Vol.50, n. 4 : pp. 587-595 July 2007
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Isolation and Mycelial Growth of *Diehliomyces microsporus*: Effect of Culture Medium and Incubation Temperature

José Soares do Nascimento[1]* **and Augusto Ferreira da Eira**[2]
[1]*Universidade Federal de Pelotas; Instituto de Biologia; Departamento de Microbiologia e Parasitologia; C.P. 354; jose@ufpel.tche.br; 96010-900; Pelotas - RS - Brasil.* [2]*Universidade Estadual Paulista; FCA; Departamento de Produção Vegetal (Módulo de Cogumelos); C.P. 237; 18603-970; Botucatu - SP -Brasil*

ABSTRACT

The false truffle is one of the main problems in the production of the Agaricus brasiliensis in Brazil and the control of this fungal competitor has been rather difficult due to difficulties in the isolation and cultivation of this pathogen. This experiment was conducted in three stages, the first consisting of the isolation of Diehliomyces microsporus *starting from portions of the fruiting body and through the ascospores suspension; second,* D. microsporus *cultivated* in vitro *at 15, 20, 25, 30 and 35°C in six different culture media (CSDA, OCDA, PCDA, ODA, PDA, CDA); third,* D. microsporus *was inoculated on sterilized compost for formation of the fruiting body. The colony formation from tissue of* D. microsporus *starting from portions of fruiting body was more efficient than germination of the ascospores. Compost medium (CDA) allowed a larger diameter of the* D. microsporus *colony, followed by the medium made up of compost and potato mixture, favoring a denser composition. The largest mycelial growth speed of* D. microsporus *occurred when the culture was incubated at 28 and 30°C. Incubation temperatures lower than 15°C or above 35°C inhibited the mycelial growth of* D. microsporus *completely. The fruiting bodies were obtained easily in sterilized compost and later inoculated along with mycelial competitor.*

Key words: *Agaricus brasiliensis*, false truffle, culinary-medicinal mushrooms, culture media, mushroom disease, competitor

INTRODUCTION

Brazil is one of the main producers of the mushroom *Agaricus brasiliensis* (Murrill) S. Wasser, popularly denominated "Royal-sun-Agaricus" in Brazil and Himematsutake in Japan. The growth of this mushroom started in Brazil in the 1990s, raising great interest in the Asian and North American markets due to its medicinal properties, especially its anti-oncogenic activity. *A. brasiliensis* is grown in the hot months, especially during the spring, summer and autumn. Because it is a mushroom of hot climate and low technology systems, those conditions favor the emergence of pest, such flies, nematodes and colembolas. The bacteria and mainly the fungi act as competitors or causes of diseases, resulting in low yields (Nascimento and Eira, 2003b; Nascimento, 2003).

The problems that affect the yields of *A. brasiliensis* are countless because it is a recent culture. Lately, the appearance of false truffle has been suggested (*Diehliomyces microsporus* Diehl and Lamb. Gilkey) as a major problem for the cultivation, mainly when the appropriate technology is not used (Nascimento and Eira, 2001).

* Author for correspondence

In 2000, farmers from the Brazilian states of São Paulo and Paraná suggested the presence of structures similar to deformed origins, commonly called "popcorns", during the fruiting period. When these structures appear, *A. brasiliensis* does not produce and more severe recurrences take place in subsequent cultivations. *A*fter identification studies, the contamination was found to be caused by *D. microsporus* (Nascimento and Eira, 2001). This fungus is considered as of the most critical competitor in the cultivation of *A. brasiliensis* (Nascimento and Eira, 2003a).

