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Scanning Electron Microscope Studies of Interactions between *Agaricus bisporus* (Lang) Sing Hyphae and Bacteria in Casing Soil

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Relationships between the hyphae of *Agaricus bisporus* (Lang) Sing and bacteria from the mushroom bed casing layer were examined with a scanning electron microscope. Hyphae growing in the casing layer differed morphologically from compost-grown hyphae. Whereas the compost contained thin single hyphae surrounded by calcium oxalate crystals, the casing layer contained mainly wide hyphae or mycelial strands without crystals. The bacterial population in the hyphal environment consisted of several types, some attached to the hyphae with filamentlike structures. This attachment may be important in stimulation of pinhead initiation.

It is well established that bacteria present in the casing soil considerably affect the growth and morphogenesis of *Agaricus bisporus* (5, 8, 9, 12, 18). Morphological changes conferred by one microorganism on another have been described for many cases. For example, mycelial strands are formed in the pathogenic fungus *Sclerotium rolfsii* by *Trichoderma harzianum* (7), sclerotial formation in *Rhizoctonia solani* is induced by the bacterium *Bacillus subtilis* (10), production of sporangia in *Phytophthora cinnamomi* is favored by *Pseudomonas* spp. and *Chromobacterium violaceum* (2), and production of rhizomorphs in *Armillaria mellea* is enhanced by *Aureobasidium pullulans* (13).

In some cases a morphogenetic stimulation requires a direct contact (7), while in other cases it may take place from a distance (10).

A. bisporus is affected by the bacteria of the casing layer, which consist mainly of pseudomonads (4) which stimulate pinhead production. Bacteria are found in the hyphal environment of *A. bisporus* in large numbers (15), and their population composition is found to be affected by the presence of the hyphae. It is not yet known whether this effect requires direct contact between the two microorganisms. However, it has been reported that *Pseudomonas tolaasii* isolated from the casing layer was strongly attached to the hyphae (14).

This work describes scanning electron microscope (SEM) studies of interactions between *A. bisporus* hyphae and bacteria, as well as morphological changes of the hyphae occurring in the casing layer.

MATERIALS AND METHODS

Fungal strain and growth conditions. *A. bisporus* (Lang) Sing var. 53 was obtained from Somycel Co., Mesnil-le-Roi, France. The fungus was grown by the Halbschalentest method (6) on the compost layer of a petri dish (15-cm diameter) containing sterile compost and peat side by side, each in half of the dish. Two weeks after the compost spawning, the peat layer was inoculated with bacteria isolated from the casing layer taken from an ordinary mushroom-growing room. Bacterial suspension (1 ml) was taken from a 1:10 dilution of wet peat in phosphate buffer. Control plates were not inoculated with bacteria. The plates were incubated at 25°C for 3 days and then were transferred to a dark growing room equipped with a ventilation system and adjusted to a temperature of 16°C and a relative humidity of 90%. The plates were incubated until initiation of pinhead production took place 3 weeks later. Samples were then removed for SEM observation. The samples were taken from different locations in the peat layer, representing different hyphal ages.

The fungus was also grown on malt extract agar (Oxoid Ltd., London, England) under axenic conditions at 25°C for 2 weeks. Then a suspension of *P. tolaasii* (isolated from the commercial casing layer by the white-line method of Preece and Wong [14]) was added to the fungus in the malt extract agar, and samples, both washed and unwashed, were taken. The samples were washed three times (1 min for each washing, in a vortex mixer (Reax 2000; Heidolph Co.) with a maximum shaking frequency of 40 Hz) in sterile phosphate buffer (0.1 M, pH 7.0). Agar blocks (0.5-cm diameter) were taken for SEM 30 min after inoculation.

Preparation of samples for SEM. Samples for SEM were fixed with 5% glutaraldehyde for 4 h in a refrigerator. The fixative was then dehydrated in the following series of acetone-water mixtures: 50% acetone for 30 min, 70% acetone for the night, 100% acetone for 1 h, and again 100% acetone for 30 min (3). After dehydration, the samples were critical point dried with liquid CO_2, adhered to stubs, coated with a gold-palladium layer, and observed under SEM (JEOL JSM 35C, Japan).

