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ALLERGENIC AND TOXIGENIC FUNGI IN THE COMPOST OF CULTIVATION OF *Agaricus brasiliensis*

FUNGOS ALERGÊNICOS E TOXIGÊNICOS NO COMPOSTO DE CULTIVO DE *Agaricus brasiliensis*

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ABSTRACT

The mushrooms *Agaricus bisporus* and *Agaricus brasiliensis* are grown on substrate obtained through a composting process that involves a complex and little known microbial succession. Sporulating and rapid-growth ascomycetes, and in special *Aspergillus fumigatus* have been reported among the different species isolated from the *A. bisporus* compost, indicating that pathogenic or potentially toxigenic species can remain in the compost at the end of the pasteurization process. However, in Brazil, there are no reports of isolation and identification of fungi in the growing compost of *A. brasiliensis* mushrooms after the pasteurization process. This study aimed to evaluate, through serial dilution technique, the presence of contaminant fungi in two kinds of *A. brasiliensis* growing compost (1- sugar cane bagasse/coast-cross hay; 2- cotton residue/ coast-cross hay). Species of *Aspergillus, Emericella* and *Penicillium* genera have been identified. Toxigenic fungi, as *Aspergillus ochraceus, Aspergillus terreus and Penicillium oxalicum*, were isolated from the formulations of growing compost of *A. brasiliensis. Aspergillus fumigatus* was found in both formulations; however, an intense spore production on the surface was observed in compost produced from cotton residue; which caused its use to be non-viable, for safety reasons. Considering that *A. fumigatus* is a human pathogen, preventive measures should be taken by workers involved in the production of compost, in order to prevent successive inhalation of spores.

Key-words: Composting; *Aspergillus*; aspergillosis; edible and medicinal mushroom.

RESUMO

Os cogumelos *Agaricus bisporus* e *Agaricus brasiliensis* são cultivados em substrato obtido por um processo de compostagem que envolve uma sucessão microbiana complexa e pouco conhecida. Dentre as diferentes espécies isoladas do composto de *A. bisporus*, foram relatadas espécies de ascomicetos esporulantes e de crescimento rápido e, em especial, *Aspergillus fumigatus*, indicando que espécies patogênicas ou potencialmente toxigênicas podem permanecer no composto ao final do processo de pasteurização. Entretanto, não há relatos no Brasil acerca do isolamento e identificação desses fungos a partir do composto de cultivo do cogumelo *A. brasiliensis* após o processo de pasteurização. Este estudo teve como objetivo avaliar, por meio da técnica de diluição seriada, a presença de fungos contaminantes em dois tipos de compostos de cultivo de *Agaricus brasiliensis* (1- bagaço de cana/capim Coast cross; 2- resíduo de algodão/capim coast-cross). Foram identificadas espécies pertencentes aos gêneros *Aspergillus*, *Emericella* e *Penicillium*. Fungos toxigênicos como *Aspergillus ochraceus, Aspergillus terreus e Penicillium oxalicum* foram isolados a partir das formulações de composto de cultivo do cogumelo *A. brasiliensis*. *Aspegillus fumigatus* foi encontrado nas duas formulações, porém, no composto produzido com resíduo de algodão, verificou-se produção intensa de esporos na sua superfície, inviabilizando a sua utilização, por questões de segurança. Considerando que *A. fumigatus* é um patógeno humano, medidas preventivas devem ser tomadas pelos trabalhadores envolvidos na produção do composto para evitar a inalação sucessiva de esporos.

Palavras-chave: Compostagem; *Aspergillus*; aspergilose; cogumelos comestíveis e medicinais.

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INTRODUCTION

Agaricus brasiliensis mushrooms have medicinal properties; for that reason, they have been cultivated in Brazil and several other countries. In countries like Japan, Korea, China and Taiwan they are also considered functional foods (Firenzuoli et al., 2007).

Like other mushrooms, this species is grown on substrate obtained after a composting process (with the participation of different species of bacteria, fungi and actinobacteria) that depends on microbial succession while it happens (Chang & Miles, 2004). The study of this microbiota is very important to show which species have the desirable characteristics for achieving a better quality product as well as those that may compromise the quality of the mushroom cultivation substrate or that may be harmful to humans and should therefore be monitored or controlled.