The isolation and the culture conditions for cultivation of *D. microsporus* have been described by many authors. From its first isolation, accomplished by Diehl and Lambert (1930), it was deduced that oats medium and soil extract favored the mycelial growth. Zaayen and Pol-Luiten (1977 and 1978) obtained the best mycelial growth in malt and potato medium; however, this growth demonstrated an instability in the formation of the colony. There have been several studies in relation to incubation temperature. According to Bisset et al. (1982), *D. microsporus* needs high temperatures (22-30^{o}C) for germination of ascospores; however, the mycelium also grows at low temperatures (16^{o}C), but the ideal temperature for the mycelial growth is 26^{o}C. Sharma (1998) submitting the ascospores to 45^{o}C, followed by incubation at 33^{o}C, reduced the germination of ascospores significantly (4%) when compared in relation to incubation at 27^{o}C. The lowest temperature presented 70% germination after a 72 h incubation period. Thus, this research had as objectives: establish a methodology for the isolation of *D. microsporus*; to study the cultivation conditions for *D. microsporus* (medium and temperatures) and to obtain ascostroms of *D. microsporus* on sterilized composed.

MATERIAL AND METHODS

Isolation of *D. microsporus*

The ascostroms of the false truffle (competitor fungus) were collected from *A. brasiliensis* cultivations. In aseptic conditions, the fungus was isolated on PDA and CDA media. For obtaining PDA medium, 150g potato was added to 1L distilled water and then boiled for 15 minutes. The medium was filtered in gauze and 10g liquid dextrose, 15g agar was added and the volume for 1L was completed with distilled water. This medium was sterilized at 121^{o}C for 20 minutes. The same procedure was followed with CDA medium, in which 20g of dry and ground compost prepared for the cultivation of *A. brasiliensis* was used. The culture media were poured in to Petri dishes (90mm x 15mm) (15mL/plate).

The treatments consisted of two different ways of *D. microsporus* isolation with 10 replications. For both, ascostroms were used within 24h after collection. For the first isolation form, the ascostroms were opened manually in aseptic conditions to remove the inner mass with a nickel-chrome needle in the shape of an "L" and transferred to a central surface medium. The other isolation form consisted of softening the inner mass of several fruiting bodies, which was thereupon diluted in sterile water. The suspension was filtered throng sterilized cotton wool to remove asci and mycelial fragments, thus obtaining the ascospores. The suspension of spores previously prepared was on the surface of the medium, at a concentration of 4.8 x 10^{3} ascospores mL^{-1}. Incubation was performed in Petri dishes at 25^{o}C in the absence of light for 12 days. The evaluation consisted of estimating the number of colonies formed by *D. microsporus* and the index of contamination for other microorganisms.

In vitro cultivation condition for *D. microsporus*

A culture of *D. microsporus* (DMI 00/01) was multiplied in other culture media (Table 1) on Petri dishes (90mm x 15mm). These culture media (Table 1) were prepared from the extracts. After distribution the media in Petri dishes, the culture was transferred and the plates were incubated at 25^{o}C. After mycelial growth, 0.5cm diameter culture discs were transferred to the center of other Petri dishes which contained the same culture medium and incubated according to the treatments. The treatments consisted of the interaction of the temperature (15, 20, 25, 30 and 35^{o}C) and the culture medium (Table 1) in a completely randomized factorial design with seven replications. The evaluation consisted of daily measurements of mycelium growth (colony diameter) of *D. microsporus*.

Cultivation of *D. microsporus* on sterilized compost

Parts of the mycelium obtained after the isolation of *D. microsporus* were transferred to sterilized compost and arranged in flasks with 250g of humid compost in order to be cultivated until

fruiting bodies production in 10 replications. The top of the flasks was sealed with cotton to enable gaseous changes with the environment and incubated at 25°C in the absence of light for 12 days. After the mycelial growth, the fruiting bodies of *D. microsporus* were observed in an optical microscope (in sections stained with lactofenol and Amann's blue).

Table 1 - Composition of medium used on *Diehliomyces microsporus* cultivation *in vitro*.

