TOXIN PRODUCTION BY *PSEUDOMONAS TOLAASII* PAINE*

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Abstract

Evidence is presented for the production of toxin *in vitro* and *in vivo* by *P. tolaasii*. Nutrient broth suspensions of *P. tolaasii* placed on detached mushroom sporophores but separated by a dialysis membrane caused brown discoloration and slightly sunken lesions. These were identical to the symptoms caused directly by *P. tolaasii* in commercial houses. The observation that cell-free culture filtrate of the bacterium also caused the browning of mushroom sporophores actively growing under conditions similar to those in commercial mushroom growing units on farms points to the importance of toxin production by *P. tolaasii* on its ecology in mushroom compost and casing peat on mushroom beds. The toxin appears to be of relatively low molecular weight, is heat stable, and is active in browning mushroom tissues over a range of pH from 3 to 9.

Introduction

Brown blotch of the cultivated mushroom *Agaricus bisporus* (Lange) Sing. caused by *Pseudomonas tolaasii* Paine occurs on most farms in New South Wales (Nair 1969). The disease causes brown, slightly sunken spots and blotches to develop on the maturing pileus. The browning appears to involve only the superficial mushroom tissues. In our studies of the ecology of microorganisms associated with the cultivated mushroom the mechanism of infection of the mushrooms by *P. tolaasii* has been investigated. This may lead to the development of a method of control of the brown blotch disease by biological means (Nair and Fahy 1972).

Lelliott *et al.* (1966) in a study of the fluorescent groups of the genus *Pseudomonas* suggested that *P. tolaasii* could be regarded as a normal constituent of the microflora of mushroom beds which, under certain conditions produced a metabolite toxic to mushrooms. As far as we are aware, there is no published record of the production of toxin by *P. tolaasii*. The present communication reports evidence of this.

Methods

P. tolaasii was isolated from blotched mushrooms from crops in and around Sydney. Cultures were maintained on nutrient agar (beef extract 0·3%, Bacto peptone 0·5%, agar 1·5%) at 25°C. Preliminary experiments on the production of extracellular toxin were carried out as follows.

Glass cuvettes 14 mm long, 18 mm diameter, and enclosed by a dialysis membrane (size 22/32 cellulose tubing with a pore radius of less than 20 Å obtained from Union Carbide Corporation,

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U.S.A.) were placed on the undamaged surface of mushroom sporophores. Maturing sporophores with an intact veil ("cup" stage of growth) were selected for all experiments.

The sterilized cuvettes were filled with 3·5 ml of nutrient broth and inoculated with *P. tolaasii*. The inoculated cuvettes and the sporophores were placed in sterile glass containers. After 24 hr the cuvettes were removed and the condition of surface tissue of the sporophores was noted. Uninoculated broth was used as control.

Cell-free extracts and culture filtrate were obtained by growing *P. tolaasii* at 25°C in a mineral base medium of Palleroni and Doudoroff (1972) which contained sodium–potassium phosphate buffer, pH 6·8, M/30; NH_4Cl 0·1%; $MgSO_4.7H_2O$ 0·05%; ammonium citrate 0·005%; and $CaCl_2$ 0·0005%. Glucose (0·2%) was added as the utilizable source of carbon.

Cultures were grown at 25°C in a rotary shaker for 20 hr and then centrifuged at 10,000 *g* for 10 min. The supernatant was filtered through a 0·22 μm Millipore membrane filter and concentrated 100 times in a rotary evaporator at 37°C. Uninoculated medium similarly concentrated was used as control. The presence of the toxin was determined by placing drops of the filtrate on 3·5 cm^3 blocks of mushroom sporophore tissues and on sporophores actively growing under environmental conditions similar to those in mushroom growing units on commercial farms.

Effect of culture filtrate on mycelial growth of *A. bisporus* was studied by placing sterilized antibiotic assay disks (Schleicher and Schuell No. 740-E, 12.7 mm diameter) impregnated with cell-free filtrate, 5 mm from the periphery of actively growing mycelium on malt agar plates.

Live cultures of *P. tolaasii* were also streaked on agar plates 5 mm from the periphery of actively growing mycelium. Inhibition of growth of the mycelium was recorded.

The production of toxin was determined at 25°C in 100-ml shake cultures of the medium of Palleroni and Doudoroff (1972) at intervals from 0 to 20 hr. Live cell counts were made by the Miles and Misra (1938) drop plate technique. Dry weight of cells was obtained by centrifuging the cell suspension at 10,000 *g* for 10 min, resuspending the pellet in small quantities of water, and evaporating to constant weight at 60°C. Concentrated cell-free extracts of each culture were bioassayed by a modification of the tissue block method of Gandy (1968).

