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Effect of bacteria associated with mushroom compost and casing materials on basidiomata formation in *Agaricus bisporus*

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The pattern of bacterial colonization of mushroom compost and casing layers and the effect of bacteria on initiation of basidiocarps of *A. bisporus* (= *A. brunnescens*) were determined. After spawning, higher bacterial populations were found in the upper layers of the mushroom compost than at lower depths. Bacterial populations were lower in the compost than in the casing. Addition of spawned compost to the casing layer resulted in a significant increase in bacterial populations, in the numbers of basidiocarps formed, and in the numbers of fruitbodies harvested compared to unamended casing. Some of the bacteria that inhibited the vegetative growth of mushroom mycelium in culture also induced strand development and basidiocarp formation. Initiation of basidiocarps was significantly higher when some of the above bacteria were added to the casing.

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L'évolution de la colonisation bactérienne du compost et des couches du matériel de gobetage ainsi que l'influence de bactéries spécifiques sur l'initiation des basidiocarpes d'*Agaricus bisporus* (= *A. brunnescens*) ont été établies. Suite à l'ensemencement, des populations bactériennes plus élevées ont été dénombrées dans les couches supérieures du compost comparativement aux couches plus profondes. Les populations bactériennes étaient moindres dans le compost que dans le matériel de gobetage. L'addition de compost ensemencé au matériel de gobetage a résulté en une augmentation significative des populations bactériennes, du nombre de basidiocarpes formées et de fructifications récoltées par rapport à un matériel de gobetage non traité. Quelques-unes des bactéries inhibant en culture la croissance végétative du champignon provoquent également la stratification du mycélium et la formation de basidiocarpes. L'initiation de basidiocarpes était significativement plus importante lorsque certaines des bactéries précitées étaient ajoutées au matériel de gobetage.

Fungal sporulation and the factors that modify the process are of vital importance in plant disease epidemiology. There are marked differences among the various pathogenic fungi in the conditions required for sporulation and many factors, internal and external, appear to be involved (Cochrane 1958). Some fungi sporulate readily in nature (and in culture) while others do so sparingly and only after certain specific combinations of environmental factors (Cochrane 1958). Often the same organism may remain in the vegetative phase for a longer or shorter period of time depending on the external environment. There is no universal set of conditions which lead to fructification in all fungi (Cochrane 1958). Of the many factors that appear to regulate fungal reproduction, temperature, moisture, light, and other abiotic factors have received most attention (Cochrane 1958, Dahlberg & Van Eten 1982). Effects of biological factors, especially the role of microorganisms in regulating fungal reproduction, has been less studied even though in nature microbial associations and interactions are virtually universal. That microorganisms may play a role in regulating pathogen reproduction has been reported for *Rhizoctonia solani* (Stretton et al. 1964), *Alternaria* sp., *Helminthosporium* sp., and other fungi (Lilly & Barnett 1951). An apparently essential role of microorganisms in basidiomata initiation has been postulated for the commercial mushroom, *Agaricus brunnescens* Peck, better known as *A. bisporus* (Lange) Imbach (Eger 1972, Flegg et al. 1985).

In commercial mushroom cultivation, experience has shown that to induce mushrooms to form in any quantity it is necessary to cover the vegetative growth of *A. bisporus*, which takes place in the compost (consisting of composted straw, horse dung and other plant derived materials), with a layer of soil, neutralized peat moss, ground calcium carbonate, or various combinations of similar materials. The application of these materials, termed the casing layer, is essential for initiation of the basidiomata (Eger 1972, Flegg et al. 1985). Studies have shown that some of the bacteria that were associated with the compost and casing materials exerted significant effects on growth and basidiocarp initiation of the mushroom (Eger 1972, Hayes 1981, Ingratta & Patrick 1987). For example, *A. bisporus* grown axenically on agar media or on the regular compost substrates continued its vegetative growth without forming