Medium	Composition
CSDA	10g compost (after phase II) and 10g soil were added to 1L distilled water, boiled for 15min and, to the resulting liquid extract, 10g dextrose and 15g agar were added. After that, the volume was made 1L was completed with distilled water. The medium was sterilized at 121°C for 30 minutes.
OCDA	10g oat and 10g of compost were added to 1L distilled water, boiled for 15min and 10g dextrose and 15g agar were added. The volume was then made 1L with distilled water. The medium was sterilized at 121°C for 30min.
ODA	20g of oat was added in 1L distilled water, boiled for 15min and 10g dextrose and 15g agar were added to the liquid extract. Subsequently, the volume of 1L was made with distilled water. The medium was sterilized at 121°C for 30min.
PDA	150g chopped potato was added to a liter of distilled water, boiled for 15min and 10g dextrose and 15g agar were added to the liquid extract. Then the volume for 1L was made with distilled water. The medium was sterilized at 121°C for 30min.
PCDA	75g chopped potato and 10g compost were added to 1L distilled water, boiled for 15min and 10g dextrose and 15g agar were added to the liquid extract. After, the volume for 1L was made with distilled water. The medium was sterilized at 121°C for 30min.
CDA	20g compost was added to 1L distilled water, boiled for 15min and 10g dextrose and 15g agar were added to liquid extract. The volume for 1L was made with distilled water. The medium was sterilized at 121°C for 30min.

RESULTS AND DISCUSSION

Isolation of *D. microsporus*

False truffle contaminations became evident during the cultivation of *A. brasiliensis* by the formation of ascostroma of *D. microsporus.* Usually signs of the false truffle were observed in the final phase of mycelial growth or more commonly during the cropping of the mushrooms (Fig. 1). The young ascostroma had firmer texture and when they ripened they were fragile and desintegrate. At this point, spores were disseminated through air.

When the ascospores were separated from the ascostroma, they had a low percentage of germination and when the isolation was made starting from portions of the recently picked young ascostrom, there was a largest colony formation (Table 2). In view of these results, it was believed that the colony formed the *D. microsporus* fruiting bodies were due to vegetative propagation (asexual reproduction). In this type of isolation, contamination also occurred and the results were not always positive, because it depended on the life time of ascostroma and on casing soil and compost that were involved during their formation. The ascostroma is kidney-shaped and formed by anastomosis, keeping the incoming particles from the external casing soil. Also, during the growth phase of ascostroma, those closer are joined, forming a larger structure. When the ascostroma were washed with either water or antiseptic, they became more fragile for manipulation and the propagation rate decreased. Therefore, in this experiment, the result of isolation was more satisfactory when the ascostroma had been recently picked and not washed, taking portions of the ascostroma and transferring them into the compost medium (CDA).

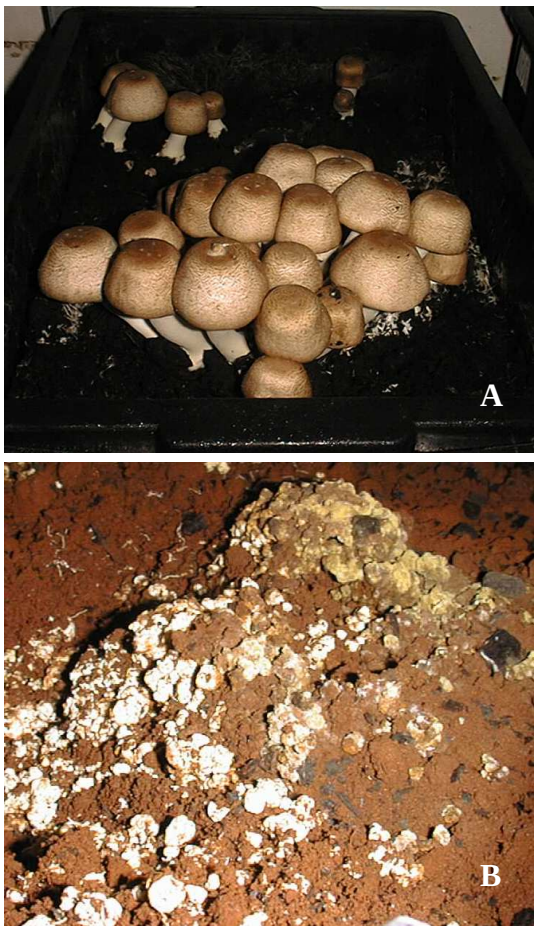

Figure 1 - Fruiting bodies of the *Agaricus brasiliensis* (A) and *Diehliomyces microsporus* (B) in Brazil.

