

## Electron microscopy of the infection process of rapid soft rot disease of the edible mushroom *Agaricus bitorquis*

P. T. ATKEY, T. R. FERMOR AND S. P. LINCOLN

Microbiology and Crop Protection Department, Horticulture Research International, Littlehampton, W. Sussex, BN17 6LP

*Pseudomonas gladioli* pv. *agaricicola* the cause of rapid soft rot disease of *Agaricus bitorquis*, produced large brown wet lesions on the surface of the pileus, rendering the mushroom unmarketable. Electron microscopy has been employed to study the development of artificially induced lesions over a 33 h period. Degradation of hyphae leaves a compressed layer of collapsed wall debris behind the advancing bacteria. After initial penetration of the surface of the pileus the bacteria rapidly spread sideways, indicating a degree of resistance to attack in the pileipellis which is not present in the underlying hyphae.

Commercial production of *Agaricus bitorquis* (Quélet) Saccardo, has a number of advantages over *Agaricus bisporus* (Lange) Imbach, particularly in tropical countries, as it will fruit at 23–30 °C (Raper, 1976; Smith & Love, 1989), has a good post-harvest shelf life (Nichols & Hammond, 1976) and is resistant to the viruses infecting *A. bisporus* (Van Zaayen, 1976) making it useful as a 'virus breaker' in a virus-infected *A. bisporus* cropping cycle (Atkey, 1985).

The *A. bitorquis* basidiome is, however, susceptible to bacterial soft rot disease caused by *Pseudomonas gladioli* Sereini pv. *agaricicola* Lincoln, Fermor, Stead & Sellwood (Lincoln *et al.*, 1991), which produces slimy brown lesions on the surface of the pileus.

This paper describes the development and spread of lesions formed from the application of droplets of bacterial suspension of known concentration.

### MATERIALS AND METHODS

#### Bacterial culture

The soft-rotting bacterium (RR3) was isolated at Horticulture Research International, Littlehampton, U.K. from a rotting basidiome of *Agaricus bitorquis* (ATCC 32675) growing at 28 °C. The bacterium was identified as *Pseudomonas gladioli* pv. *agaricicola* pv. nov. (Lincoln *et al.*, 1991) and has been deposited at the National Collection of Plant Pathogenic Bacteria, Harpenden, UK, accession number NCPPB 3580. The bacteria were maintained on King's B agar slopes (King, Ward & Raney, 1954) at 4° or stored in liquid nitrogen at –196° using the technique of Challen & Elliott (1986).

#### Cultivation of *Agaricus bitorquis*

*Agaricus bitorquis* (ATCC 32675) was grown in wheat straw/chicken manure GCRI Formula 2 compost (Randle, 1974). Wooden trays, 0.9 × 0.6 × 0.15 m deep, were filled

