Determination of *Pseudomonas tolaasii* Threshold Concentrations Required to Produce Symptoms of Bacterial Blotch Disease in *Pleurotus eryngii*

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Abstract: *Pseudomonas tolaasii* causes bacterial blotch disease of many cultivated fungi including *Agaricus bisporus* and *Pleurotus* spp. Concentration and amount of bacterial inoculum are critical for symptom development. A minimal concentration of 2×10^6 cells per inoculation site has been reported for pilei surfaces of *A. bisporus*. However, the same concentration of bacteria was not sufficient to produce symptoms on basidiomata of *P. eryngii*. To determiné the effect of inoculum concentration on disease development, we produced *P. eryngii* (WC-888) on a mixture (contained in 1050 ml polypropylene bottles) of cottonseed hulls (62%), ground soybean (6%), oak sawdust (27%), corn distiller's waste (4%), and calcium sulphate (1%). Mushrooms were harvested at the same developmental stage (margins and pileus completely flat) for disease assays.*Pseudomonas tolaasii* was grown for 40 hours in King's B broth. The bacterial suspension (20 ml) was centrifuged (6,500 x g) and rinsed twice with sterile distilled water. From this stock concentration, several bacterial suspensions, 0.1 - 2.0 OD_{450}, were prepared. The external layer of the pilei and freshly cut pilei tissue of the *P. eryngii* basidiomata were inoculated (50 µl). Mushroom pilei were incubated in individual moist chambers at 16°C for 48 hours. Presence or absence of disease symptoms was recorded. A minimal concentration of 5 x 10^6 bacterial cells (0.3 OD_{450}) per inoculation site was necessary for symptom expression on surfaces of cut pileus tissue. However, a nearly 5-fold higher concentration of bacteria [2.6 x 10^7 (1.6 OD_{450})] was required to produce symptoms on the intact surface of the pileus. Bacterial concentration and tissue type were critical factors in disease development and symptom expression.

Key words: Bacterial blotch disease, *Pleurotus eryngii, Pseudomonas tolaasii,* bacterial inoculum size, symptom expression, inoculation site

1 Introduction

Bacterial blotch disease causes crop losses *(A. bisporus)* estimated at 8-10% worldwide[1] Economically important losses also are reported on *Pleurotus* spp., *Lentinula edodes, Flammulina velutipes,*[2] and *A. bitorquis.*[3] On *Pleurotus* eryngii, the unsightly brown lesions make the mushrooms unmarketable with crop losses estimated at about 25-35% per year.[4] None of the commercial strains of *P. eryngii* are resistant to bacterial blotch. Symptoms of bacterial blotch disease vary among species. In *P. eryngii,* infection may cause single, depressed and dispersed spots of some 1-4 mm in diameter. Other symptoms may include cracks on the pileus that may extend to the stipe. The lesions may be yellow-orange, dark hazel-brown, or rusty-orange in color and may be both diffused and uniform. Over time the lesions may become darker to reddish-brown, dark rusty-red or brownish. On the stipe, the infection may produce long or short striations that are sometimes depressed. Under high humidity, a thin viscous layer of bacteria may cover the whole pileus. Mushrooms of any development

stage may become infected. The infected basidiomata may stop expanding, rot, and produce a foul odor that may attract flies. Bacteria may be present in the substrate during spawn run and fructification.[5]
The role and the mode of action of *P. tolaasii* strains in the development of brown blotch disease have been studied in detail for the *A. bisporus* system and, to a lesser extent, in *P. ostreatus.* Little is known about the interaction between *P. tolaasii* and *P. eryngii,*[4, 6] and attempts to inoculate *P. ostreatus* fruiting bodies with *P. tolaasii* have not reliably been successful.

2 Materials and Methods

2.1 *Pleurotus eryngii*

Strain WC-888 (spawn laboratory, Penn State University) was used for this experiment. Mushrooms were produced on substrate containing ingredients as follows: cottonseed hulls (62%), ground soybean (6%), oak sawdust (27%), corn distiller's waste (4%), and calcium sulphate (1%). Mushrooms were harvested at the same developmental stage (margins and pileus completely flat) for disease assays.

2.2 *Pseudomonas tolaasii*

The bacteria strain (I5) used in this experiment was isolated from an infected *P. eryngii* basidiocarp following Koch's postulates. The infected mushroom was obtained from a mushroom farm located in Kennett Square, PA. PCR (primers Pt-1D1 and Pt-1A)[2, 7] was used to ensure identity of the isolate.