Table 2 - Formation of colonies and contaminations starting from *Diehliomyces microsporus* (DMI) isolation *in vitro*

Treatments	Colony formed (DMI)	Contamination (%)	No growth
Ascostroma portion	60	10	30
Ascospores suspension	20	30	50

In vitro* cultivation conditions of the *D. microsporus

The average mycelial growth data of *in vitro* cultivation the *D. microsporus* in different culture media and temperatures are shown in Table 3. At 15 and 35°C, there was no colony formation in any of the culture media tested. The incubation at 20, 25 and 30°C on the CDA media (compost based) provided a significantly larger diameter of the colony than any other media. This referred mainly to the starch based ones (ODA and PDA), in which the growth had its peak at 30°C. The mixed media, made up of the mixture of compost with other such materials as oats, soil or potato favored the mycelial growth of *D. microsporus*. On the other hand, at 20, 25 and 30°C, the culture media that favored the largest growth, such as CDA, CSDA and OCDA yielded a less dense mycelium, with predominance of fluffy mycelium, while PCDA favored the mycelial occurrence with

largest density (visualized, but not quantified data) and a colony with a smaller diameter.

Diehl and Lambert (1930) tested several combinations of media from wheat, oat, rice, corn, soil extract to manure extract in the cultivation of *D. microsporus*, obtaining better growth for oat and soil extract based media. That was partly different from the results obtained in this experiment, because the oat based media presented a significantly smaller mycelial growth. However, when testing several culture media for mycelial growth of *D. microsporus*, Zaayen and Pol-Luiten (1977) obtained better regular growth in the malt-agar and PDA media. Nevertheless, in most cases, there was formation of sector and fluffy mycelium, what made the measurements difficult. In the mixed medium made up of potato and compost for *A. brasiliensis*, this did not happen and there was a typical radial growth.

A critical point in the cultivation of *A. brasiliensis*, in relation to *D. microsporus* was that both presented faster growth in the same temperature range, differently from the *A. bisporus*, in which the temperature could drop below 18°C during the body fruiting phase, limiting the progression of the false truffle in this phase of the cultivation.

The diameter of the colony of *D. microsporus* in the several culture media and incubation temperature can be expressed through a sixth degree polynomial equation, with a peak at around of 30°C (Fig. 2).

The CDA media yielded a largest peak of mycelial growth of the *D. microsporus*, as showed in Table 3. In all the media, the null diameter at 15°C increased gradually according to a temperature increase from 20 to 25°C. It reached its maximum peak at around 30°C, after which it decreased quickly when incubated at 35°C, when the colony diameter was null again. Different results were presented by Zaayen and Pol-Luiten (1977), who obtained maximum growth of *D. microsporus* at 26°C. The estimated temperatures by the regression equation would result in the maximum peaks of colony diameter in the respective media are: 29.7°C (CSDA), 31.0°C (PCDA), 28.5°C (OCDA), 29.3°C (CDA), 30.8°C (PDA) and 29.8°C (ODA).

Table 3 - Colony diameter (cm) of *Diehliomyces microsporus* cultivated in different culture media *in vitro* and incubated at temperatures of 15, 20, 25, 30, 35°C for 7 days.

Culture medium	**Incubation temperature, °C**				
	15	20	25	30	35
CSDA	0 a	1.06 c	2.03 b	3.35 b	0 a
PCDA	0 a	0.73 d	1.98 b	2.64 c	0 a
OCDA	0 a	1.54 b	2.61 a	3.35 b	0 a
CDA	0 a	2.50 a	2.68 a	3.82 a	0 a
PDA	0 a	0.64 ed	1.34 c	2.38 cd	0 a
ODA	0 a	0.33 e	1.59 c	2.33 d	0 a
CV%	-	17.69	8.63	6.29	-
DMS	0	0.32	0.28	0.3	0

Tukey's test: means followed by different letters in the column are significantly different at 5%.