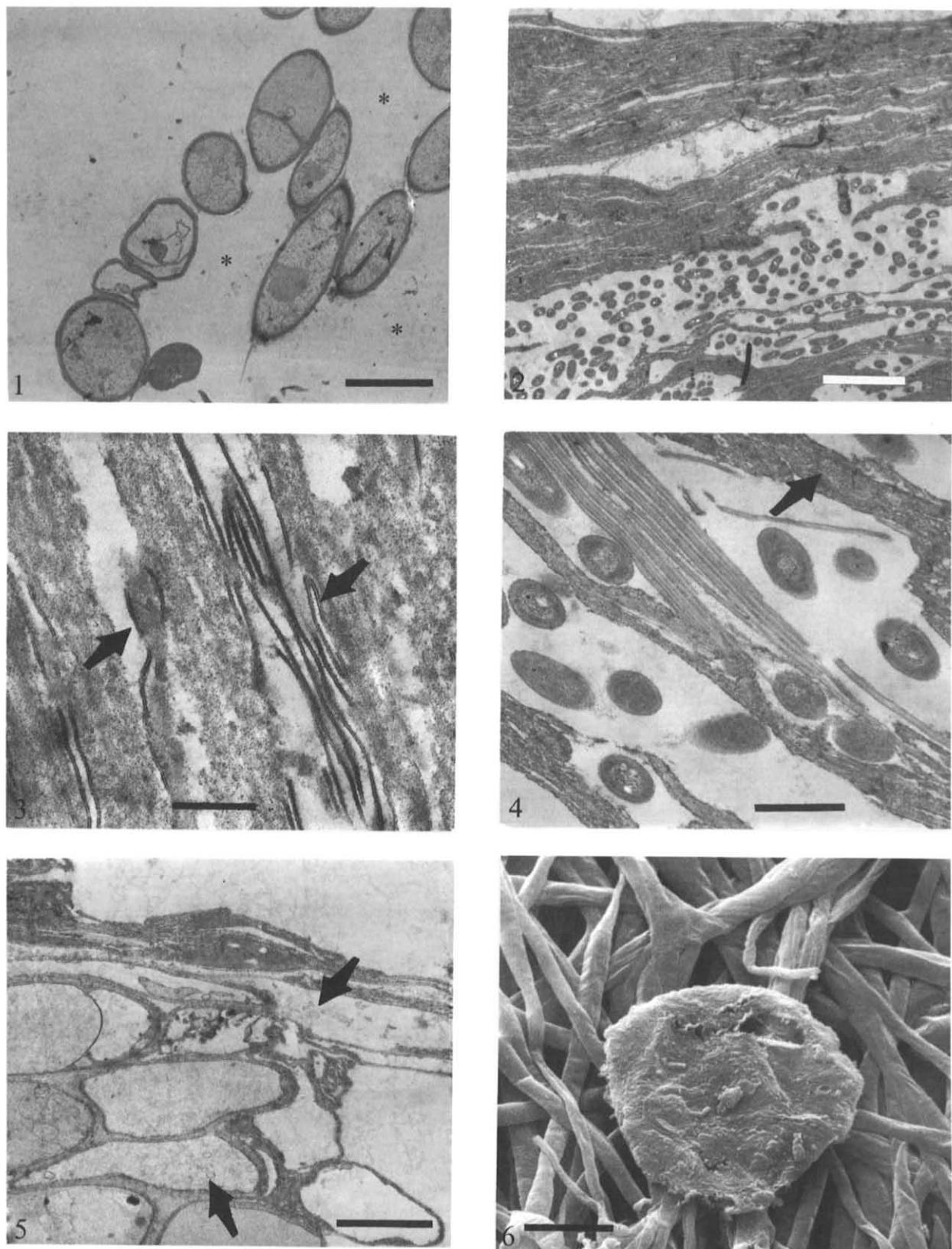
with compost, inoculated with *A. bitorquis* and placed in a controlled environment chamber at 28°. Fourteen days after inoculation the compost was overlaid with a 'casing layer' (2 cm deep) of peat and chalk (1:1 v/v). Air temperature was maintained at 28°, relative humidity at 85% and CO<sub>2</sub> at 800–1000 ppm throughout the mushroom cropping period.

#### Inoculation of mushroom sporophores with bacterial pathogen

Soft rot bacterium RR3 was grown in King's B broth incubated at 28°, shaken at 200 rpm and harvested at the end of logarithmic growth phase. The bacterial culture was diluted with sterile distilled water to a final concentration of 3.5 × 10<sup>3</sup> cfu ml<sup>-1</sup> as confirmed by dilution plates (Miles & Misra, 1938). Seven 1 µl droplets, each containing ca 3–4 c.f.u., were deposited equidistantly on the pileal surface (2–3 cm diam) of each of fifteen growing basidiomes. The position of each droplet was marked by adding Phenol red (0.018% w/v), to the bacterial suspension. Individual basidiomes were picked at 3 h intervals.