RESULTS AND DISCUSSION

Morphological types of hyphae and their relationships with bacteria in the casing layer. It was of interest to monitor the relationship between the mycelium of *A. bisporus* and the bacteria in the casing soil. In the noninoculated casing soil, the mycelium consisted mostly of thin (1 to 2 μm wide) single hyphae with calcium oxalate crystals surrounding them (Fig. 1). This type of vegetative hypha is typical of the fungus

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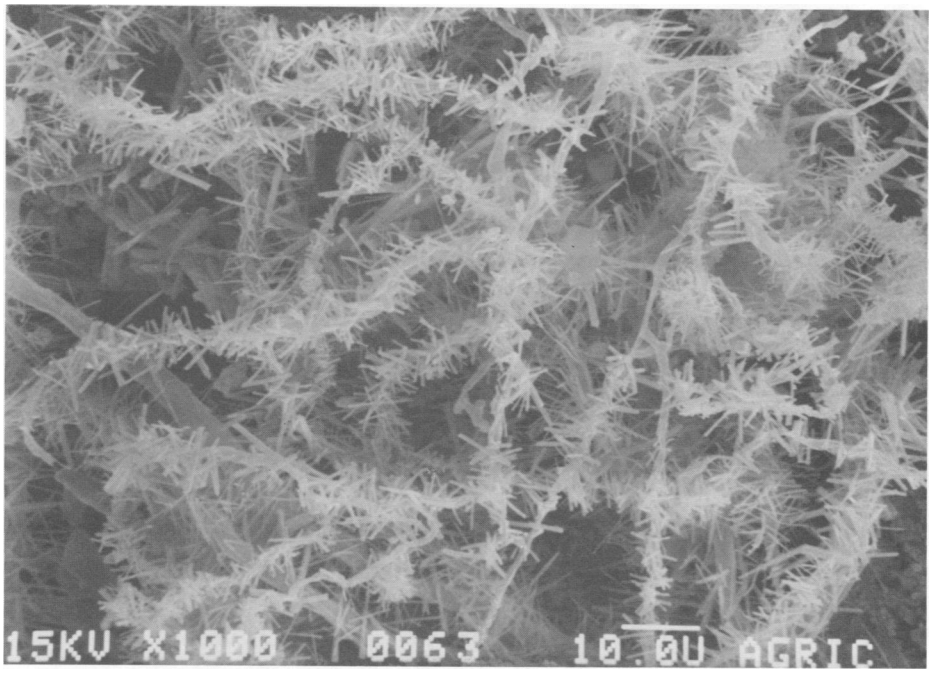

FIG. 1. Vegetative growth of mushroom mycelium in the casing soil. The hyphae are thin and are surrounded by calcium oxalate crystals. Sample was taken 2 cm from the compost layer. Magnification, ×1,000. Bar, 10.0 μm.

growing in compost (17). Upon entering the productive stage in the inoculated peat, the hyphae appeared to be relatively free of calcium oxalate crystals, and there were many wide (4- to 6-μm cross section) single hyphae (Fig. 2) and multihyphal mycelial strands (Fig. 3). It is likely that the calcium oxalate crystals interfere with strand formation, which is the initial pinhead-forming stage and precedes the formation of fruiting bodies. The pinhead shown in Fig. 4 also consists of crystal-free interwoven hyphae.

Bacterial population in the casing layer. Observation of the bacteria associated with hyphae in the inoculated peat revealed rods, vibriolike rods, and cocci (Fig. 5). Some