CSDA: compost-soil-dextrose-agar; PCDA: potato-compost-dextrose-agar; OCDA: oat-compost-dextrose-agar; CDA: compost-dextrose-agar; PDA: potato-dextrose-agar; ODA: oat-dextrose-agar.

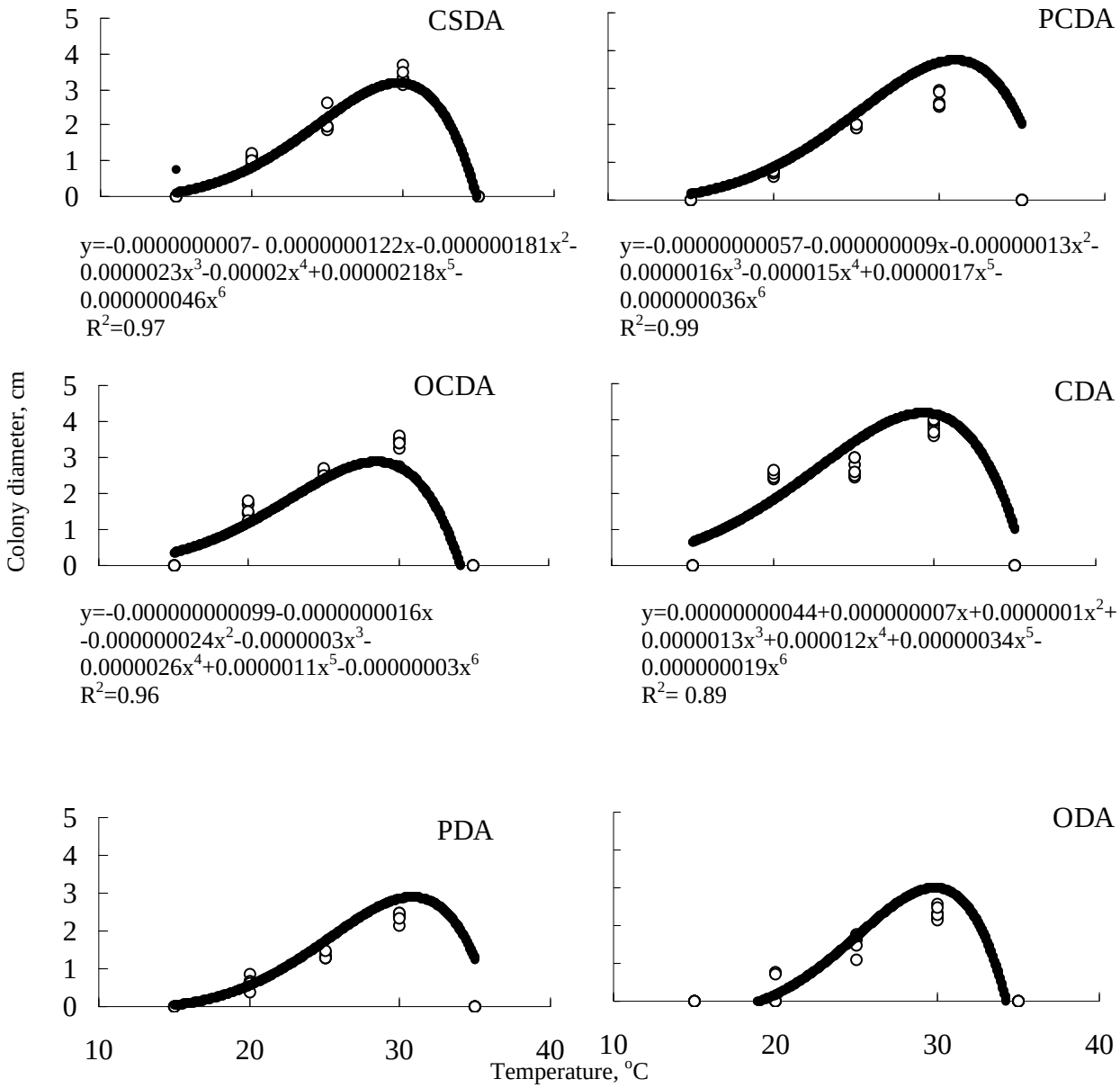

Figure 2 - Colony diameter of *Diehliomyces microsporus* in different *in vitro* culture media incubated at temperatures of 15, 20, 25, 30, 35°C for 7 days.
CSDA: compost-soil-dextrose-agar; PCDA: potato-compost-dextrose-agar; OCDA: oat-compost-dextrose-agar; CDA: compost-dextrose-agar; PDA: potato-dextrose-agar; ODA: oat-dextrose-agar.

Cultivation of *D. microsporus* in sterilized compost

The fruiting bodies of *D. microsporus* were checked approximately 30 days after the inoculation of the prepared compost for the cultivation of *A. brasiliensis*. This medium was sterilized and inoculated with the competitor only (Fig. 3A). The fruiting bodies of the false truffle were shown to be similar to a great amount of deformed pin or "popcorn-like" structures, as it was called by the growers.

Biotic and abiotic factors such as temperature, humidity, presence of the host or other microorganisms, are still not certain. They can favor the fruiting bodies of the false truffle in the cultivation of mushrooms or even the ones that they can contribute in the performance of the fungus as a parasite in the system.

