Discoloration and tyrosinase activity in *Agaricus bisporus* fruit bodies infected with various pathogens

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Accepted 28 April 1999.

The discoloration of *A. bisporus* after infection by *Pseudomonas tolaasii*, *P. gingeri*, *P. agarici*, *P. reactans*, *Verticillium fungicola* and *Trichoderma harzianum* can be distinguished by chromametric measurements. Infection with *P. tolaasii* caused a specific colour change, independent of the bacterial concentration applied ($\Delta L = 54.7$, $\Delta a = 16.6$, $\Delta b = 28.7$%). The colour change provoked by *P. gingeri* had a characteristic profile different from *P. tolaasii* ($\Delta L = 36.3$, $\Delta a = 14.1$, $\Delta b = 49.5$%). *P. agarici* and *P. reactans* provoked only a slight increase of red colour. Response to *T. harzianum* was orange, and to *V. fungicola* brown, similar to *P. tolaasii* but paler and more yellowish. All pathogens except *P. reactans* provoked degradation of total tyrosinase to variable extents. Activation of tyrosinase was only observed after infection with *P. tolaasii* (to almost 7%) and *V. fungicola* (to 3%).

White and off-white *Agaricus bisporus* still have by far the largest market share, in particular in the western hemisphere (80%). Brown discoloration usually is perceived as a symptom of senescence (decreased freshness) or microbiological deterioration (spoilage). Brown discoloration in mushrooms results from polyphenol oxidase action. In *Agaricus bisporus* U1 fruit bodies, only tyrosinase activity can be detected. This enzyme is normally found in a latent form. When activated, it can oxidase phenolic compounds into brown melanins (Boekelheide *et al*., 1979; Soulier & Arpin, 1993; Jolivet *et al*., 1995). It was activated during natural senescence (Burton, 1988), and also during infection by *Pseudomonas tolaasii* or exposure to its toxin, tolaasin, causing brown blotch disease symptoms (Soler-Rivas *et al*., 1997).

The symptoms of brown blotch disease are sunken, dark and brown spots (Olivier, Guillaumes & Martin, 1978). *P. tolaasii* is not, however, the only pathogen able to induce browning. *P. agarici* was described as responsible for the yellow blotch or drippy gill disease. This disease is characterized by pale brown discoloration. After only 24 h of infection, mushrooms showed a brownish marbled appearance (Geels, Hesen & Griensven, 1994). *P. gingeri* initially causes a pale yellowish-red discoloration which finally becomes a reddish ginger coloured spot (ginger blotch disease) covering the entire mushroom surface (Wong *et al*. 1982). Later *P. reactans* was described on the basis of its reaction with *P. tolaasii* in the White-Line test of Wong & Preece (1979). It was considered as non-pathogenic for mushrooms, but Wells *et al*. (1996) described a *P. reactans* strain as the cause of a mild dark, purple-light brown discoloration on mushrooms producing also a slight surface depression that, when the lesions became older, were deeper and darker.

Under certain conditions, *Trichoderma harzianum* strains induce a loss of mushroom crop yield. Fruit-body morphology is aberrant and affected mushrooms have dry brownish specks or lesions on the stem, especially near the base (Steane, 1978). *Verticillium fungicola* is responsible for dry bubble disease. The mushrooms show brown coloured spots or streaks on the basal or upper regions of the stem and on the developing caps (Dragt *et al*., 1996).

All these pathogens were shown to induce brownish discoloration. Only *P. tolaasii* and *V. fungicola* have been found to activate latent tyrosinase (6–8% in Soler-Rivas *et al*. (1997) and 'slightly' in Thapa & Jandaik (1989)). In this paper the discoloration induced by various pathogens is compared and the level of tyrosinases in infected mushrooms is measured to check whether the activation of tyrosinase is a generic response of the mushroom to variable microbial challenge.

MATERIAL AND METHODS

Mushroom strain

Agaricus bisporus (J. E. Lange) Imbach strain U1 (Somycel) (ATCC 62462) was used. The Mushroom Experimental

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Station, Horst, The Netherlands, supplied colonized compost beds, a few days before the onset of fruit-body production. Fruit bodies were grown at 18 °C and 80–85 % r.h.