The genus *Aspergillus* constitutes species of high importance in nature for being saprophytic organisms, and in the industry for the production of enzymes and other products used in the production of beverage and food. For being a saprophytic fungus, important to nutrient recycling, this fungus has already been isolated from different environments such as soil, compost and agricultural waste composting (Straatsma et al., 1994; Latgé, 2001; Phutela et al., 2005).

Besides, some species are also very important since they cause diseases known as aspergillosis, although the fungus usually acts as an opportunist pathogen. The species *Aspergillus fumigatus* is responsible for allergic bronchopulmonary aspergillosis, which has important implications in the human health, mainly in immunosuppressed patients. Allergic diseases caused by *A. fumigatus* occur due to repeated exposure to *Aspergillus* conidia or antigens, and there was no mycelial colonization of the respiratory tract. Therefore, the problem is usually solved when the patient is removed from the environment of exposure to spores (Latgé, 1999). These allergic diseases are known as farmer's lung and mushroom worker's lung, as they are common among farm workers who are exposed to spores of mushrooms or other fungi, besides other particles from the growing environment (Mori et al., 1998; Latgé, 1999; Tanaka et al., 2000).

Syndromes involving mycelial growth of *A. fumigatus* in the body, on the other hand, require a therapeutic intervention, because in immunosupressed patients the evolution of the disease can lead to death. Respiratory aspergillosis are difficult to diagnose and can occur in different ways in the body. The allergic bronchopulmonary aspergillosis (ABPA) is considered the most severe manifestation, and it may lead to the destruction of the lungs. Formation of masses of hyphae in lung or nasal cavities – the aspergilloma – results in rupture of blood vessels, causing cough followed by expectoration of blood. Invasive aspergillosis has become an increasing cause of death in immunosuppressed patients (AIDS, cancer and transplant), in which the disease may progress rapidly, leading to death in two weeks. Besides lung aspergillosis, invasive aspergillosis may still reach the mucosa, cartilage, brain and other organs (Latgé, 1999).

The compost for the *Agaricus* mushrooms cultivation is gotten by a composting process from agricultural residues and/or different grasses. Some species of fungi have already been isolated from the compost of *Agaricus bisporus*; nevertheless, there aren't many studies about this (Straatsma et al., 1994). In Brazil, considering the different environmental conditions, with different kinds of soil and a wide biodiversity, probably there's a large diversity of fungi in the material used for composting and, it is likely that different species of *Aspegillus* are also present. In addition to the spores of fungi that cause allergic diseases in workers who are involved in mushroom cultivation, harvesting and packing stages; particles from the compost that contain different kinds of spores, are also responsible for these diseases (Sakula et al., 1967; Mori et al., 1998; Latgé, 1999; Tanaka et al., 2000).

Also considering that there are no reports of *Aspergillus* isolation from the *A. brasiliensis* mushroom compost, the main objective of this work was the isolation and identification of *Aspergillus* and related species from the *Agaricus brasiliensis* compost.

MATERIAL AND METHODS

Compost production

Two compost formulations were used for the isolation and identification of fungi. For compost 1, crushed sugarcane bagasse [*Saccharum officinarum* L.] (426 kg), coast-cross hay [*Cynodon dactylon* (L.) Pers] (459 kg), wheat bran (100 kg), limestone (20 kg), gypsum (20 kg), ordinary superphosphate (10 kg) and urea (1.55 kg) were used. For compost 2, cotton textile mill waste was used [*Gossypium hirsutum* L.] instead of sugarcane bagasse, in the same amount, keeping the other ingredients in the same proportion, except urea, whose concentration in the compost was defined to adjust the initial concentration of nitrogen in 1%, similar to compost 1. For the compost preparation, standard procedures were used (Chang & Miles, 2004) except that phase I and phase II took both 14 days.