#### Specimen preparation for Scanning Electron Microscopy (SEM)

Uninoculated pilei and pilei 1, 3, 6, 9, 13, 15, 18, 21, 25, 27, 30 and 33 h after inoculation were examined. The 3 h sampling interval was broken at 13–25 h due to difficulty with the working day. Pieces of pileus approximately 5 × 5 × 3 mm thick, and containing lesions, cut from the surface were fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) at room temperature for 12 h, rinsed in buffer, post-fixed in aqueous osmium tetroxide for 6 h, rinsed, dehydrated through an ethanol series and critical-point-dried using liquid CO<sub>2</sub> as the intermediate fluid (Anderson, 1951). Specimens were then sputter-coated with gold/palladium and examined in a Jeol



**Figs 1–5.** TEM *A. bitorquis* pileus. **Fig. 1.** Transverse section of the surface layer of a healthy basidiome showing large intercellular spaces (\*). Bar mark = 5 µm. **Fig. 2.** Transverse section of a rapid soft-rot lesion showing compressed, degraded fungal elements at the surface with pockets of bacteria below. Bar = 5 µm. **Fig. 3.** Degraded hyphae in lesion containing many membrane fragments (arrows). Bar = 0·25 µm. **Fig. 4.** Bacterial cells among degraded hyphae, showing laminar bodies in T.S. and electron-dense material (arrow). Bar = 1 µm. **Fig. 5.** Bacterial 'front' of lesion 21 h after inoculation, showing electron-dense debris and sparse bacterial penetration into cells (arrows) ahead of the dense region. Bar = 4 µm. **Fig. 6.** SEM. A droplet-like bacterial colony on the pileal surface 3 h after inoculation. Bar = 10 µm.

T330 SEM at various accelerating voltages. All scanning micrographs were recorded on Polaroid type 665 positive/negative film.

#### **Transmission Electron Microscopy (TEM)**

Cubes ( $1\text{ mm}^3$ ) cut from well-developed lesions were fixed for 4 h in 2·5% (w/v) glutaraldehyde, rinsed in 0·2 M sodium cacodylate buffer (pH 7·2) and post-fixed in 2·0% aqueous osmium tetroxide for 4 h at room temperature. After further rinsing the specimens were dehydrated through an ethanol series and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections were cut on a Reichert-Jung ultracut E ultramicrotome using glass or diamond knives, contrasted with uranyl acetate followed by lead citrate (Reynolds, 1963), and examined in a Jeol 100S TEM at 80 kV accelerating voltage. Electron micrographs were recorded in Kodak 4489 electron microscope film.

## **RESULTS**

### **TEM**

*Healthy pileus.* The pileus of the *Agaricus bitorquis* basidiome consisted of hyphal elements similar to those of *A. bisporus* (Wood *et al.*, 1985). They were very loosely packed near the surface of the pileus with large intercellular air spaces and a number of degenerated cells (Fig. 1).

*Infected pileus.* Vertical sections of lesions (Fig. 2) showed that a layer 10–180  $\mu\text{m}$  thick below the surface consisted of degenerated and densely compressed hyphae. This layer, which contained few bacterial cells, consisted mainly of fungal wall material in which electron-dense fragments and aggregates of bilaminar membrane were prominent (Fig. 3). The next layer in the lesion was formed largely of pockets up to 8  $\mu\text{m}$  deep, containing large numbers of bacteria (Fig. 2). The side of the bacterial layer proximal to the surface of the lesion bordered directly on to the compressed first layer, whereas the distal side consisted of highly degraded and loosely packed fungal walls. This level of the lesion contained bundles of grey laminar bodies interspersed with bacterial cells (Fig. 4) and was behind the deepest part of the lesion, which consisted of a layer of collapsed hyphae often containing electron-dense material (Fig. 5). Below this layer the *A. bitorquis* elements appeared healthy.

#### **Development of lesions (SEM)**

Specimens from all sampling times were examined in the SEM. The following are described: 1, 3, 9, 13, 15, 21 and 25 h after inoculation. Those taken at 18, 27 and 30 h after inoculation are not described, for the sake of brevity, as the changes from the previous sampling time were not noteworthy.

*1 hour.* No surface damage was observed. Groups of two or three bacterial cells were found after prolonged searching near the point of inoculation.