The concentrated filtrates from shake cultures at different periods of growth (viz. 2, 4, 6, 8, 10, 11, 12, 13, 14, 16, 18, and 20 hr) were serially diluted and 0·05 ml of each dilution placed on a 3·5 cm^3 block of mushroom sporophore tissue; five replicates were used for each dilution. Degree of browning of the mushroom tissues was recorded after 24 hr. The toxicity index was calculated by taking the total number of tissue blocks showing browning at all the dilutions for each period of growth.

Heat stability of the toxin was tested by autoclaving concentrated culture filtrate at 121°C for 15 min. Tolerance to pH was tested by adjusting the pH of the concentrated culture filtrate with HCl or NaOH and placing drops of the solution onto mushroom blocks. Acid or alkali at each pH was placed on mushroom blocks as controls.

Results

Nutrient broth suspensions of *P. tolaasii* placed on detached mushroom sporophores but separated by a dialysis membrane caused brown discoloration and slightly sunken lesions in 6 hr. These were identical with the symptoms caused directly by *P. tolaasii* in commercial houses. No browning occurred when only the nutrient broth medium was used. Browning of mushroom tissue blocks and growing sporophores was also produced by cell-free culture filtrates.

The relationship between log number of bacterial cells and degree of browning is shown in Figure 1. The logarithm of the number of cells and dry weight of cells were found to increase during the log phase of growth from 4 to 16 hr. A corresponding increase in browning of mushroom tissues was also observed between 4 and 12 hr.

Autoclaving the cell-free culture did not prevent it from browning the mushroom tissues. The cell-free culture filtrate was also found to be active in browning mushroom tissues over a range of pH from 3 to 9.

Disks of cell-free culture filtrates placed at the periphery of a mushroom mycelial colony inhibited mycelial growth. Similar inhibition was also noticed when live cells were streaked alongside a growing mycelial colony.

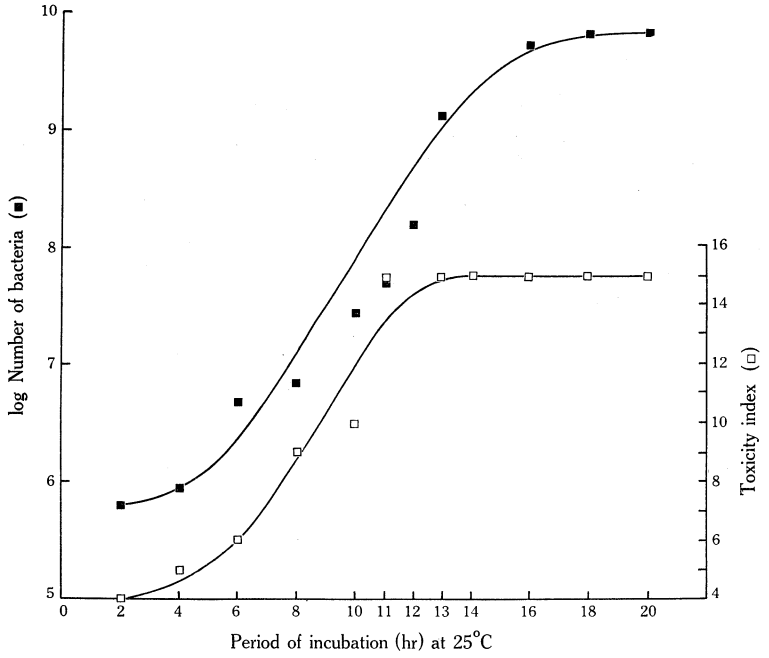

Fig. 1.—Relationship between log number of bacterial cells and toxin production.

Discussion

Browning of mushroom tissue blocks when nutrient broth suspensions of *P. tolaasii* separated by a dialysis membrane were placed on them points to the likelihood that an extracellular toxin was the cause of the browning. Since the toxin could pass through a membrane with a pore radius less than 20 Å, a substance of relatively low molecular weight is indicated. The production of a toxin was further confirmed when cell-free culture filtrates caused the browning of mushroom tissue blocks and sporophores actively growing under environmental conditions similar to those in commercial mushroom growing units on farms.

The relationship between growth of *P. tolaasii* and toxin production shows that the toxin is produced at the log phase of its growth. The toxicity index, as determined by bioassay using mushroom tissue blocks, did not show any further increase beyond the log phase into the stationary phase of growth of the bacterium.

The production of toxin *in vitro* and *in vivo* by *P. tolaasii* has significant bearing on its ecology in mushroom compost and casing peat on mushroom beds. The ability of the toxin to cause browning of mushroom tissues in a relatively short period of 6 hr points to the efficiency of its mechanism of infecting mushrooms. The observation that toxin inhibits mycelial growth of the mushroom is relevant to commercial growing as *P. tolaasii* may inhibit the growth of mycelium at the "spawn growing" stage of mushroom cultivation.

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