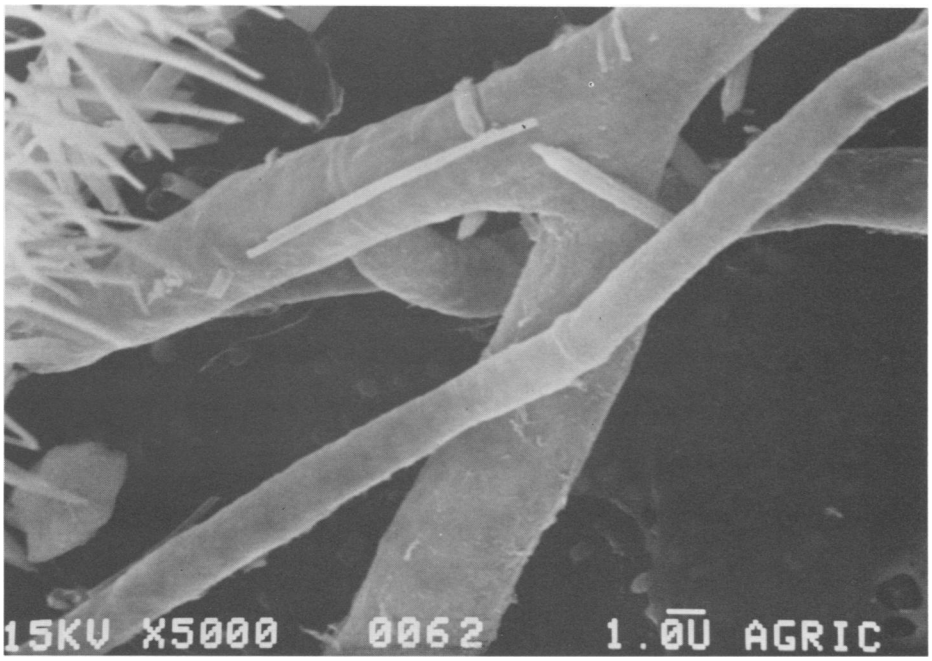

FIG. 2. Wide hyphae (crystal free) from the casing soil at the time of pinhead initiation. Sample was taken 4 cm from the compost layer. Magnification, ×5,000. Bar, 1.0 μm.

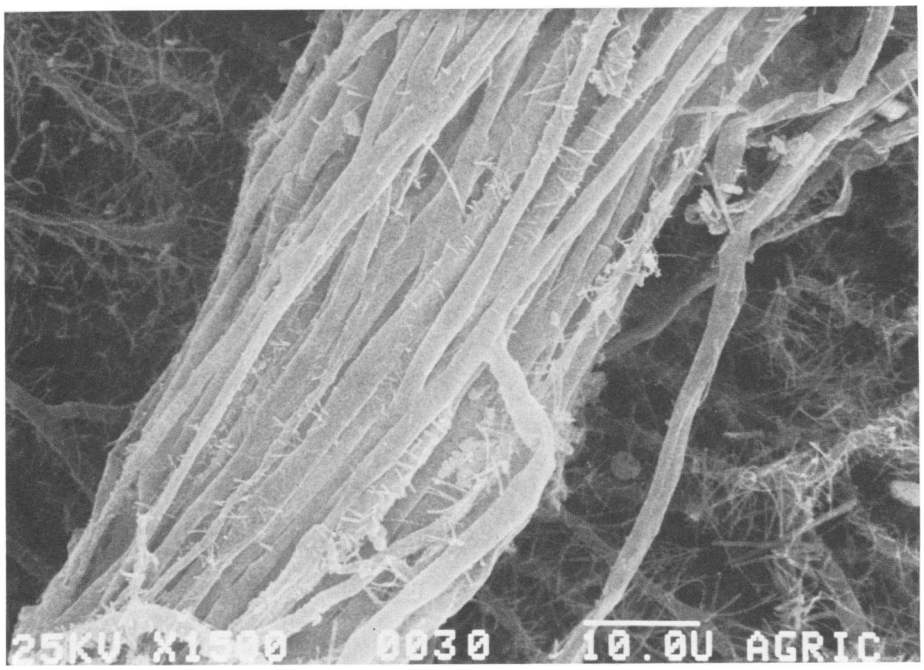

FIG. 3. Multihyphal mycelial strand from the casing soil at the time of initiation. Sample was taken 5.5 cm from the compost layer (2.0 cm from the dish wall at the peat layer). Magnification, ×1,500. Bar, 10.0 μm.

bacteria were very close to the hyphae, and some were attached to hyphae and to each other by filamentlike structures which were morphologically different from flagella. These structures were observed on washed hyphae in association with attached rodlike bacteria (Fig. 6) which appeared alone, in pairs, or in aggregates on the hyphal surface (Fig. 7). These attached bacteria were observed more often on young hyphae in the sample taken nearer to the dish wall than on older hyphae in the sample taken nearer to the compost layer (compare Fig. 5 with Fig. 2). Possibly the hyphae exude metabolites which cause the bacteria to be attracted by chemotaxis to the hyphal surface and to multiply there. According to Stanek (15), bacteria which grow in the hyphosphere or on the hyphae are affected by hyphal

