1 and ITS4) as described by White et al. (1990) were screened against all isolates. Each amplification was done in a final volume of 25 µL containing 1.0 unit of *Taq* polymerase, 10 ng total DNA, 100 mM each dNTP, 0.2 µM of each primer, 50 mM of KCl, 2.0 mM of $MgCl_2$, and 10 mM Tris HCl (pH 8.3). Amplification was performed in a PTC-100 Thermocycler (MJ Research) after an initial denaturation at 94°C, for 3 minutes, and subjected to 40 cycles through the following temperatures/times profiles: 50°C for 1 minute for annealing and 72°C for 2 minutes for extension. In the final cycle extension was for 10 minutes.

Subsequently, the PCR-amplified DNA products were ethanol extracted, precipitated, dried, and resuspended in water MilliQ. Each strain of PCR product was digested with three different restriction enzymes, *Alu* I, *Hha* I, and *Hinf* I. Restriction digests were then eletrophoresed in 2.0% (w/v) agarose gels stained with ethidium bromide (0.6 µg. mL^{-1}). Restriction patterns were subsequently visualized under UV light, photographed, and recorded in an Eagle Eye system (Stratagen). DNA fragment sizes were estimated using as a standard the DNA from the ϕX 174 phage digested by the *Hae* III enzyme.

DNA analysis by RAPD

For the RAPD analysis, a total of 10 primers (OPF-01, 02, 04, 06, 08, 09, 10, 12, 13, 14) were used for amplification, which was performed in a PTC-100 Thermocycler (MJ Research) and subjected to a PCR program, which included 40 cycles with the following sequence of time and temperature: 15 seconds at 94°C, 30 seconds at 35°C, and 1 minute at 72°C. In the final cycle, the extension step was for 7 minutes at 72°C, and finally temperature was reduced to 4°C up to the recovery of the samples. Amplified products were then eletrophoresed in 1.5% (w/v) agarose gels stained with ethidium bromide (0.6 µg.mL^{-1}). DNA fragment sizes were estimated using as a standard the DNA from the ϕX 174 phage digested by the *Hae* III enzyme. Patterns were then visualized under UV light for photography and recorded in the Eagle Eye system.

For data analysis, each strain was scored using codes 1 and 0 for the presence and absence of each specific fragment on the gel with the 10 RAPD primers. Genetic distance between isolates was assessed as defined by Nei and Li (1979). The cluster

analysis of the data was based on a similarity matrix calculated using the GENES package (Cruz, 1997). A dendrogram was then constructed after genotypes were grouped according to the method of UPGMA (unweighted pair group method with arithmethic average) developed by Sokal and Michener (1958), based upon the coefficients of similarity to provide insights into the relationships among strains.

A variability tree based upon band sharing of all possible pairs in analysis was constructed using a similarity matrix.

RESULTS AND DISCUSSION

All isolates produced basidiomata, which were identified as *Agaricus brasiliensis*. Vouchers of the dried fruit bodies were registered under the code numbers 11,794, 11,795, 11,796, 11,797, 11,798, and 11,799 and deposited at FLOR Herbarium, Universidade Federal de Santa Catarina, Florianopolis, Santa Catarina, Brasil. Neves (2000) detected discrepancies in several characteristics between the Brazilian fruit bodies and American fruit bodies based on Murrill's (1945) and Freeman's (1979) descriptions of *A. blazei*. In 2002, Wasser et al. reevaluated the taxonomy of *A. blazei* Murrill and suggested that *A. blazei* ss. Heinem. from South America (Brazil) was a new species designated *Agaricus brasiliensis* Wasser et al. On the other hand, *A. blazei* ss. Murrill is a North American (Florida) endemic species, known from three localities and not existing in culture.