Isolation and identification of filamentous fungi

At the end of the composting procedure, samples in different points of the compost were collected in order to get composite samples, with 5 samplings per sample. For each sample, 20 g of compost were weighed, which were mixed with 180 cm^3 of peptone 0.1% and, later, grinding in blender for 10 s in order to have a better disintegration. Serial dilutions were done, following plating in triplicates of dilutions 10^{-4}, 10^{-5} and 10^{-6} in Dichloran 18% Glycerol Agar medium (DG-18) (per 1000 cm^3 of distilled water: glucose, 8 g; peptone, 4 g;

KH_2PO_4, 0.8 g; $MgSO_4$, 0.4 g; glycerol 95%, 185 cm^3; dichloran 0.1%, 1 cm^3; chloramphenicol, 10 mg; agar, 15 g). The plates were incubated at 25 ºC for 7 days. After counting, the data were transformed to log and subjected to analysis of variance (ANOVA).

After morphological characterization, the different morphotypes were isolated in Malt Agar medium (MA) (per 1000 cm^3 of distilled water; malt extract, 12 g; soybean peptone, 0.5 g; agar, 15 g). After that, they were incubated for 7 days at 25 ºC and at 37 ºC in Czapeck Yeast Agar medium (CYA) (per 1000 cm^3 of distilled water: $NaNO_3$, 3 g; K_2HPO_4, 1 g; KCl, 0.5 g; $MgSO_4\ 7H_2O$, 0.5 g; $FeSO_4\ 7\ H_2O$, 0.01 g; yeast extract, 5 g; sucrose, 30 g; agar, 15 g; pH 6.0 - 6.5) and also in Malt Extract Agar medium (MEA) (per 1000 cm^3 of distilled water, malt extract, 20 g; soybean peptone, 1 g; glucose, 20 g; agar,15 g; pH 5.5) for 7 days at 25 ºC.

The identification of species belonging to genus *Aspergillus* and *Penicillium* was made, as it's described in the guides Identification of Common *Aspergillus* Species (Klich, 2002) and A Laboratory Guide to Common *Penicillium* Species (Pitt, 2000). We observed the macroscopic characteristics of colonies in the three culture media described above, at temperatures of 25 ºC and 37 ºC. Afterwards, microscopical characteristics were examined, and from 8 to 19 morphological characteristics of reproductive structures, depending on the genus, were observed.

RESULTS AND DISCUSSION

Isolation and identification of *Aspergillus fumigatus*

During the preparation of the compost made with cotton and coast-cross hay, it was difficult to uniformly mix cotton residue with coast-cross hay, resulting in the formation of small cotton clusters throughout the windrows. These cotton clusters promoted higher moister accumulation than in regions where they were not present.

At the end of phase II composting, it was verified that the cotton textile mill waste based compost had an intense sporulation by a green mold on its surface. Below this layer the compost showed a normal aspect, with the presence of actinomycetes. The green mold was later identified as *Aspergillus fumigatus*, which grows well in higher temperatures and probably resists to compost pasteurization. This characteristic, associated with high humidity in the superficial layer of the cotton textile mill waste based compost, seems to have helped this uncommon colonization in the mushroom compost. This abnormal growth of *A. fumigatus* on the surface of the compost shows imbalance in the microbiota and makes impossible the use of this formulation under the tested conditions.

This layer with intense sporulation was carefully removed and thrown away, only being used the part of the compost with normal appearance to isolation and quantification of fungi, for comparing it with compost 1, which didn't show any abnormal growth of fungi on the surface. This was done to check if the intense sporulation on the surface was affecting the fungi population below the surface. In the total counting of fungi, it was found for compost 1, a population of 3.8×10^7 CFU g^{-1} of fresh compost and 6.7×10^7 CFU g^{-1} for compost 2, not observing significant differences between both ($p > 0.05$). These results indicate that the abnormal colonization by *A. fumigatus* on the surface of the compost actually happened as a result of excessive water accumulation in cotton textile mill waste. Therefore, if this residue is to be used in cultivation compost of *A. brasiliensis* mushroom, it must be mechanically mixed with every other ingredient to allow uniform mixing of cotton and coast-cross hay.

The presence of *A. fumigatus* in the composting process has already been described for the mushroom *Agaricus bisporus* in other countries (Chang & Miles, 2004; Straatsma et al., 1994); thus, it can be considered normal, as long as its occurrence is in balance with other microorganisms that are present in the compost. Therefore, the cotton waste-based substrate did favor an abnormal production of *A. fumigatus* spores; however, it is necessary to note that their presence may be common in any kind of compost, since this species widely occurs in nature and plays an important role in recycling organic matter (Furtado et al., 2005).