*3 hours.* Roughly circular bacterial colonies  $\approx 40\text{ }\mu\text{m}$  diam. were seen on the surface of the pileus (Fig. 6). These colonies were mucilaginous and had small numbers of bacterial cells –

usually less than 10 – on their surfaces. No damage was visible and bacteria were restricted to the colony, none being observed on the hyphae.

*9 hours.* The colonies had enlarged up to 150  $\mu\text{m}$  diam. and became less regular in shape (Fig. 7). They were composed of a dense mass of bacteria which were spilling from the edge of the colony on to the surrounding pileus (Fig. 8). Flaking of the hyphal surface was apparent, and aggregates of laminar structures were frequently seen (Fig. 9).

*13 hours.* Lesions, approx. 1 mm diam., on the surface of the pileus appeared depressed. Disrupted hyphae in the lesion were aggregating, and the intercellular spaces were large filled with granular and stranded material and bacteria (Fig. 10). Detached flakes with a tendency to curl were common on hyphae adjacent to the lesion.

*15 hours.* Hyphae around the lesions appeared to be coalesced, with some bundles of aggregated hyphae radiating from the affected area (Fig. 11). Bacilli occurred frequently on the surface of the hyphae (Fig. 12).

*21 hours.* Bacteria were closely associated with many hyphae either in pockets between aggregated elements (Fig. 13) or associated with cracks in the wall (Fig. 14).

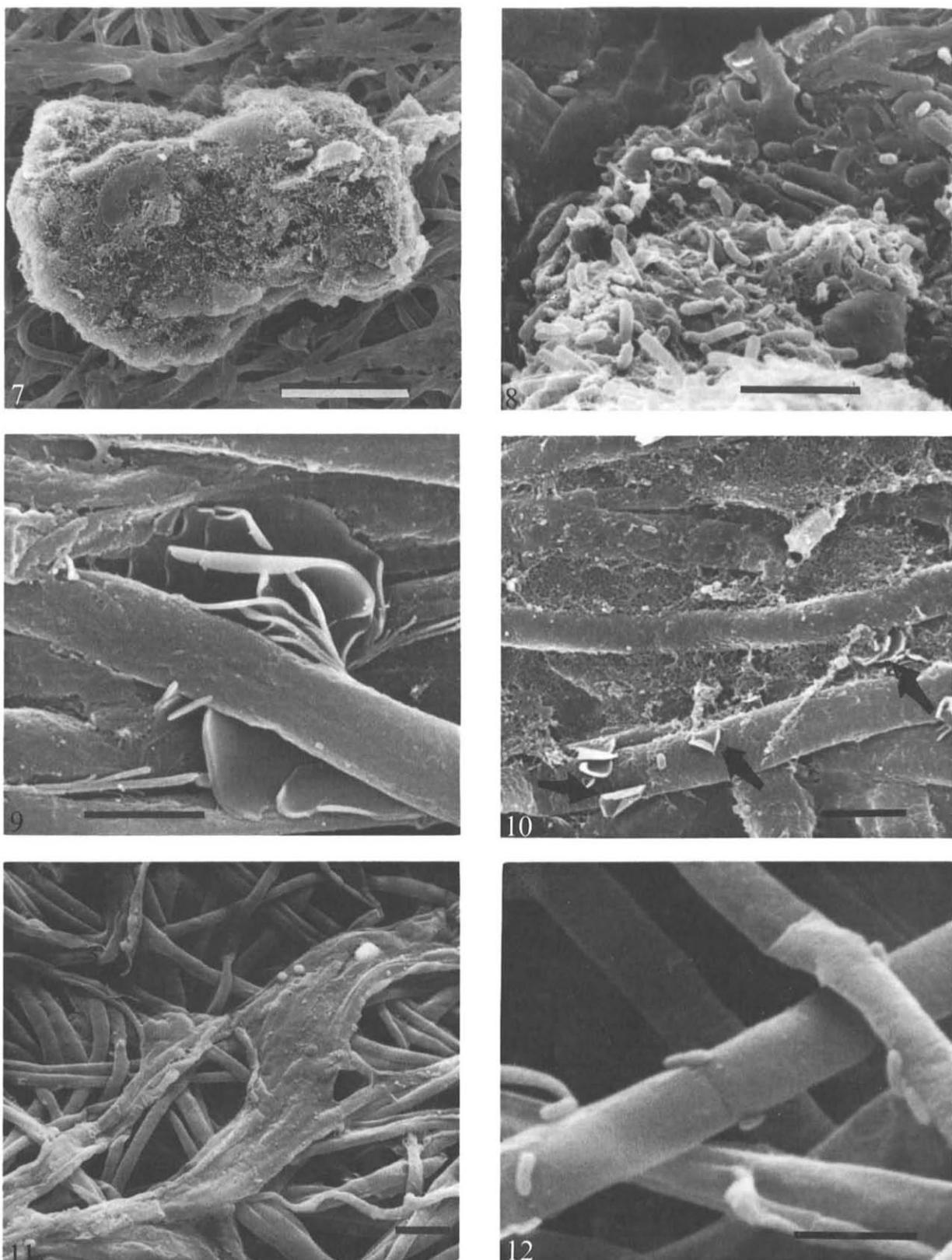
*25 hours.* Lesions on the surface of the pileus appeared crater-like (Fig. 15). The floor of the lesion crater was slightly concave and consisted of flattened and aggregated hyphae (Fig. 16). The vertical wall of the crater appeared similar, with an overhanging lip at the pileal surface. Numbers of bacteria were present on all the surfaces of the lesion.