basidiocarps (Eger 1972, Hayes 1981, Ingratta & Patrick 1987, Park & Agnihotri 1969 a,b; Peerally 1979). Fruiting occurred, however, if axenic conditions were not maintained and the casing layer was allowed to become recolonized by microorganisms (Eger 1972, Wood 1976). Although, the precise role of microorganisms in basidiocarp initiation in *A. bisporus* is not clear and is somewhat controversial (Flegg et al. 1985, Sinden 1982, Tschierpe 1973, Visscher 1978), the findings are intriguing and relevant to plant pathology since microbial associations are also part of the environment of pathogenic fungi in nature. More information on the involvement of microorganisms in fungal fructification is needed and is of considerable theoretical and practical importance.

The overall objectives of the present studies were to identify some of the complex microbial interrelationships that occur in mushroom growing substrates and determine their possible role in regulating fructification. Specifically, the general pattern of bacterial populations associated with the compost at varying depths and during different stages of growth of *A. bisporus* were examined. We also studied the bacterial populations in the casing layer and in peat moss and ground calcium carbonate, the two major components of the casing layer. Some bacteria were isolated and their influence on the vegetative growth and basidiocarp initiation of various commercial strains of *A. bisporus* were determined.

Materials and methods

Experimental growth facilities for mushroom production. Some of the experiments were conducted at the mushroom research facility (MRF) of the Horticultural Research Institute of Ontario, Vineland Station, where semi-commercial facilities exist. The facility at MRF has four production rooms, each containing 50 trays (0.61 × 0.61 × 0.15 m) with independently controlled environments. The laboratory petri dish and pot trials were conducted in the Botany Department, University of Toronto. Pot and petri dish trials were conducted in growth cabinets with temperature and humidity controls (Model 1-35 LLV1, Percival, Boone, Iowa, USA). The trays and pots used were capable of holding 23 kg, and 450 g of spawned compost, respectively. The compost was a horse manure-wheat straw-based substrate commonly used by the commercial mushroom industry. The moisture content of the compost at spawning was 65-70%. The compost was spawned with a hybrid strain of *A. bisporus* (C-4, Canadian Spawn and Supply, Richmond Hill, Ontario). The spawned compost was placed in trays or pots as required in the tests. For vegetative mycelial development (spawn growth), temperature was maintained at 24-25°C and relative humidity at 90-95% in the dark. The spawned compost was covered with a polyethylene sheet and the containers were watered regularly to create optimum conditions required for mycelial growth. After 14 days growth the colonized compost was covered with a 3-5 cm casing layer of neutralized peat moss to provide a suitable environment for fruiting. The casing layer usually consists of sphagnum peat and ground calcium carbonate (lime) mixed in a ratio of 1:1 on dry weight basis to give pH 7.5. Water is applied to the casing layer to its maximum water holding capacity. After application of the casing layer and after the mycelium had reached the surface of the casing, the room was ventilated, the temperature was lowered to 18°C, and full ventilation was maintained until basidiocarps appeared.

Harvesting of basidiocarps usually commenced 19-24 days after application of the casing layer. There were four replications per treatment in a given experiment, and each experiment was repeated at least two times unless otherwise noted.

Enumeration of bacterial populations. Three series of experiments were conducted to determine levels of bacterial populations associated with the compost, peat moss, calcium carbonate, casing layer, and mushroom mycelium in the appropriate trays or pots during the experiments.

Experiment 1 — Pot trials were conducted in controlled growth cabinets, as described earlier. The bacterial populations in the spawned compost at varying depths were determined. The samples were collected at 7-day intervals up to 56 days after spawning at three depths (bottom, middle and top) in a 15 cm diam pot.

Experiment 2 — The bacterial populations in the two components of the casing layer, namely peat moss and ground calcium carbonate, were determined in the dry state before mixing. The peat moss and calcium carbonate were then mixed in a ratio of 1:1 (w/w) on a dry weight basis, watered to reach maximum water holding capacity, and bacterial populations in the prepared casing soil were determined immediately after preparation. The proportions of Gram-positive and Gram-negative bacteria were estimated by testing 20-50 colonies selected at random from each replication as described by Suslow et al. (1982).