Among the identified species, the *A. fumigatus* was also the fungus with higher incidence in the traditional compost, showing its presence is not conditioned to the use of cotton textile mill waste (Table 1). The *A. fumigatus*, for being broadly disseminated and having high capacity of sporulation, has its spores frequently inhaled by human beings. Normally these spores are eliminated efficiently by innate immune mechanisms; however, for people with debilitated immunological system and farmers the fungi represent a real danger (Latgé, 2001). *A. fumigatus* is an important human pathogen which causes several diseases like allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis (Hong et al., 2005). Because of this, it is important that safety policies be adopted by part of the workers involved in the production and compost management, once these workers will be exposed repeatedly and consecutively to *A. fumigatus* spores having more probability to develop the disease, since the reduced size of the spores makes it easier for the pulmonary alveoli to be reached and the disease to be established (Latgé, 1999). In addition to *A. fumigatus* and other thermophilic fungi that are present in *Agaricus* mushroom growing composts, actinobacteria spores have also been reported as a causing agent of respiratory allergies in workers of composting and mushroom farms (Van Den Bogart et al., 1993). These respiratory allergies, also known as Mushroom worker's lung are traditionally associated with the spores of mushrooms or other particles that are present in the compost and in the mushroom houses (Mori et al., 1998) which make necessary to cease work on the mushroom farm so that the symptoms can cease. Clinical cases have been reported for a long time, which shows us it is an old problem (Sakula et al., 1967) and it must be considered for projects related to installation of composting and mushrooms farms, in order to avoid continuous replacement of sensitive workers, as well as legal (compensation) issues.

TABLE 1 - Species of filamentous fungi isolated from the *Agaricus brasiliensis* compost.

Species	Number of isolates per compost	
	Compost 1[(1)]	Compost 2[(2)]
Aspergillus fumigatus	10	13
Aspergillus niveus	4	4
Aspergillus ochraceus	2	3
Aspergillus terreus	ND[(3)]	3
Emericella nidulans	2	1
Penicillium funiculosum	1	ND[(3)]
Penicillium oxalicum	3	1

[(1)] Compost 1: sugarcane bagasse (426 kg), coast-cross hay (459 kg), wheat bran (100 kg), limestone (20 kg), gypsum (20 kg), superphosphate (10 kg) and urea (1.55 kg);
[(2)] Compost 2: cotton textile mill waste (403 kg) coast-cross hay (402 kg), wheat bran (100 kg), limestone (20 kg), gypsum (20 kg), superphosphate (10 kg) and urea (3.9 kg).
[(3)] ND: not detected.

Considering the results obtained in this work, it is recommended that workers involved in the production and compost management wear masks to protect them against *Aspergillus fumigatus* and other allergenic fungi.

Isolation and identification of different species of *Aspergillus* and *Penicillium*

Other *Aspergillus* and *Penicillium* species were isolated from the compost (Table 1) and are recognized as potential mycotoxin producer. Although these fungi are not good competitors in the compost colonization in relation to *Agaricus brasiliensis* and *A. bisporus* mushrooms and also don't cause diseases to it, their presence in the compost cultivation is important, once they can contaminate the mushrooms and stay in the final dehydrated or canned product. Therefore, it's important that these fungi be evaluated in the dehydrated mushroom, once inadequate dehydration conditions and storage can cause an increase of their contamination. Since *A. brasiliensis* mushrooms, once dried, can be stored for one or more years, it is important that they be free of potentially toxigenic fungi to guarantee final quality.

Non-sporulating fungi

Besides the species with conidia production, several isolates that didn't have any kind of sporulation in solid medium were observed. Among these isolates there can be important fungi species for enzyme production or important fungi in the composting process for the mushroom cultivation. These species must later be identified through molecular techniques, once their identification by traditional morphological techniques isn't possible.

CONCLUSION

The use of cotton textile mill waste for growing compost of *Agaricus brasiliensis* caused abnormal *Aspergillus fumigatus* spore production on the surface of the compost at the end of the pasteurization and conditioning phase.

Compost produced according to traditional formulation also showed the presence of *Aspergillus fumigatus*; however, no abnormal growth was observed.

Different species of potentially toxigenic fungi were isolated from two composts analyzed at the end of the process.

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