Our descriptions agreed with Wasser et al. (2002) even when the properties typical of *A. brasiliensis*, such as the inability of the mycelium to survive at temperatures below 4 °C and the ability to fruit in sterilized substrates forming purely white fruit bodies, were observed in our laboratory (personal communication, Margarida de Mendonça). These observations were also made by Wasser et al. (2002)

Physiological Variability of *Agaricus brasiliensis* Strains

Mycelial growth on different media (MEPA and PDA) at incubation temperatures (20°, 25°, 28°, and 30 °C) of the six strains is shown in Figure 2. Five strains (21, 23, 24, 33, and 34) colonized completely the MEPA media on the Petri dishes, reaching a colony at least 9 cm in diameter after 10 days of incubation at 28 °C (Fig. 2c) and at 30 °C (Fig. 2d). Growth of all strains did not differ ($\alpha = 0.05$) in both media at temperature of 20 °C. Differences in growth rates occurred when strain 22 was compared with all other strains on both media at 30 °C, but no differences were observed at 28 °C. However, strain 22 was the slowest growing among the six on MEPA and PDA media. Plates were only completely covered with strain 22 mycelia after 20 and 14 days on media MEPA and PDA, respectively. Strain 22 showed a constant tendency to grow more slowly under most substrates and temperatures tested. All strains, except 22, presented a similar pattern and rate of growth, with slight differences, throughout the 10-day incubation. Optimal growth temperatures for all strains varied with media composition and reached 28 °C on PDA or 30 °C on MEPA media.

Lowest growth diameters were obtained at 20 °C incubation temperatures, confirming results obtained by Eguchi et al. (1994) for *A. blazei* ss. Heinem. These authors defined after *in vitro* studies that minimal temperature for *A. blazei in vitro* growth on media, which included yeast extract, malt extract, and saccharose, was 20 °C. Maximal temperature for growth was defined to be 35 °C, whereas the optimal temperature range for growth was between 22 °C and 26 °C. In our experiments it was observed that all strains except 22 showed optimal growth temperatures at 28 °C and 30 °C. It is possible that these strains may have adapted to higher temperatures, typical of the regions where the species has been cultivated in Brazil. Another possibility is related to the taxonomic differences between strains being used in Japan and Brazil.

It was observed that among the six strains selected, two groups were defined in relation to the influence of temperature on growth in two media. One group included strains 21, 23, 24, 33, and 34, and the other group included strain 22.

Similar growth patterns for all six strains were observed at each pH (6.0, 7.0) as observed in Figure 3. At pH 6.0 and 7.0, strain 22 showed a consistent tendency toward the lowest mycelial growth rate. At