Experiment 3 — The changes that occurred in bacterial populations in the compost during the spawn run were determined. Also, the bacterial populations in the casing layer amended with spawned compost as practised in commercial

mushroom culture, and in casing without the added spawn were determined. The bacteria associated with the vegetative mushroom mycelium also were determined. Samples of the mycelium were obtained from a) spawned compost at day 14 after spawning, b) casing layer at day 10 after casing, and c) casing layer at day 21 after casing.

The method used in the enumeration of the bacterial populations associated with the above treatments was the standard dilution plate technique (Johnson & Curl 1972). Samples of compost, casing with or without spawned compost, peat moss, calcium carbonate, and mycelium were collected from the replicated treatments using at least five random core samples (1 cm diameter) per treatment which were then consolidated into one composite sample from each replication. All samples were processed within 48 h of sampling and were plated onto the surface of previously solidified Thornton's agar (TA) (Johnson & Curl 1972) in triplicate. The plates were incubated at 25°C and the bacterial colonies enumerated after 3-4 days. All counts were converted to colony forming units (CFU) per gram dry weight (g/DWT) of the original sample.

In the mycelial samples, approximately 0.5 g (fresh weight) of mycelium from each replicate treatment was placed into a 250 mL flask containing 10 mL sterile 0.1 M $MgSO_4$, the bacteria were dislodged by agitation for 20 min and populations determined by the procedure described above. Colony counts were recorded as CFU/g fresh weight (FWT) of mycelium.

Isolation of predominant bacterial groups. Random isolations were made of representative colony types present in greatest numbers on TA plates. The selected colonies were streaked onto fresh potato dextrose agar (PDA) or nutrient agar (NA), incubated at 25°C and checked for purity. All the isolates were maintained on PDA or NA prior to characterization. Gram-staining reactions, morphology, motility, and fluorescent reaction were tested by the standard methods (Schaad 1980). Pure cultures of the isolates were tested on King's B media (King et al. 1954) to determine their fluorescence capability. The fluorescence was determined under a Black-Ray lamp (Model UVL-21, UV Products, San Gabriel, California, USA). The organisms were grouped according to source and colony morphology. No attempts were made to identify them to genus or species. An isolate of *Pseudomonas putida* GR12-2, obtained from Allelix Inc., Mississauga, Ontario, was also included in the tests. Various isolates of *P. putida* have been associated with mushroom cultivation and shown to have a stimulatory influence on basidiocarp formation in *A. bisporus*.

Influence of bacterial isolates on growth and basidiocarp formation in commercial strains of *A. bisporus*. The effect of selected bacterial isolates on growth and basidiocarp formation were conducted on four different commercial strains of *A. bisporus*. The strains are designated as C-4, Campbell, C-2, and M-1 (Canadian Spawn and Supply Co.). For the tests 5-mm-diam cores of mycelium were placed in the centre of a previously solidified PDA plate (100 × 15 mm). The cores were taken from the edge of an actively growing colony of *A. bisporus* with a No. 2 cork borer. Two 3.5 cm long streaks of the bacterium were made 2 cm apart on two sides of the fungal mycelium plug. Plates were incubated in the dark for up to 15 days at 25° C, and the fungal radial growth determined and compared to the controls, which were not challenged by the bacterial isolates. Tests with each bacterial isolate and each strain of *A. bisporus* were replicated five times and repeated twice.