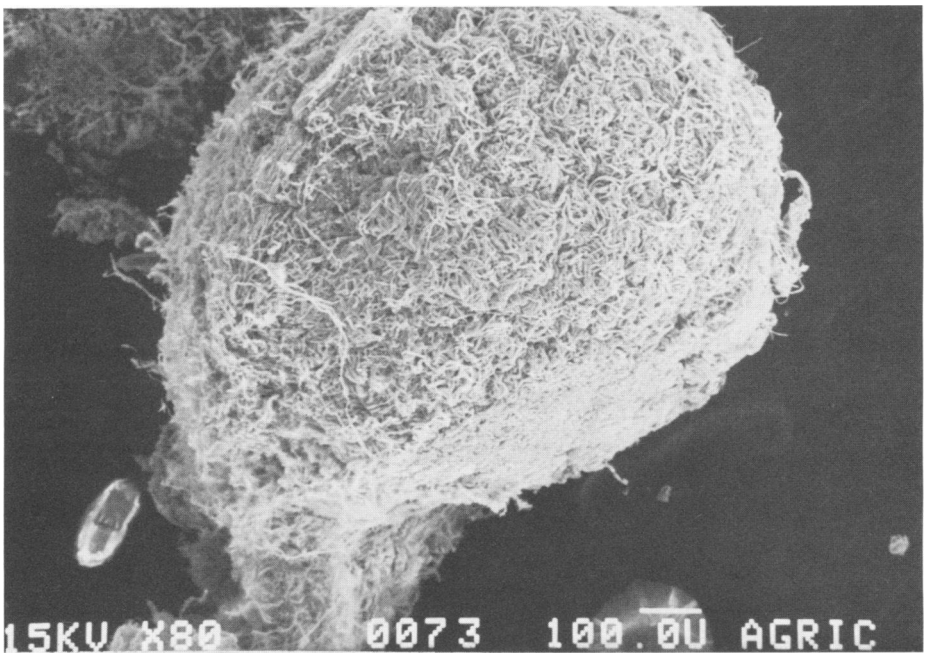

FIG. 4. Young pinhead from the casing soil consisting of interwoven crystal-free hyphae. Sample was taken 7.0 cm from the compost layer (0.5 cm from the dish wall). Magnification, ×80. Bar, 100.0 μm.

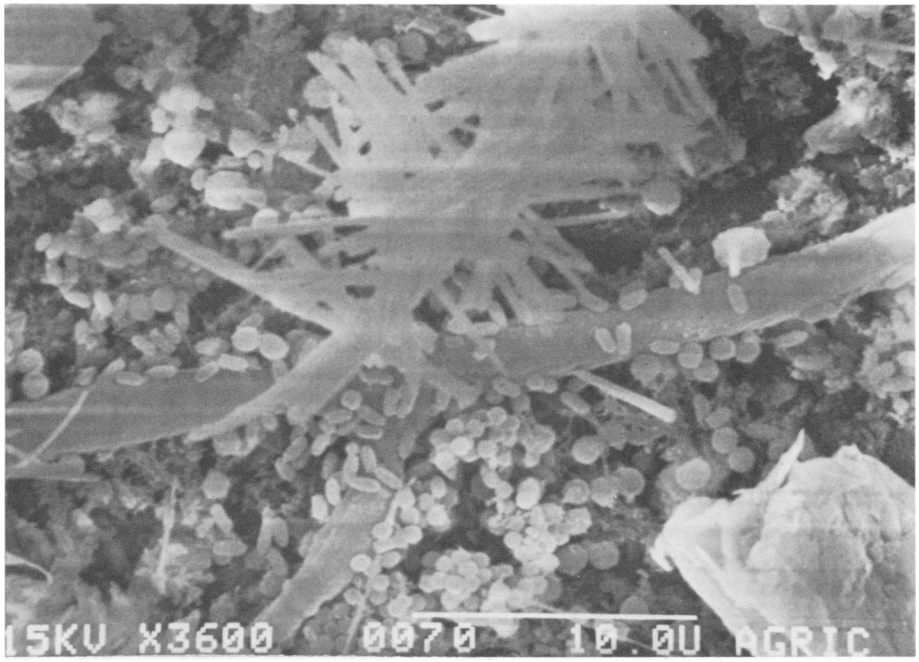

FIG. 5. Colonies of bacteria in the hyphal environment of mycelium taken from the casing soil in which mycelial strands were observed. Sample was taken 5.5 cm from the compost. Magnification, ×3,600. Bar, 10.0 μm.

exudates, since they exhibit different nutritional requirements when compared with bacteria isolated from a more distant location.