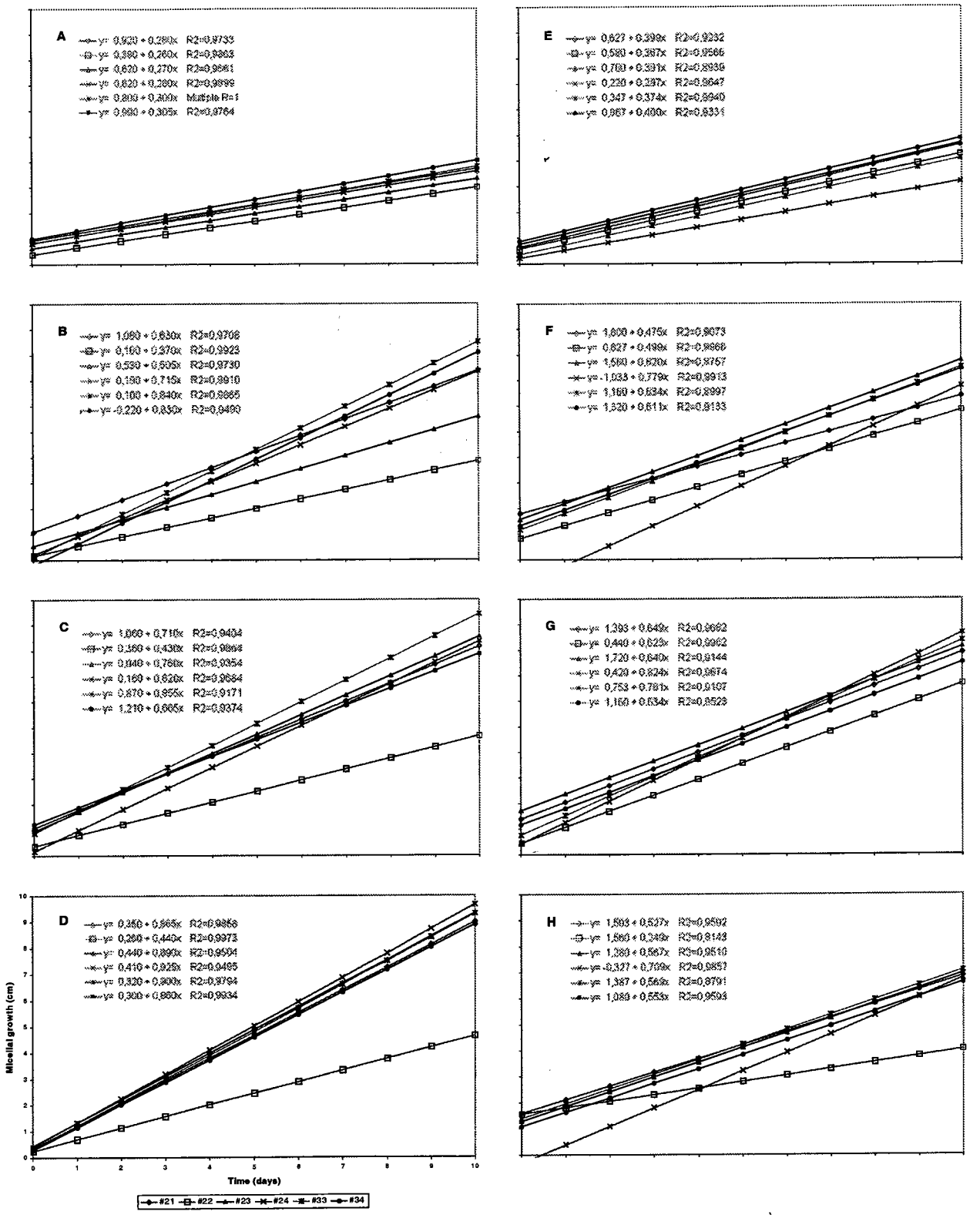

FIGURE 2. *Agaricus brasiliensis* mycelial growth in Petri dishes containing different culture media: malt extract peptone agar media (MEPA) incubated at 20°C **(A)**; at 25°C **(B)**; at 28°C **(C)**; at 30°C **(D)**; and potato dextrose agar media (PDA) incubated at 20°C **(E)**; at 25°C **(F)**; at 28°C **(G)**; and at 30°C **(H)**.

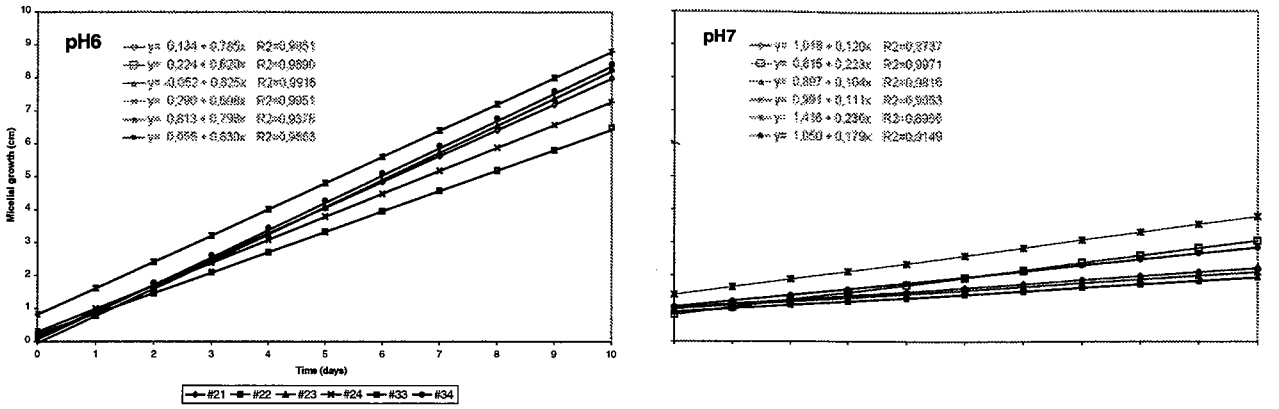

FIGURE 3. Mycelial growth rates of six strains of *Agaricus brasiliensis* in culture media with different pH (6.0 and 7.0).

pH 8.0, no isolate was able to grow, showing that better pH levels for *A. brasiliensis* strains ranged from pH 6.0 to 7.0. The use of pH around 7.0 for substrate (compost and casing layer) preparation will favor the establishment of *A. brasiliensis* while reducing fungal competition, particularly *Trichoderma* spp., a common substrate contaminant that is usually present under tropical temperatures and proliferates at low pH levels (5–6).