Laboratory tests were also conducted to determine whether any of the above bacterial isolates influenced basidiocarp formation under more controlled conditions. The methods were similar to those designated as "Halbschalentest" by Eger (1972). The experiments were conducted in standard glass petri dishes (100 × 15 mm, Pyrex brand, Canlab). Half the lower part of each petri dish was filled with approximately 15 g of the compost and sterilized for 1 h at 121°C on each of 3 consecutive days. After cooling, the plates were inoculated with each of the four mushroom strains (2-3 spawn grains per plate) and were placed in a temperature and humidity controlled cabinet in the dark. After spawning, the moisture content of the compost was 65-70%, temperature was maintained at 24-25° C and relative humidity in the cabinet was 90-95%. After 14 days of vegetative growth, sterilized casing (approximately 15 g) was placed in contact with the compost. Sterile casing material was obtained by autoclaving the casing in polypropylene autoclavable bags (20 × 30 cm) (Bel-Art Products, CanLab, Division of McGraw Supply Ltd.) for 1 h on each of 3 consecutive days. No microorganisms were detected when the autoclaved casing was plated onto PDA plates. Cultures of bacterial isolates for the tests were prepared by growing them in 1000 mL flasks containing 500 mL of sterilized nutrient broth on a reciprocating mechanical shaker for 72 h at 24-25°C. The culture was then centrifuged for 20 min at 2500 rpm to attain a bacterial pellet. The pellet was washed with sterile distilled water (SDW) and suspended in sterilized 0.1 M $MgSO_4$. The suspensions were diluted to give 10^8-10^9 cells/mL and were sprayed uniformly at the rate of 10 mL/

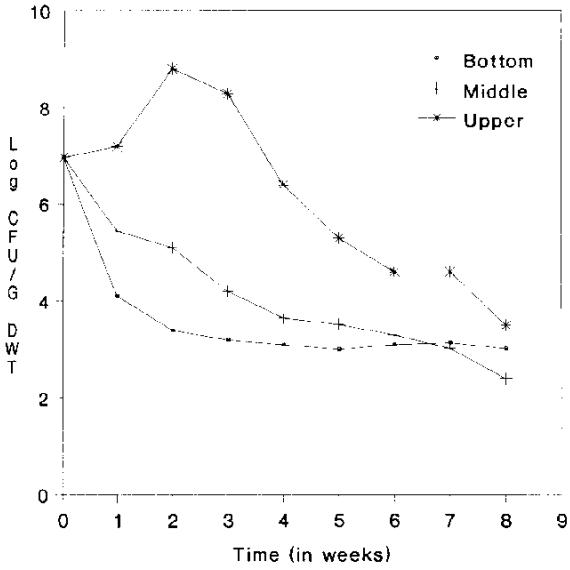

Figure 1. General pattern of bacterial populations associated with the compost at varying depths during spawn run in pot trials conducted in experiment 1.

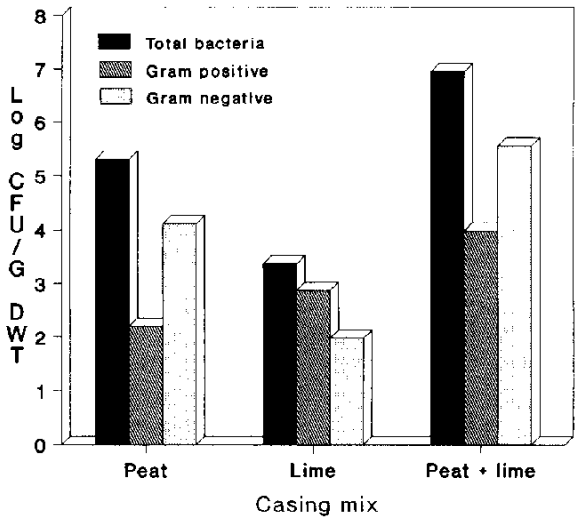

Figure 3. Populations of general bacterial types associated with peat moss and ground calcium carbonate (lime), the two major components of the casing layer in experiment 2.

plate over the surface of the casing in each plate. Comparisons were made using nonsterilized and sterilized casing treatments without the addition of the test bacterial suspension. There were 10 plates for each treatment for each mushroom strain. The experiment was repeated three times. The effects on fruiting were recorded 25 days after casing.