Bacterial strains

The bacterial strains used were *Pseudomonas tolaasii*, *Pseudomonas agarici*, *Pseudomonas reactans* and *Pseudomonas gingeri*. *P. tolaasii* SPC 8907 was a subculture of *P. tolaasii* 112 S (Preece) (NCPPB no. 3148). *P. agarici* SPC bvk01 (Br. 01) was shown to provoke blotch disease in mushroom caps (Geels *et al.*, 1994). *P. reactans* SPC 8903 produced a white line precipitate when it was plated adjacent to *P. tolaasii* SPC 8907. All strains were supplied by The Mushroom Experimental Station, at Horst, The Netherlands.

P. gingeri 5327^{T1} (NCPPB 3146), was supplied by the LMG culture-collection (Universiteit Gent, Belgium). The type T_1 was described as pathogenic for mushrooms in 1983 when it was lyophilized.

Fungal strains

The pathogenic fungi used were *Trichoderma harzianum*, isolated and supplied by The Mushroom Experimental Station, Horst, The Netherlands, and *Verticillium fungicola* isolated from French caves and identified by INRA (Institut National de la Recherche Agronomique), Station de Recherches sur les Champignons, Centre de Recherches de Bordeaux.

Bacterial and spore suspension preparation

Bacteria were cultivated on Pseudomonas agar F medium (Difco) for 2 d at 24° on Petri dishes. The suspensions were prepared according to Soler-Rivas *et al.* (1997), using optical density at 450 nm as a measure of bacterial concentration (Olivier *et al.*, 1978). The correlation of O.D. with cell concentration (cfu ml^{-1}) was calculated as in Soler-Rivas *et al.* (1997).

Fungi were cultivated on malt extract agar (Oxoid) plates for 7 d (*Trichoderma*) or 15 d (*Verticillium*) at 25° on Petri dishes. Spores were collected and counted in a counting chamber with an optical microscope before and after storage at −80°. After storage and before inoculation on mushroom caps, dilutions of the spore extracts were plated on malt extract agar to determine their viability.

Inoculation procedure

Mushrooms of 30–40 mm cap diam., at stage 2 or 3 (classification according to Hammond & Nichols, 1975) from the first flush, were harvested and prepared as in Moquet, Mamoun & Olivier (1996), inoculated with 300 µl of the bacterial suspensions, or the spore dilutions as described in Soler-Rivas *et al.* (1997) and incubated for 2 d.

Bacterial suspensions were adjusted until absorbances of 0.2, 0.4, 0.8 and 1.0 a.u. at 450 nm were obtained. The spore suspensions were dilutions (× 10) of the stock suspensions (3.20×10^7 spores ml^{-1} for *T. harzianum* and 4.36×10^7 spores ml^{-1} for *V. fungicola* before viability text). Six mushroom caps per inoculum level were used. Mushrooms treated with 300 µl water were used as a control.

Browning measurements and tyrosinase activity

The natural colour of *A. bisporus* fruit bodies can be quantified by chromametry (Smith, Love & Burton, 1993; Sapers *et al.*, 1994). Colour changes were measured with a Chroma Meter (Minolta CR-200, 1 cm slot) using the *L*, *a*, *b* system as in Soler-Rivas *et al.* (1997). Increases (Δ) were calculated by measuring the samples immediately before inoculation and after the incubation ($\Delta L = L_0 - L_f$, $\Delta a = a_f - a_0$, $\Delta b = b_f - b_0$). Samples were then prepared and treated following the procedure described in Soler-Rivas *et al.* (1997). The powder obtained was used to measure the tyrosinase activity as L-DOPA oxidation activity according to Wichers *et al.* (1984).

RESULTS

Concentrations of pathogens

The correlation between optical density at 450 nm (from 0.2 to 1.0 a.u.) and bacterial cell concentration varied for each species (Fig. 1), but, all showed a similar correlation curve, characterized by an initial non-proportional phase, a linear phase and a saturated phase where the bacterial concentration is too large to be estimated by O.D. Optical density *v.* cell density-relations from the linear part of each curve were used to quantify bacterial cell concentration.