After 33 h the lesions were extensive, up to 3·2 mm wide  $\times$  1·8 mm deep, with overhanging sides tending to collapse inwards.

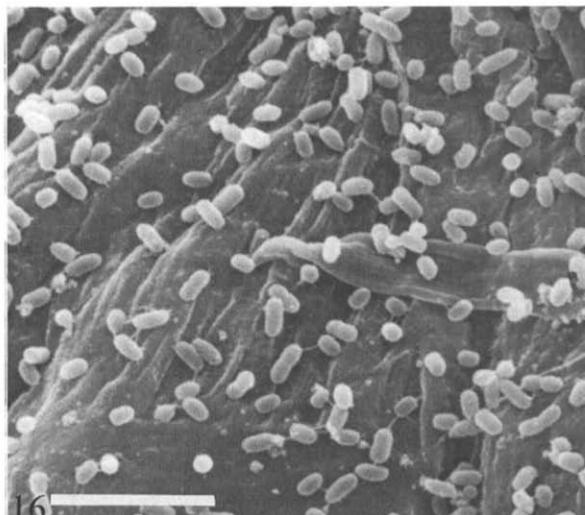
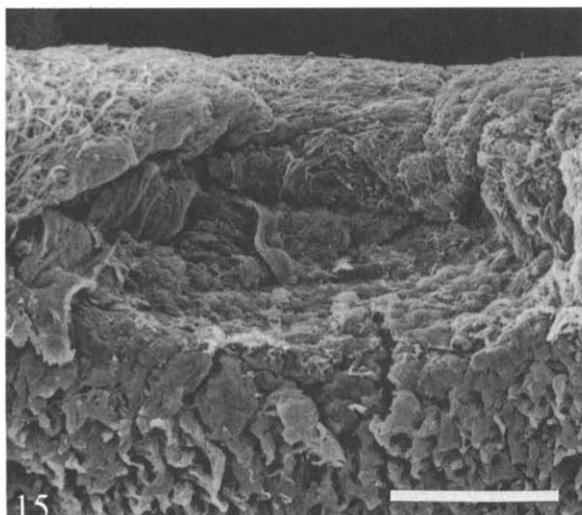
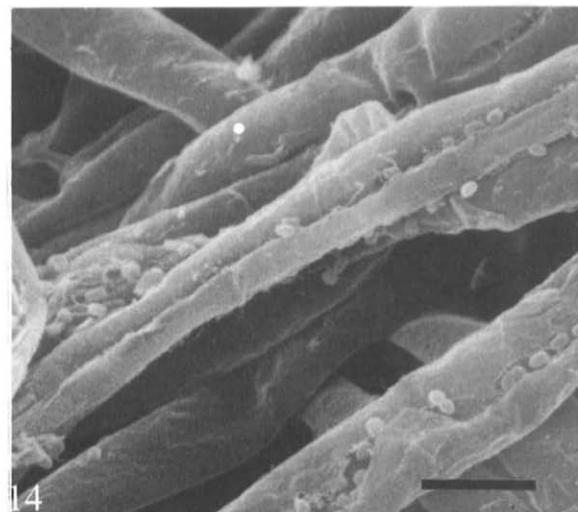
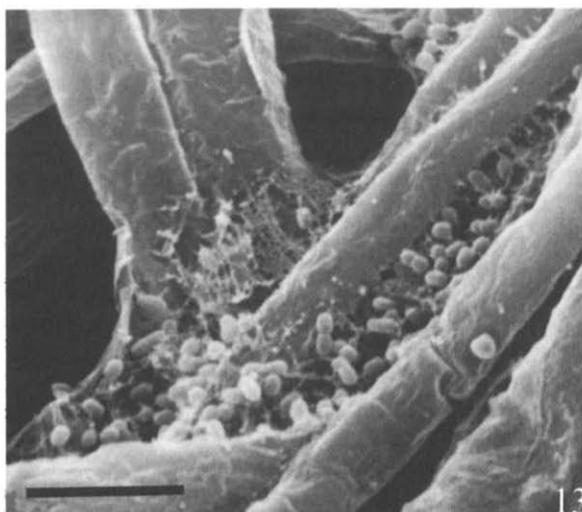
## **DISCUSSION**