When the *D. microsporus* was inoculated in the sterilized compost, the fruiting bodies of *D. microsporus* occurred independently of the presence of the *A. brasiliensis* (Fig. 3A). Parallel to this, during the experiment, the farmers of *A. brasiliensis* observed ascostroma of the false truffle before placement of the casing layer (Fig. 3B), and the most significant incidence of the ascostroma occurred during, or after the initial phase of fruiting bodies of *A. brasiliensis.* When grown in transparent plastic bags, a regular procedure in mushroom farms, the largest ascostroma concentration was observed at the edge of the plastic. Initially, in the surface of the casing layer, there appear cracks where a lot of the ascostroma are concentrated in the interface compost/casing, usually with a decrease or absence of production of *A. brasiliensis* (Fig. 3C). These observations also apply to the cultivation of *A. bisporus* and *A. bitorquis*, according to Kligman (1944), Zaayen and Pol-Luiten (1978), Sharma (1998).

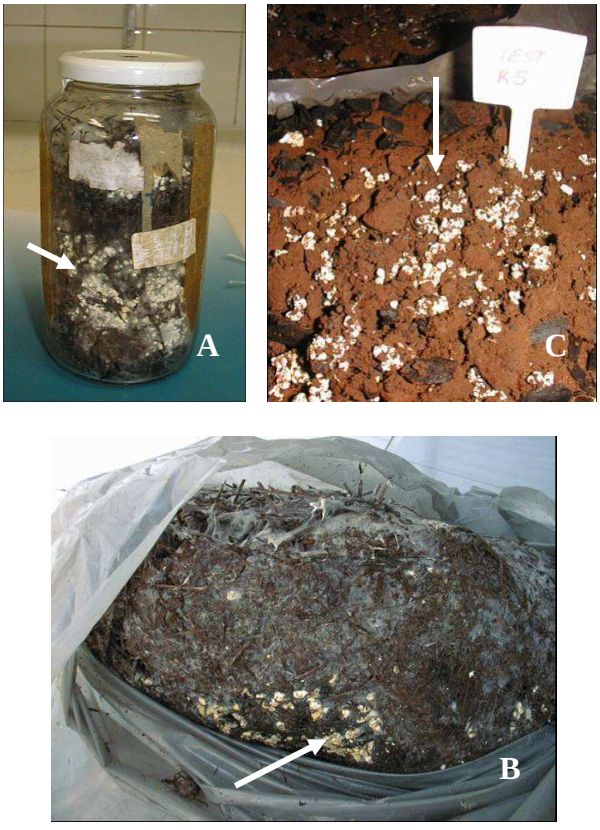

Figure 3 - Fruiting bodies of *Diehliomyces microsporus* (indicated by the arrows) with material incubated at the laboratory and supplied by the farmers of *Agaricus brasiliensis* (bags). (A) ascostroma in sterilized compost incubated at 28°C for 35 days; (B) fruiting bodies in the compost before the casing with approximately 30 days; (C) superficial blooming of the ascostroma in the casing soil.