Most (94.5 %) of the *V. fungicola* spores were viable. Mushrooms were inoculated with dilutions from 4.12×10^7 to 4.12×10^4 viable spores. For *T. harzianum* only 83.2 % of the

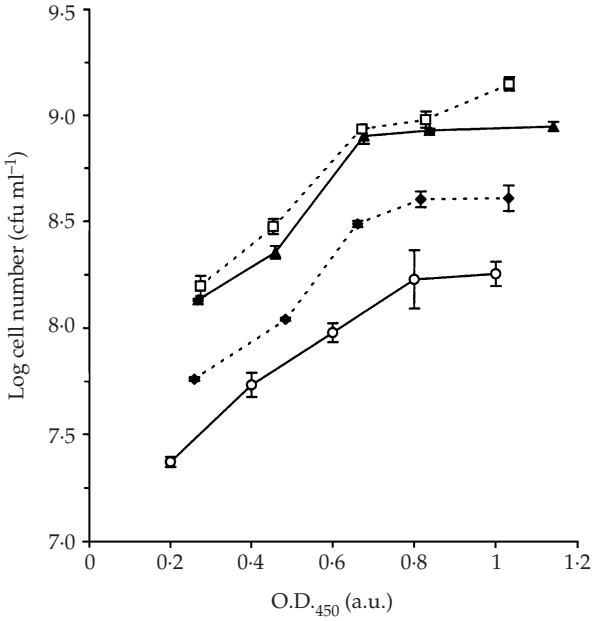


Fig. 1. Correlation curves for quantification of bacterial concentration by measuring O.D. at 450 nm. (—○—) *P. tolaasii*, (-- ◆ --) *P. gingeri*, (—▲—) *P. agarici* and (-- □ --) *P. reactans*.

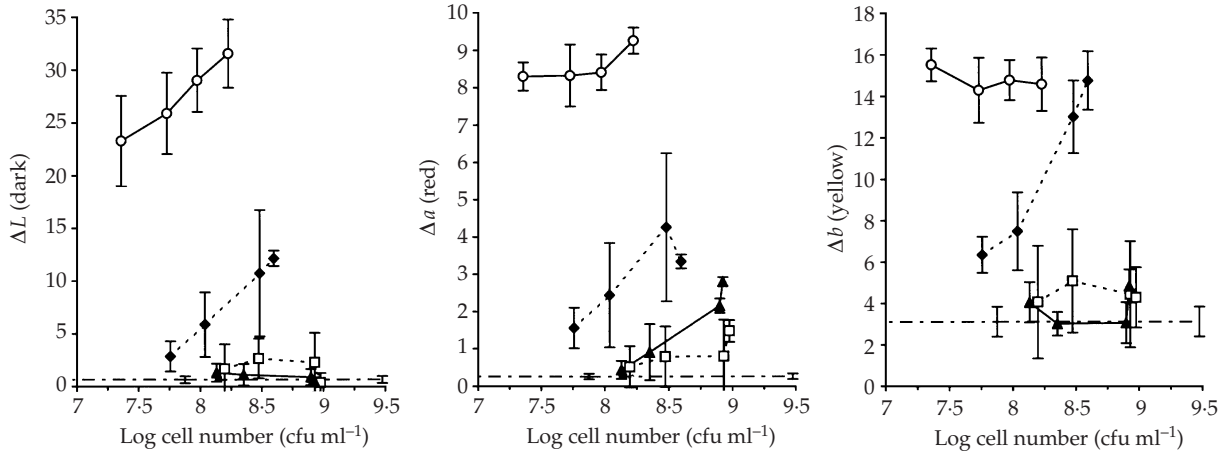


Fig. 2. Colour changes in mushroom caps after 2 d incubation with (—○—) *P. tolaasii*, (---◆--) *P. gingeri*, (—▲—) *P. agarici*, (--□--) *P. reactans* and (-—-) water (control). Decrease of brightness is represented as increase of darkness (Δ*L*). Change to red as (Δ*a*) and change to yellow as (Δ*b*).