Cole & Skellerup (1986) reported that *Pseudomonas toluae* Paine infection of *A. bisporus* caused progressive degeneration of the hyphae, many of which became depressed and contained electron-dense material. The degeneration of *A. bitorquis* cells appeared to be much more rapid with very few intermediate stages of breakdown. There was, however, considerable evidence of electron-dense material and cell compression (Fig. 2), which supports the idea (Cole & Skellerup, 1986) that this may form a physical barrier which delays the infection process. *Pseudomonas gladioli* pv. *agaricicola* is able to kill healthy *A. bitorquis* tissue in isolation, indicating that it is the causal agent of rapid rot disease (Lincoln *et al.*, 1991).

From 13 h after inoculation onwards the lesions increased in depth and diameter up to 3·2 mm wide and 1·8 mm deep at 33 h. The side walls of the lesions were usually undercut (Fig. 16) which caused the sides to collapse into the lesion pit. This undercutting suggests that the internal hyphae of the pileus are more susceptible to bacterial degradation than the surface layer. In *A. bisporus* the pileipellis can be readily peeled from the underlying trama, from which it is chemically distinct. The pileipellis has greater tyrosinase activity, protein and phenol levels than the trama (Burton, 1988). As the situation in *A. bitorquis* seems likely to be similar it may represent a biochemical system of resistance to bacterial



Figs 7–12. SEM showing development of bacterial lesion on *A. bitorquis* pileus at various times after inoculation. Fig. 7. An enlarged bacterial colony; 9 h. Bar = 50 µm. Fig. 8. A high density of bacterial cells which are 'spilling' over edge of colony on to pileal surface; 9 h. Bar = 5 µm. Fig. 9. A hypha showing aggregated laminar material, adjacent to a bacterial colony; 9 h. Bar = 5 µm. Fig. 10. Surface of lesion with mucilage aggregating hyphae; 13 h. Note also laminar material (arrows). Bar = 5 µm. Fig. 11. A ribbon of aggregated hyphae leading away from lesion; 15 h. Bar = 10 µm. Fig. 12. Bacteria on the surface of hyphae; 15 h. Bar = 5 µm.