Pseudomonas fluorescens and *Pseudomonas putida* were reported to be attracted to fungal propagules by chemotaxis (1). A similar phenomenon could have occurred in our system.

The bacteria found to be attached to the hyphae were similar to those isolated from casing soil by Preece and Wong (14), which those authors claimed to be *P. tolaasii*. Our results indicate that there is a difference between the bacteria attached to the hyphae in the casing layer and the *P. tolaasii* that we added to sterile hyphae (Fig. 8). The added bacteria (previously isolated from infected fruiting bodies)

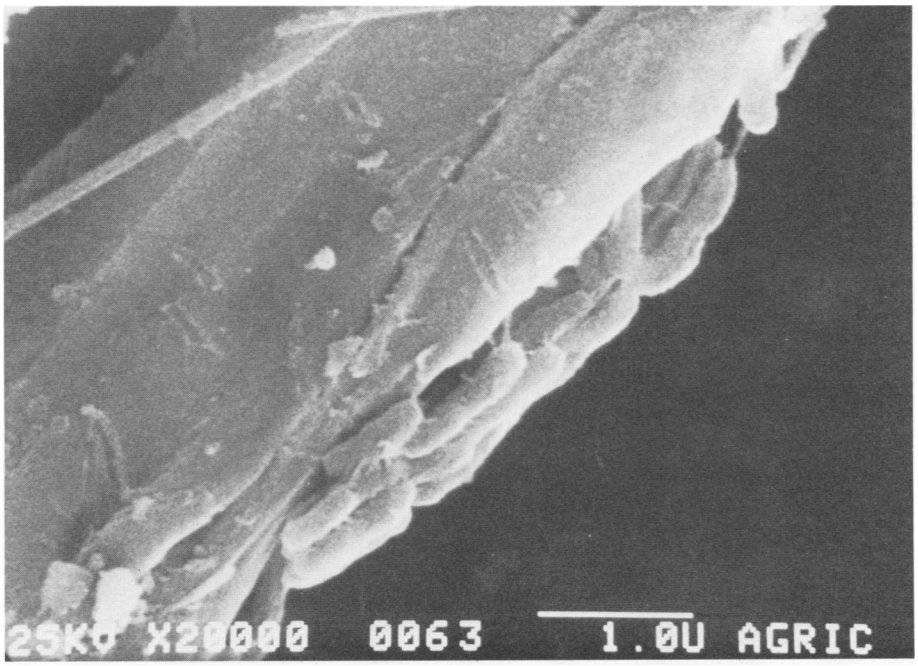

FIG. 6. Rodlike bacteria attached to the hyphae in the casing soil with filamentlike structures. Sample was taken 6.5 cm from the compost layer. Magnification, ×20,000. Bar, 1.0 μm.