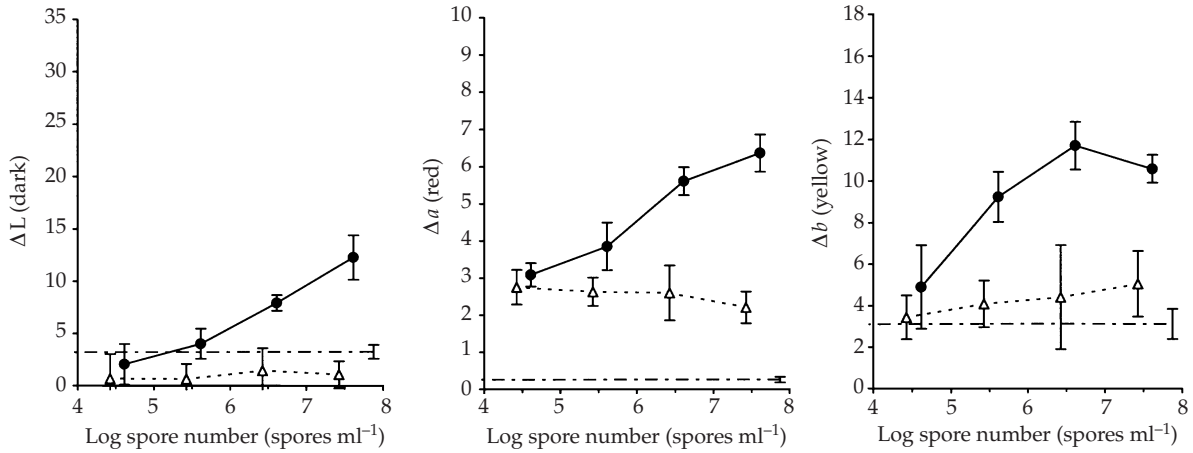
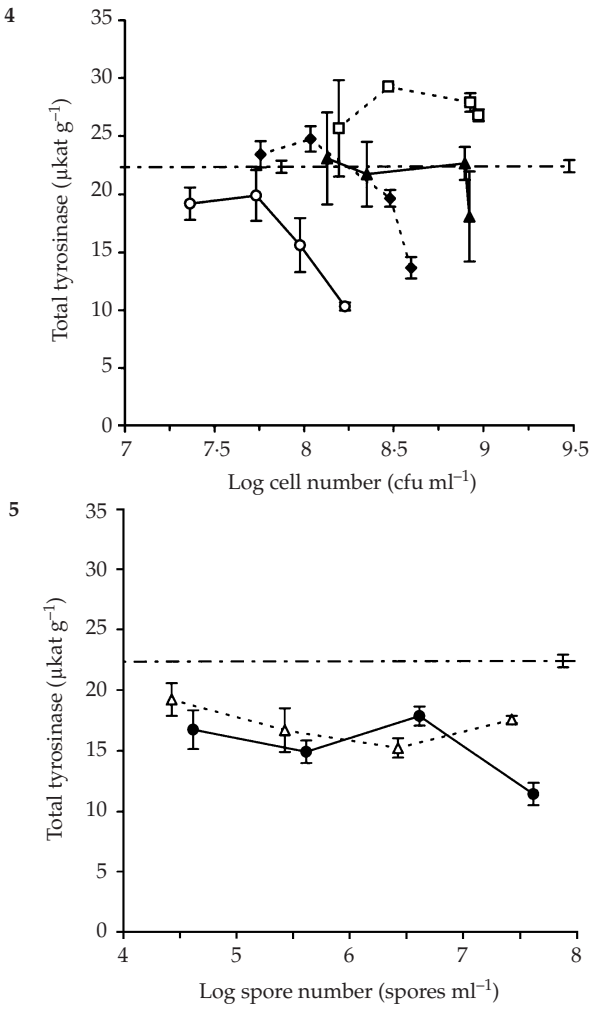


Fig. 3. Colour changes in mushroom caps after 2 d incubation with (—●—) *V. fungicola*, (--△--) *T. harzianum* and (-—-) water (control). Decrease of brightness is represented as increase of darkness (Δ*L*). Change to red as (Δ*a*) and change to yellow as (Δ*b*).