The ascostroma of *D. microsporus* presented variations in size from 5 to 30mm in diameter, had cephalic forms, initially from a yellowish-white coloration to brown in the maturation phase (Fig. 4A). When they grew very closely some ascostroma joined, forming larger and deformed structures (Fig. 4B). These characteristics confirmed the observations made by Diehl and Lambert (1930), Kligman (1944), Zaayen and Pol-Luiten (1977), Wood and Fletcher (1991), Yadav et al. (2000), during the cultivations of *A. bisporus* and *A. bitorquis.*

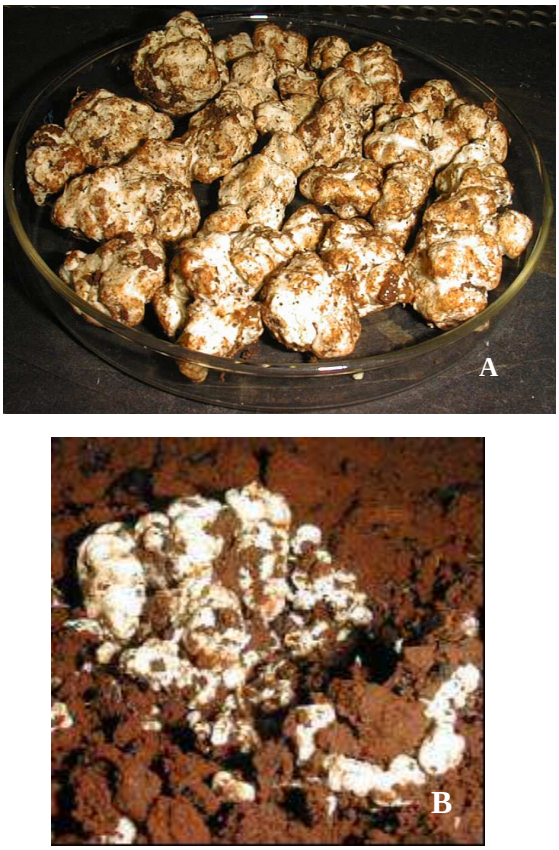

Figure 4 - Ascostroma of *Diehlioyces microsporus*: (A): ascostroma collected in Petri dish; (B) ascostroma union on casing soil.

CONCLUSIONS

The isolation of *D. microsporus* from fragments of recently collected ascostroma was more efficient than the germination of the ascospores. The culture media made from a compost base (CDA) provided largest colony diameter of the *D. microsporus*, followed by the media made up of the compost and potato mixture, favoring a denser mycelia. The maximum growth speed of *D. microsporus* occurred when the culture was incubated between 28 and 30^{o}C. Low (15^{o}C) or high (35^{o}C) incubation temperatures completely inhibited the mycelial growth of *D. microsporus*. The fruiting bodies of *D. microsporus* occurred before the placement of the casing layer, and during the phase of fruiting bodies of *A. brasiliensis*.

ACKNOWLEDGMENTS

FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) supported this research.