**Fig. 13.** Bacteria packed between hyphae; 21 h. Bar = 5 µm. **Fig. 14.** Bacteria associated with longitudinally cracked hyphae; 21 h. Bar = 5 µm. **Fig. 15.** Lesion showing an overhanging lip; 25 h. Bar = 500 µm. **Fig. 16.** Bacteria on floor of lesion; 25 h. Bar = 5 µm.

infection. As the pileipellis forms a somewhat 'skinlike' layer, it may be expected that the cells are physically stronger than those of the trama and could therefore also offer an additional physical barrier. Transmission electron microscopy did not reveal any physical difference, however (Fig. 1).

No attempts were made to count bacterial cells in the lesion pits containing liquid, as much of this was washed out during the preparation for SEM; the remaining cells were largely those with attachment to the substrate. Careful monitoring and control of environmental conditions in the growing rooms should lessen the potential impact of soft rot disease on the marketable output of *A. bitorquis*, as has been the case with *A. bisporus* and its associated bacterial blotch disease (Fermor, 1987). Further work is in progress to compare the spread of soft rot disease on basidiomes with that of bacterial blotch. The production of exoenzymes by the bacterium and their relationship to the breakdown of *Agaricus* hyphae should be investigated using immunoelectron-microscopic techniques.

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## REFERENCES

- Anderson, T. F. (1951). Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. *Transactions of the New York Academy of Sciences* **79**, 130–134.
- Atkey, P. T. (1985). Viruses. In *The Biology and Technology of the Cultivated Mushroom* (ed. P. B. Flegg, D. M. Spencer & D. A. Wood), pp. 241–260. Chichester, U.K.: J. Wiley.
- Burton, K. S. (1988). The effects of pre- and post harvest development on mushroom tyrosinase. *Journal of Horticultural Science* **63**, 255–260.
- Challen, M. P. & Elliott, T. J. (1986). Polypropylene straw ampoules for the storage of micro-organisms in liquid nitrogen. *Journal of Microbiological Methods* **5**, 11–23.
- Cole, A. L. J. & Skellerup, M. V. (1986). Ultrastructure of the interactions of *Agaricus bisporus* and *Pseudomonas tolaasii*. *Transactions of the British Mycological Society* **87**, 314–316.
- Fermor, T. R. (1987). Bacterial diseases of edible mushrooms and their control. In *Cultivating Edible Fungi* (ed. P. J. Wuest, D. J. Royse & R. B. Beelman), pp. 361–370. (Developments in Crop Science no. 10.) Amsterdam: Elsevier Science.
- King, E. O., Ward, M. K. & Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine* **44**, 301–307.
- Lincoln, S. P., Fermor, T. R., Stead, D. E. & Sellwood, J. E. (1991). Bacterial soft rot of *Agaricus bitorquis*. *Plant Pathology* **40**, 136–144.

- Miles, N. G. & Misra, S. S. (1938). The estimation of the bacterial power of blood. *Journal of Hygiene* **38**, 732.
- Nichols, R. & Hammond, J. B. W. (1976). Storage of *Agaricus edulis* (Syn. *A. bitorquis*) sporophores in pre-packs. *Mushroom Journal* **45**, 286–288.
- Randle, P. E. (1974). Compost. In *Annual Report of Glasshouse Crops Research Institute 1973*, 82–84.
- Raper, C. A. (1976). The biology and breeding potential of *Agaricus bitorquis*. *Mushroom Science* **9**, 1–10.
- Reynolds, D. S. (1963). The use of lead citrate at a high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208.
- Smith, J. F. & Love, M. E. (1989). A tropical *Agaricus* with commercial potential. *Mushroom Science* **12**, 305–315.
- Spurr, A. R. (1969). A low viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* **26**, 31–34.
- Van Zaayen, A. (1976). Immunity of strains of *Agaricus bitorquis* to mushroom virus disease. *Netherlands Journal of Plant Pathology* **82**, 121–131.
- Wood, D. A., Craig, G. D., Atkey, P. T., Newsam, R. J. & Gull, K. (1985). Ultrastructural studies on the cultivation processes and growth and development of the cultivated mushroom *Agaricus bisporus*. *Food Microstructure* **4**, 143–164.

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