Table 1. Discoloration resulting from *Pseudomonas tolaasii* and *P. gingeri* infection. Increase in the *L*, *a* and *b* parameters. Percentage of colour related to the total sum of the three parameters.

O.D.$_{450}$	cfu ml^{-1}	Δ*L*	Δ*a*	Δ*b*	SUM	Δ*L*(%)	Δ*a*(%)	Δ*b*(%)
P. tolaasii								
0.2	2.3×10^7	23.3±4.3	8.3±0.4	15.5±0.8	47.1	49.4	17.6	32.9
0.4	5.4×10^7	25.9±3.8	8.3±0.8	14.3±1.6	48.5	53.4	17.2	29.5
0.6	9.5×10^7	29.0±3.0	8.4±0.5	14.8±1.0	52.2	55.6	16.1	28.3
0.8	1.7×10^8	31.6±3.2	9.3±0.3	14.6±1.3	55.5	57.0	16.7	26.3
1	1.8×10^8	32.1±5.8	8.4±0.3	14.7±1.8	55.2	58.2	15.2	26.6
Average						54.7	16.6	28.7
S.D.						3.5	0.9	2.7
P. gingeri								
0.2	5.7×10^7	2.9±1.4	1.6±0.5	6.4±0.9	10.9	26.6	14.7	58.7
0.4	1.1×10^8	5.9±3.1	2.4±1.4	7.5±1.9	15.8	37.2	15.5	47.3
0.6	3.0×10^8	10.8±6.0	4.3±2.0	13.0±1.8	28.1	38.4	15.3	46.3
0.8	4.0×10^8	12.2±0.7	3.3±0.2	14.8±1.4	30.3	40.2	11.0	48.8
1	3.9×10^8	11.9±2.4	4.3±1.4	14.1±1.4	30.3	39.3	14.2	46.5
Average						36.3	14.1	49.5
S.D.						5.6	1.8	5.2



Figs 4–5. Total tyrosinase activity in mushroom caps after 2 d incubation with **Fig. 4.** (—○—) *P. tolaasii*, (--◆--) *P. gingeri*, (—▲—) *P. agarici*, (--□--) *P. reactans*, (—●—) and (-—-) water (control). **Fig. 5.** (—●—) *V. fungicola*, (--△--) *T. harzianum* and (-—-) water (control).

spores were viable. Mushrooms were inoculated with 2.67×10^7 to 2.67×10^4 viable spores. These concentration ranges were similar to those used by Mamoun & Olivier (1995) where 10^5 spores ml^{-1} provoked symptoms after 54 h at 20°.

Changes in colour

Before inoculation, mushroom colour was described by a brightness (L) of 93.7 ± 0.4 U, a redness (a) of -0.4 ± 0.4 U, and a yellowness (b) of 7.5 ± 0.5 U. After inoculation and 2 d incubation values changed depending on the pathogen applied to the cap. The changes of colour due to water-treatment were almost insignificant in this incubation period.

The lowest bacterial concentrations were applied to mushrooms inoculated with *P. tolaasii*, but even at the lowest concentration the brightness changes were more intense than for any other bacterial pathogen and increased with increasing bacterial density (Fig. 2, ΔL). *P. gingeri* infection produced a less severe decrease of brightness (represented in Fig. 2 as increase of darkness, ΔL). *P. agarici* and *P. reactans* infected samples did not differ from the controls, even at the highest bacterial concentrations.

P. tolaasii-infected mushrooms were the reddest, with the level constant, only increasing with the highest inoculum (Fig. 2, Δa). The redness provoked by *P. gingeri* was less intense than for *P. tolaasii*. The red-values of *P. agarici* and *P. reactans*-infected samples increased only at the highest bacterial concentrations, in particular for *P. agarici*. The redness from these two last pathogens was not intense, when compared with *P. tolaasii* infection.

In the case of yellow colour changes, *P. tolaasii*-infected mushrooms also showed the highest values (Fig. 2, Δb), which were constant at all bacterial concentrations. *P. gingeri* caused a very steep and marked linear increase of yellowness, with increasing bacterial concentration. The highest concentrations produced values similar to those from *P. tolaasii* infection. *P. agarici* or *P. reactans* did not result in yellow-values that differed significantly from the control.