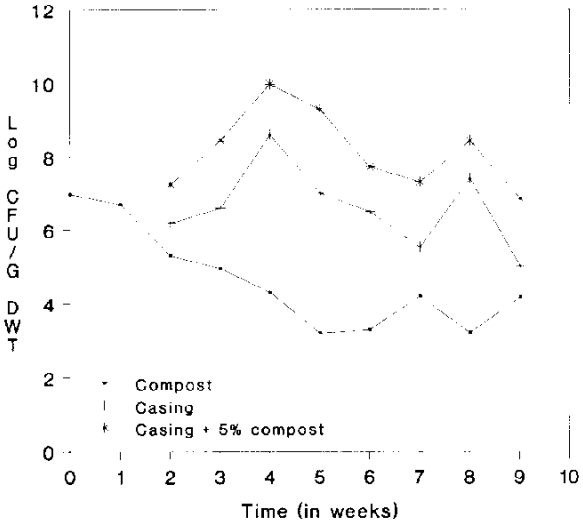

Figure 2. General pattern of bacterial populations associated with the compost during spawn run and in the casing layer nonamended or amended with spawned compost during different stages of growth of *Agaricus bisporus* in experiment 3.

Results

Enumeration of bacterial populations. Bacterial populations in the bottom, middle, and upper zones of the spawned mushroom compost during a cropping cycle were estimated at 7-day intervals up to 56 days after spawning (Fig. 1). The initial bacterial population in the compost immediately after spawning averaged log 6.98 CFU/g DWT of the compost (zero day). As the spawn continued to grow in the compost, the bacterial populations in the bottom and middle zones declined, starting at about day 7, and continued to fall. The bacterial numbers in the top layers of compost increased slightly from day 7 to day 21, then started to decline from day 28 to the end of the experiment. However, the bacterial population remained higher in the top layer than in the bottom or middle layers. There were generally higher bacterial populations in the upper parts of the compost than the middle or bottom layers. Peak bacterial activity occurred in the top layers approximately 14 days after spawning.

The bacterial populations in the compost and the casing layer are shown in Fig. 2. The effects of adding spawned compost to the casing layer are also shown from initial spawning to the time of sporophore initiation and fruit development. As shown in Fig. 2, the bacterial populations associated with the compost were significantly ($P = 0.05$) lower compared to those in the casing layer. Bacterial populations in the casing were significantly higher when spawned compost was

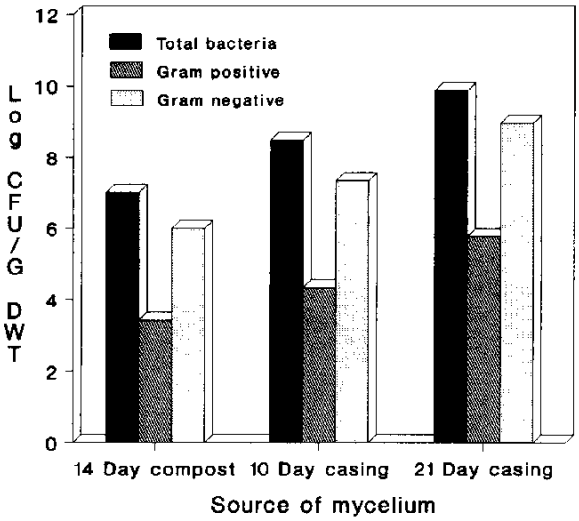

Figure 4. General pattern of bacterial types present in immediate vicinity of vegetative mycelium of *Agaricus bisporus* at various stages.

added to the casing. There were two peaks of bacterial activity in the casing layer, one at approximately 14 days and the other at 42 days following the application of casing.