2.3 Bacterial suspension

Bacteria were grown for 40 hr in King's B broth (50 ml) contained in 250 ml Erlenmeyer flasks. Aliquots (20 ml) were centrifuged at 6,500 x g for 10 minutes. The media was discharged and the remaining bacteria pellet resuspended in sterile water using a vortex. This process was done twice to ensure the removal of any media residue. The bacterial suspension was adjusted to different concentrations by measuring the optical density at 450 nm in a Beckman spectrophotometer DU 640B. Bacterial inocula concentrations ranged from 0.1 to 2.0 OD_{450}. Bacterial number was determined by dilution and plateing. Concentrations of 0.3 OD_{450} yield approximately 10^8 cfu/ml.

2.4 Evaluation of disease severity *in vitro*

Inoculation of basidiomata with bacteria was carried out in two ways: 1) inoculation of the external layer of the pilei and 2) inoculation of a cut surface of the pileus tissue (Figure 1). Aliquots of 50 µl were used in both cases. Presence or absence of blotch symptoms was recorded after incubation of the samples for 48 hours in individual moist chambers (9.5cm x 7.5cm Petri dishes with filter paper and 6 ml of sterile water) at 16°C.

3 Results

3.1 Inoculation of pileus cuticle

Bacterial suspensions ranging from 0.1 to 1.5 OD_{450} did not cause disease on intact pilei. However, lesions were observed at bacterial concentrations of 1.6 OD_{450} and higher. A concentration of 1.6 OD_{450} yields approximately 2.6 x 10^7 bacteria per inoculation site (per 50 µl).

3.2 Inoculation of cut pileus tissue

Bacterial suspensions of 0.1 and 0.2 OD_{450} did not cause blotch. However, concentrations of 0.3 OD_{450} (5×10^6 bacteria per inoculation site) and higher produced disease symptoms.

3.3 Lesion morphology

Lesions observed on the pileus cuticle and fleshly cut tissue differed in morphology. Severe sunken, water-soaked lesions (Fig. 2) were produced on cut surfaces when the bacterial concentration was higher than 0.5 OD_{450}. Slightly sunken yellowish lesions or spots were observed at bacterial concentrations lower than 0.5 OD_{450} (Fig. 3). However, lesions on the cuticle of the pilei external layer of the pilei were not water-soaked, even at concentrations as high as 2.0 OD_{450} (Fig. 4).

Fig. 1: Longitudinal cut of a pileus of *P. eryngii* showing exposed context and inoculation site

Fig. 2: Sunken, water-soaked lesions on exposed surfaces of P. eryngii pilei produced by inoculation with *P. tolaasii* (1.0 OD_{450})

Fig. 3: Yellow lesions on cut surfaces of *P. eryngii* pilei produced by inoculation with *P. tolaasii* at 0.3 (top) and 0.4 (bottom) OD_{450}

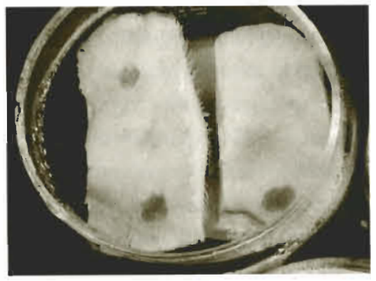

Fig. 4: Sunken lesions on intact surfaces of *P. eryngii* pilei produced by inoculation with *P. tolaasii* (2.0OD_{450})

4 Discussion

The browning reaction or discoloration of *A. bisporus,* related to tissue ageing and to bacterial disease caused by *P. tolaasii,* is well known.[8-10] The degree of discoloration of tissue of *A. bisporus* caused by *P. tolaasii* may be related to the level of active tyrosinase present in mushroom tissue. Tyrosinase activity is on average three times greater on the outer surface than in the underlying tissue. This could explain why browning is more prevalent at the surface.[11] In fact, histochemical and immunochemical studies on the location of tyrosinase in *A. bisporus* show that the enzyme is localized in the stipe, lamellae, and cuticle of the pileus and less in the pileus context.[12]

The process leading to discoloration of tissue in *Pleurotus* spp. caused by *P. tolaasii* is not known. However, our results show that disease development and symptom expression differs on different tissues of the basidiomata. This suggests that presence or levels of some compounds or enzymes in the mushroom tissue may be different and may influence severity of symptoms of bacterial blotch disease. Tyrosinase has not been identified in *P. eryngii* tissue, so a first step for further research may be to determine if this enzyme is present and, if so, at what concentration and how it is distributed in the basidiomata.

Bacterial blotch of *P. eryngii* remains a serious problem for some growers of this mushroom. The determination of factors that influence disease development and severity may be a first step in helping growers manage this disease.

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