Of the fungal pathogens, *V. fungicola* also reduced the level of brightness in inoculated mushrooms (Fig. 3, ΔL). The darkness increased with higher spore number until levels similar to those caused by *P. gingeri* were reached. *T. harzianum* infection did not show any change of brightness differing from the control.

V. fungicola provoked a clear and marked increase in the red colour correlated to spore concentration of higher intensity as induced by *P. gingeri* (Fig. 3, Δa). *T. harzianum* infections caused also a reddish discoloration on the caps of a similar magnitude as *P. gingeri* infection, but not related to increasing spore concentrations.

V. fungicola inoculated mushrooms showed a considerable increase in the yellow-value (Fig. 3, Δb). *T. harzianum* provoked only a small increase in the yellow-value in samples treated with the highest concentrations.

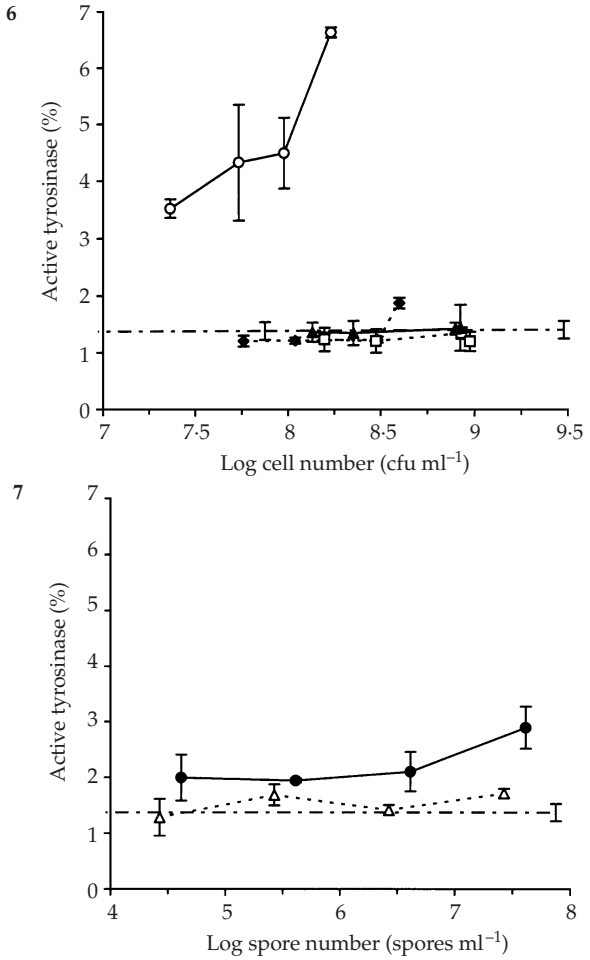
The sum of ΔL, Δa and Δb was calculated for each pathogen/inoculum concentration and each parameter was expressed as percentage of the sum. The percentage was found constant within the different bacterial concentrations in samples infected with *P. tolaasii* and *P. gingeri* (Table 1).

Changes in tyrosinase activity

The total tyrosinase activity (latent and active) levels in mushrooms infected with bacteria are shown in Fig. 4. A clear decrease in the values upon increase of bacterial concentrations for all strains was observed. *P. tolaasii* infection causes the largest decrease in total tyrosinase. *P. gingeri* infection also resulted in a decrease in total tyrosinase activity, albeit less intense. Some degradation of tyrosinase was also observed when mushrooms were treated with the highest *P. agarici* concentrations.

V. fungicola and *T. harzianum* infected mushrooms showed values of total tyrosinase lower than the control (Fig. 5). The degradation level for *V. fungicola* seemed not to be correlated with the increase of inoculum. *T. harzianum* also induced degradation of total tyrosinase of similar magnitude as *V. fungicola*.