The bacterial populations associated with the peat and calcium carbonate before and after mixing are shown in Fig. 3. There were log 5.31 CFU/G DWT bacteria in the peat moss and 3.37 CFU/g DWT in the calcium carbonate (lime). In the peat moss and calcium carbonate, respectively, 36% and 64% of the bacteria were Gram-positive. When the two components of casing were mixed and wetted to 80% moisture holding capacity, bacterial populations determined 2 days after preparation were log 6.73 CFU/g soil; 25% were Gram-positive.

The bacterial populations associated with mushroom mycelium in the compost 14 days after spawning were log 7.02 CFU/g fresh weight of mycelium (Fig. 4). In the casing the populations were log 8.47 and log 9.61 CFU/g FWT of mycelium at 10 and 21 days after casing. Significantly ($P = 0.05$) higher numbers of bacteria were recovered from the mushroom mycelium that grew in the casing layer than in the compost 14 days after spawning (Fig. 4). Gram-negative bacteria were found most frequently in the vicinity of the mycelium.

Characterization of the predominant bacterial isolates and their effect on mushroom growth and basidiocarp formation. The predominant bacterial types observed on the dilution plates that were isolated from the compost, casing, and mycelium are summarized in Table 1. Five of the eight isolates were rod shaped, the others were coccoid. The rod shaped isolates produced a brilliant green fluorescence on King's B medium (King et al. 1954), indicating that they belong to the fluorescent *Pseudomonas* group. Their influence on radial growth of different strains of *A. bisporus* was determined and presented in Table 2. In general, radial expansion of mycelial growth was reduced by the various isolates in petri plate culture tests.

The results in Table 3 summarize the experiments with nine bacterial isolates that were tested for stimulatory effects on basidiocarp initiation on four strains of *A. bisporus*. As shown, different mushroom strains respond differently to the same bacterial isolate. Basidiocarp initials were visible in all casing treatments to which bacteria had been applied. They occurred 16-22 days after casing. In general, addition of bacterial suspensions to the sterilized casing layer were inhibitory to the growth of mushroom mycelium but also induced strand development and stimulated basidiocarp initiation.

Table 1. Predominant bacterial types observed on dilution plates that were prepared from mushroom compost, casing, and mycelium

Bacterial isolate[1]	Source of isolation	Gram stain	Fluorescence reaction	Shape
P-4	Casing at pinning		+	Rod
P-6	Casing at pinning	-	+	Rod
R-7	Mycelium at pinning		+	Rod
R-5	Mycelium at pinning	-	+	Rod
B-8	Vegetative mycelium	-	-	Coccoid
B-4	Vegetative mycelium	+	-	Coccoid
CO-5	Compost	+	-	Spherical
SC-5	Spawned compost	-	-	Coccoid

[1]Random isolations of representative colony types present in greatest numbers on TA plates obtained from dilutions of mushroom compost, casing, and mycelium. The bacterial isolates were grouped according to source and colony morphology.

Table 2. Influence of bacteria isolated from mushroom compost and casing on the vegetative growth of *Agaricus bisporus*

Bacterial isolates[2]	Radial growth (cm) of vegetative mycelium[1]			
	C-4	Campbell	C-2	M-1
P-4	1.87 cd	2.12 bc	2.09 bcd	1.89 cd
P-6	1.96 bcd	2.01 c	1.85 d	1.74 d
R-7	1.77 d	1.98 c	1.93 cd	1.84 cd
R-5	1.92 bcd	2.45 ab	2.36 ab	2.14 abc
B-8	2.31 ab	2.42 ab	2.31 ab	1.86 cd
B-4	2.36 a	2.01 c	2.21 bc	2.16 abc
CO-5	2.46 a	2.51 a	2.54 a	2.45 a
SC-5	2.48 a	2.55 a	2.21 bc	2.32 ab
Control	2.25 abc	2.31 abc	2.11 bcd	2.09 bc

[1]Mean radial growth of the vegetative mycelium from 5 replicate plates of potato dextrose agar incubated for 12 days at 25°C after inoculation and repeated twice. C-4, Campbell, C-2, M-1 are different commercial strains of *Agaricus bisporus*. Means within a column followed by the same letter do not differ significantly according to Student Newman-Keul's test ($P = 0.05$).