Strain 22 presented a significantly slower growth on compost rate than the other strains (Fig. 4). Our results showed that race tubes can be considered an efficient technique for screening strains with high colonization rates and competitive ability toward specific substrates. The success of mushroom compost utilization by any fungus will be determined by, among other factors, its competitive saprophytic ability and the inoculum potential of competitors. This attribute is surely a very important one if it is considered that *A. brasiliensis* is cultivated under nonsterilized (pasteurized) conditions in environments in which contaminants (such as *Trichoderma* spp.) commonly compete for nutrients.

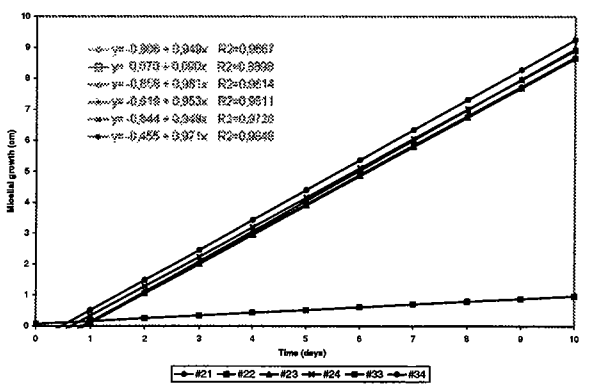

FIGURE 4. Mycelial growth rates of six strains of *Agaricus brasiliensis* on microcosms filled with commercial compost.

The success of mushroom compost utilization depends on several fungal attributes, such as the appropriate enzymatic secretion for degradation of a specific substrate, the ability of rapid colonization when stimulated by nutrients, and the ability to tolerate fungistatic substances (Garrett, 1970). Lower colonization rates of strain 22 in both sterile (e.g., culture media) and nonsterile (e.g., compost) environments suggest that the decreasing ability of degradation could be due to some metabolic change in the use of substratum components. Further studies on enzymatic activities of these strains are warranted.

Genetic Variability of *Agaricus brasiliensis* Strains

RFLPs of the ITS region did not show polymorphism among the six strains studied, suggesting that all strains belonged to the same species, *A. brasiliensis* (Fig. 5). On the other hand, RAPD profiles showed a low intraspecific genetic variability among strains

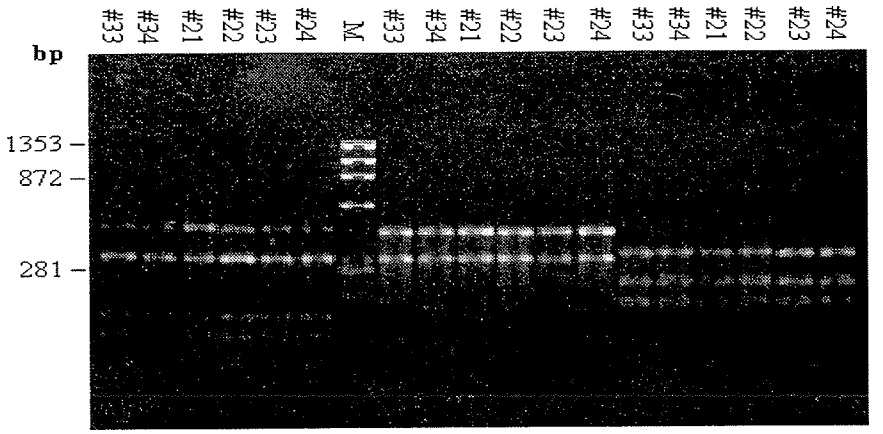

FIGURE 5. RFLP of ITS rDNA of six strains of *Agaricus brasiliensis* (21, 22, 23, 24, 33, and 34) using as restriction enzymes *Alu* I, *Hha* I, and *Hinf* I. Lane M shows the migration pattern of molecular weight marker ϕX 174 DNA/*Hae* III.