The percentage of active tyrosinase, calculated relative to



Figs 6–7. Active tyrosinase (%) in mushroom caps after 2 d incubation with **Fig. 6.** (—○—) *P. tolaasii*, (--◆--) *P. gingeri*, (—▲—) *P. agarici*, (--□--) *P. reactans*, (—●—) and (-—-) water (control). **Fig. 7.** (—●—) *V. fungicola*, (--□--) *T. harzianum* and (-—-) water (control).

total tyrosinase activity (Fig. 6) showed a very high activation in samples infected with *P. tolaasii* (3.5–6.6%). A small increase in the percentage of active tyrosinase in the sample infected with the highest *P. gingeri* concentration was found. Samples infected with *P. reactans* and *P. agarici* showed similar values to the control.

Infection with *V. fungicola* (Fig. 7) resulted in a 3% increase in the percentage of active tyrosinase. This activation was much less pronounced, however, than that caused by *P. tolaasii*. *T. harzianum* infected samples did not differ from the control.

DISCUSSION

P. tolaasii causes brown blotch disease characterized by brown coloured spots. In this study, *P. tolaasii*-inoculated samples showed a very pronounced decrease of cap brightness (L), with high and almost constant values for red and yellow. *P. gingeri*, causing ginger blotch disease, resulted in a very pronounced increase of yellowness and smaller decrease of cap brightness than *P. tolaasii*. Both types of infections could be distinguished and characterized by the chromametric measurements, because the ratios of ΔL, Δa and Δb were constant at any bacterial concentration and differing from each other. The system could be used for a fast preliminary identification when mushrooms show brown spots.

P. agarici infection caused only a small increase in redness. The strain used was not very virulent compared to *P. tolaasii* and *P. gingeri*. *P. reactans* also produced a slight increase in redness. This is perhaps what Wells *et al*. (1996) described as 'light purple brown colour'. The drawing of conclusions is hampered, however, by high variance in the measurements and a bacterial strain that is highly unstable.

The disease caused by *V. fungicola* (dry bubble) is also called brown blotch because of its similarity with the *P. tolaasii* blotch symptoms. *V. fungicola* infection showed an increase in all three parameters, to give a brown mixture of colours, different from the 'brown' of *P. tolaasii* infection, because it was paler and yellower (pale brown).

V. harzianum infection in the mushroom beds also leads to light brown spots, with a small increase in yellow and a higher and constant value for red. This resulted in an 'orange' mixture that could be considered as the pale-brown described in the literature.

The differences in colour within the different diseases might suggest that melanin is being formed through different intermediate compounds or that other pigments could interfere or play a role in some of the infections. The different bacterial or spore loading on the caps and the differences in virulence could also influence the development of the discolorations.

All pathogens that were able to provoke intense discolouration on the mushroom caps caused also a decrease in the total tyrosinase activity. Proteases excreted by these pathogens (Kalberer, 1984; Baral, Fox & O'Connor, 1995) may degrade the enzyme as proteases are known to be able to degrade tyrosinases (Burton, Love & Smith, 1993).

P. tolaasii infected samples possessed high active tyrosinase levels, even in samples infected with the lowest bacterial concentration. *V. fungicola* infection also resulted in an increase, but less than for *P. tolaasii*. These results confirm earlier observations by Thapa & Jandaik (1989) and Soler-Rivas *et al*. (1997). In these cases, their proteases may also be involved in the activation, the active form being an intermediate stage before degradation. Differences in specificity of proteases from various pathogenic organisms may explain the temporary accumulation of active tyrosinase in, for instance, *P. tolaasii* infected samples. The very specific activation in samples infected with *P. tolaasii* might be due to the effect of tolaasin. This toxin has surface active properties (Hutchison & Johnstone, 1993), and detergents are also described as able to activate tyrosinase (Yamaguchi, Hwang & Campbell, 1970; Jiménez Cervantes *et al*., 1995). In these samples, the separate effects of both protease and tolaasin could cause the high tyrosinase activation, appearing as a rather specific aspect of the response of *A. bisporus* to *P. tolaasii* infection.

ACKNOWLEDGEMENTS

This work was supported by the grants AIR 1 CT 94-5959 and FAIR CT 96-5062 from the European Commission under the framework of the Agriculture and Fisheries programmes AIR and FAIR.

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Corresponding Editor: I. C. Tommerup