[2]Bacterial isolates P-4, P-6 were obtained from the casing at the time of pinning; R-7, R-5 were from the mushroom mycelium at the time of pinning; B-8, B-4 were from the vegetative mycelium before pinning; CO-5 was from mushroom compost and SC-5 was from spawned compost.

Discussion

The results reported show that some of the bacteria which colonize the substrates normally used in commercial cultivation of *A. bisporus* have a stimulating effect on basidiomata initiation of the mushroom. The results are in agreement with laboratory studies reported by other investigators, who showed that formation of basidiomata is reduced or is absent under totally axenic conditions (Eger 1972; Flegg et al. 1985; Hayes 1974, 1981; Visscher 1978, 1979). However, because much of the information was based on the relatively

Table 3. Effect of bacteria isolated from mushroom compost, casing, and mycelium on basidiocarp formation in *Agaricus bisporus* observed in petri dish bio-assays

Bacterial isolate[2]	Basidiocarps per petri dish[1]			
	C-4	Campbell	C-2	M-1
GR12-2	29.5 a	18.2 ab	18.0 bc	16.0 c
P-4	16.2 de	12.3 c	14.6 cde	21.4 b
P-6	20.6 c	15.7 b	19.6 ab	26.9 a
R-7	25.4 b	18.8 a	15.9 bc	22.4 b
R-5	20.8 c	10.1 cd	6.6 gh	11.5 d
B-8	14.0 de	7.7 de	8.8 fg	20.2 b
B-4	12.8 ef	19.7 a	11.7 ef	12.6 cd
CO-5	4.5 f	3.1 f	2.8 h	3.7 e
SC-5	2.7 f	1.8 f	18.3 bc	2.1 e
NS-C	24.0 b	19.2 a	23.4 a	23.3 ab
S-C	2.8 g	1.9 f	2.9 h	2.4 e

[1]Numbers of basidiocarps per plate are the mean of combined data from two experiments each having 10 replications. C-4, Campbell, C-2, M-1 are different commercial strains of *Agaricus bisporus*. Means within a column followed by the same letter do not differ significantly according to Student Newman-Keul's test ($P = 0.05$).

[2]Bacterial isolates P-4, P-6 were obtained from the casing at the time of pinning; R-7, R-5 were from the mushroom mycelium at the time of pinning; B-8, B-4 were from the vegetative mycelium before pinning; CO-5 was from mushroom compost and SC-5 was from spawned compost. NS-C (nonsterilized casing) and S-C (sterilized casing) were control treatments.

artificial conditions of the laboratory, there is considerable controversy regarding the precise role and relative importance of the microbial flora in basidiocarp initiation (Flegg et al. 1985, Ingratta & Patrick 1987, Sinden 1982, Tschierpe 1973). The fact that during mushroom cultivation microorganisms are always present and totally axenic conditions are unnatural makes the questions surrounding the role of the microbial flora in basidiocarp initiation very difficult to resolve. In addition, the difficulties of creating and maintaining totally axenic conditions without introducing other abnormal environmental parameters that can also influence mushroom development, makes the problem even more complex. Despite the complexities outlined, the above findings suggest that among the many factors that are involved in the regulation of reproduction among different fungi, the biotic factors that involve microbial associations appear to be important and further studies on this aspect are needed.

The specific findings on basidiocarp initiation by the various bacterial isolates may offer possibilities of practical exploitation in commercial mushroom culture. The study showed that some of the bacterial isolates that were readily isolated from mushroom growing substrates stimulated initiation of basidiocarps in *A. bisporus*. It was interesting to note that the stimulating bacteria that were isolated directly from the surface of the mushroom mycelium growing in the casing layer were also present in the peat moss and lime, the two components of casing. The isolates tested that were obtained from the compost had no effect on basidiocarp initiation.

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