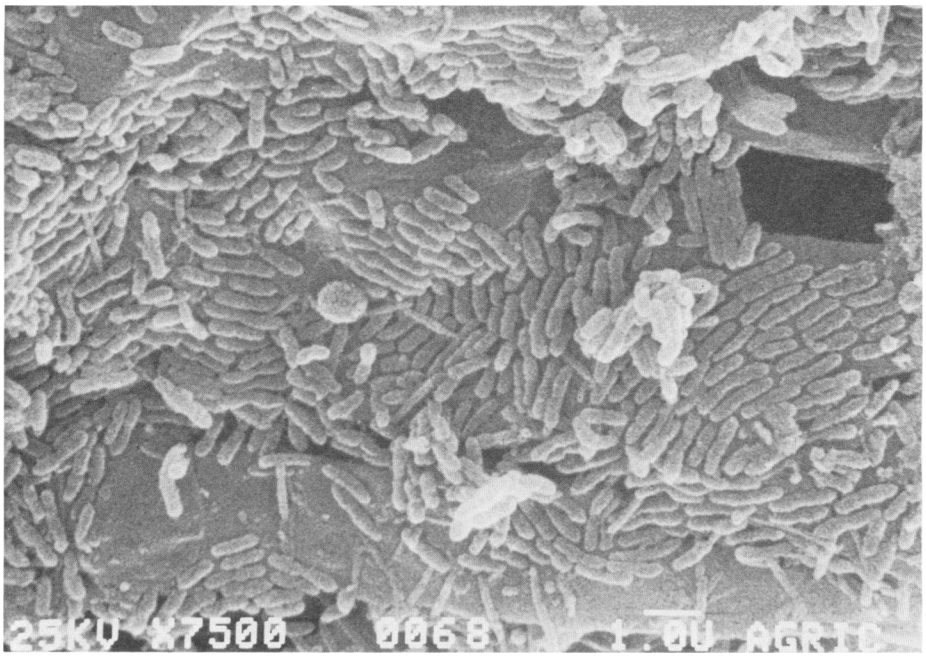

FIG. 7. Rodlike bacteria from the casing soil attached to hyphae at the time of initiation. Sample was taken 6.5 cm from the compost layer. Magnification, ×7,500. Bar, 1.0 μm.

had a smooth surface and very clear flagella that were not observed in the bacteria with the rougher surface which were attached to the hyphae in the past.

The reason for the different appearance of the bacteria is not clear, but this difference might indicate that the hypha-attached bacteria in the peat are not *P. tolaasii*.

Bacteria of the genus *Psuedomonas* inhabit the casing layer at the time of initiation (4) and are considered to be involved in the induction of the initiation process (9). Their attachment to the hyphae could further stimulate the initiation of fruiting bodies. Indeed, Hume and Hayes (11) obtained pinheads on water-agar only when the hyphae reached the bacterial colony, but the relationship between the hyphae and the bacterial colony was not examined. It should be mentioned that the attached bacteria also caused the disappearance of the calcium oxalate crystals, which may help in strand formation. The oxalate might be used under certain circumstances by the bacteria or by the

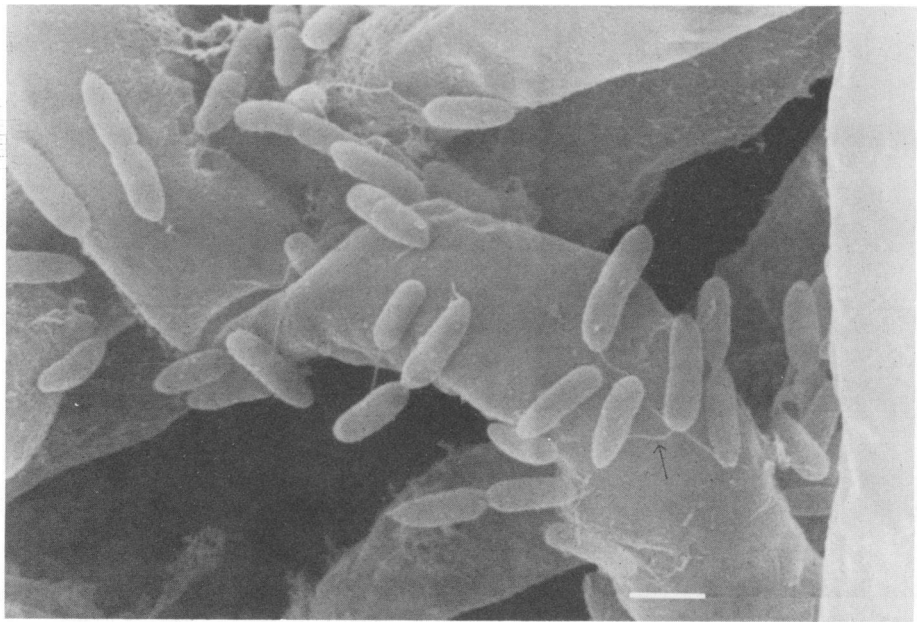

FIG. 8. *P. tolaasii* sp. attached to the hyphae of the fungus that was grown on malt extract agar. An arrow points to the flagella. Magnification, ×10,000. Bar, 1.0 μm.

fungus, as some bacteria of the genus *Pseudomonas* and fungi can use oxalate (16).

In recent experiments (S. Masaphy, unpublished data), bacteria which were attached to the mycelium were isolated and grown in culture. These bacteria stimulated initiation of fruiting bodies when inoculated into sterile casing soil.

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