RESUMO

A falsa trufa está sendo um dos principais problemas na produção do *Agaricus brasiliensis* cultivado no Brasil e o controle deste fungo competidor tem sido difícil, devido às dificuldades encontradas no isolamento e cultivo do patógeno. Este experimento foi conduzido em três etapas, sendo a primeira constituída pelo isolamento de *Diehliomyces microsporus* a partir de porções do ascostroma e através da suspensão de ascósporos; a segunda, o cultivo *in vitro* de *D. microsporus* nas temperaturas de 15, 20, 25, 30 e 35^{o}C e em seis meios de cultura (CTDA, ACDA, BCDA, ADA, BDA e CDA) e a terceira pela inoculação de *D. microsporus* no composto (pasteurizado, composto esterilizado e composto esterilizado com camada de cobertura) para formação dos ascostromas. O isolamento de *D. microsporus* a partir de fragmentos do ascostroma recém coletado foi mais eficiente do que a germinação dos ascósporos; o meio de cultura à base de composto (CDA) proporcionou maior diâmetro da colônia de *D. microsporus*, seguido pelo meio constituído da mistura de composto e batata, favorecendo um micélio mais denso; a maior velocidade de crescimento de *D. microsporus* ocorreu quando a cultura foi incubada entre 28 e 30^{o}C; temperaturas de incubação menor que15^{o}C ou a acima de 35^{o}C inibiu completamente o crescimento micelial de *D. microsporus*; a obtenção de frutificação de *D. microsporus* foi facilmente obtida em composto esterilizado e posteriormente inoculado com o competidor.

REFERENCES

Bisset, P.G., Colhoun, J., Gandy, D.G. 1982. Germination of *Diehliomyces microsporus* ascospores and determination of their thermal death point. *Transation of the British Mycological Society*, 78, 3, 540-542.

Diehl, W.W., Lambert, E.B., 1930. A new truffle in beds of cultivated mushrooms. *Mycologia*, 22, 223-226.

Kligman, A.M. 1944. Conthol of the truffle in beds of the cultivated mushroom. *Phytopathology*, 34, 376-384.

Nascimento, J.S. 2003. Etiologia, controle e demanda de energia na prevenção da falsa trufa (*Diehliomyces microsporus*) em cultivos de *Agaricus blazei* Botucatu, 115p. *Tese* (Doutorado em Agronomia/Energia na Agricultura) - Faculdade de Ciências Agronômicas, Universidade Estadual Paulista.

Nascimento, J.S., Eira, A.F. 2001. Ocorrência e prejuízos da falsa trufa (*Diehliomyces microsporus*) em cultivo do *Agaricus blazei* Murrill. Congresso Brasileiro de Micologia, 3, Águas de Lindóia. *Resumos...* São Paulo: Sociedade Brasileira de Micologia, 2001. p.37.

Nascimento, J.S., Eira, A.F. 2003a. Occurrence of false truffle (*Diehliomyces microsporus* Gilkey) and damage on the Himematsutake medicinal mushroom (*Agaricus brasiliensis* S. Wasser et al.). *Int. J. Med. Mushr.*, 5, 1, 87-94.

Nascimento, J.S., Eira, A.F. 2003b. Doenças e competidores do *Agaricus blazei*. In: Eira, A.F. *cultivo do "cogumelo-do-sol" (Agaricus blazei (Murrill) ss. Heinemann)*. Viçosa: Aprenda Fácil, p.221-249.

Sharma, V.P. 1998. Biology and management of false truffle (*Diehliomyces microsporus*) during cultivation of *Agaricus* spp. *Mushr. Res.*, 7, 1, 1-12.

Zaayen, A. Van, Pol-Luiten, B. Van Der. 1977. Heat resistance, biology and prevention of *Diehliomyces microsporus* in crops of Agaricus species. *J. Pl. Path.*, 83, 221-240.

Zaayen, A. Van, Pol-Luiten, B. Van Der. 1978. Heat resistance, some biological aspects and prevention of false truffle (*Diehliomyces microsporus*). In: Maher, M.J. (Ed.) *Science and cultivation of edible fungi.* Rotterdam: Balkema, 2, 319-336.

Wood, M.W., Fletcher, J.T. 1991. The occurrence of ascocarps of *Diehliomyces microsporus*, the cause of false truffle disease. *Mushrooms Science XII*, 1, 379-384.

Yadav, M.C., Dhar, B.L., Verma, R. N. 2000. Breeding studies on development of high yielding and quality hybrids of *Agaricus bitorquis*. *Mushrooms Science XV*, 1, 299-304.

Received: October 10, 2005;
Revised: April 20, 2006;
Accepted: March 20, 2007.

PÁGINA
EM
BRANCO