(Fig. 6). Genetic distance varied among isolates from 0% (strain 33 and 34) to 14% (strains 22 and 33; 22 and 34). Strain 22 was the most distant among the remaining strains analyzed. The dendrogram (Fig. 7), based upon data from the RAPD analysis, showed that two groups of strains were genetically different (distance 11.5%): group 1, which included strain 22 (low productivity); and group 2, which included the other five productive strains. Our results, using genetic traits, confirmed the ecological analysis that also showed that strain 22 was different when compared to the other strains. Within group

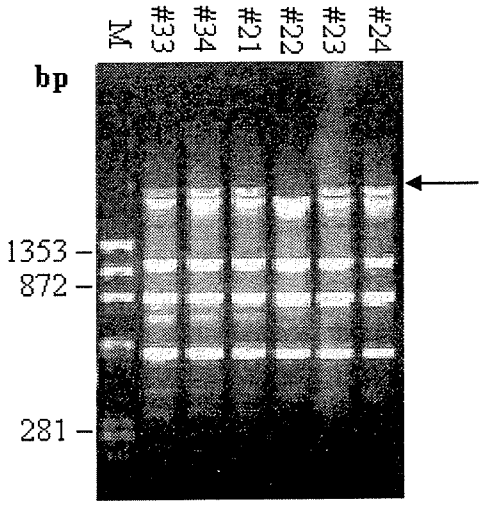

FIGURE 6. RAPD markers generated using the oligonucleotide OPF12 as primer of DNA of six strains (21,22, 23, 24, 33, and 34) of *A. brasiliensis*. The arrow indicates the band absent in strain 22 but observed on the other strains. Lane M shows the migration pattern of molecular weight marker ϕX 174 DNA/*Hinf*I.

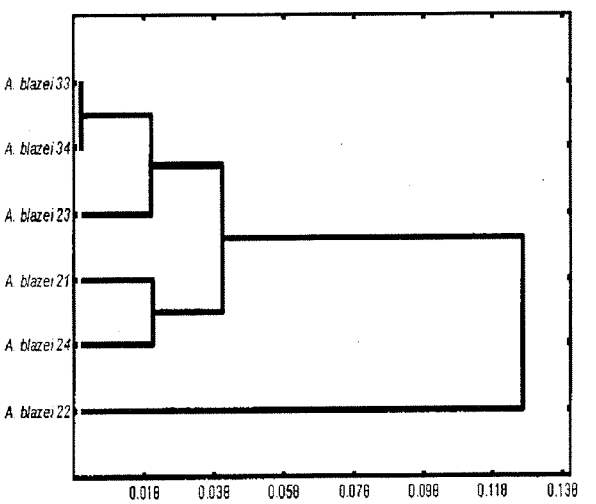

FIGURE 7. Distance tree comparison of the RAPD analysis from six strains (21, 22, 23, 24, 33, and 34) of *A. brasiliensis*. (Note: labels incorrectly cite *A. blazei*.)

2, it was possible to differentiate two subgroups: 1, which included strain 21 and 24 (2% distance); and 2, which included strains 23, 33, and 34 (distance 4%). Isolates 34 and 33 presented similar genetic pattern in the RAPD analysis.

Genetic homology was high among the strains studied, in that only one among the six showed a slight genetic distance. Even though variation was low, important traits related to productivity were involved. It may be hypothesized that strain 22 may have evolved from a common strain that has been used since the species was domesticated in the country. Based upon these results, attention should be directed to the potential widespread selection of genetically variable isolates that may be degenerative strains resultant from originally productive ones, possibly induced through mutations during the repeated propagation process. It is a common practice among some spawn production companies to use inadequate strategies for fungal propagation that favors mutations. Molecular mechanisms of degeneration of *A. brasiliensis* should be studied as a guide for strain stability and long-term preservation during the process of mushroom production.

CONCLUSIONS

Our results indicate that integrated ecological and genetic studies were important to examine intraspecific variability among the six strains of *A. brasiliensis*. Among the techniques used, growth on race tubes with compost and molecular analysis (PCR-RAPD) were the most powerful for discriminating among strains.

The close genetic relationship among commercial strains of *A. brasiliensis* also has been reported in Japan (Fukuda et al., 2003) and in Brazil (Colauto et al., 2002). The lack of variability among strains is a problem that should be addressed carefully as Brazil becomes more involved in a wider program of *A. brasiliensis* cultivation. The use of a nearly "monoculture" of *A. brasiliensis* for cultivation should be avoided. Lack of variability in *A. brasiliensis* will render the crop susceptible to pathogens and pests. Such threats to crops may spread rapidly throughout the country as a result of the frequent exchange of compost and spawn already in practice among different regions.

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