RECENT ADVANCES ON BACTERIAL DISEASES OF CULTIVATED MUSHROOMS

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ABSTRACT

Recent studies on bacterial diseases of cultivated mushrooms in southern Italy showed that brown blotch of *Agaricus bisporus* and the yellowing of *Pleurotus ostreatus,* caused by *Pseudomonas tolaasii,* are actually complex diseases since, besides *P. tolaasii,* which may be considered the main causal agent of the above diseases, also *P. reactans* and not yet characterised fluorescent pseudomonads appear to participate to the expression of the diseases symptoms. Furthermore, *P. reactans* appears to be the causal agent of *P. eryngii* yellowing. Strains of *P. tolaasii* produce tolaasin I and II (Tol I and Tol II) and five minor analogs called tolaasins A-E). Tolaasins are involved in the pathogen virulence causing membranes lysis through biosurfactant activity and transmembrane ion channel formation for which a barrel-stave mechanism has been proposed. Furthermore, strains of *P. tolaasii* produce *in vitro* an array of volatile substances which are apparently involved in the virulence of the producers and the pathogen/mushrooms interaction. Strains of *P. reactans* produce the White Line Inducing Principle (WLIP) which causes the brown discoloration of *A. bisporus* tissues though at lesser extent than tolaasin I. The loss of WLIP production by avirulent morphological variants of *P. reactans* supports its role in the pathogen virulence. The formation of avirulent variants in the cultures of *P. reactans* strains appears to be responsible for the attenuation/loss of virulence and this may tentatively explain why the pathogenicity of *P. reactans* was neglected and/or not well understood in the past. A comparative evaluation of Tol I and WLIP on blood red cells and artificial lipid vesicles demonstrated a detergent-like mechanism for WLIP. REP-PCR analysis showed that *P. reactans,* on the contrary of *P. tolaasii,* is not a genetically uniform group.

Keywords: *Agaricus bisporus*; *Pleurotus ostreatus*; *Pleurotus eryngii*; *Pseudomonas tolaasii*; *Pseudomonas "reactans".*

INTRODUCTION

World production of cultivated mushrooms is growing and, in fact, from 1,500,000 tons in 1985 [1] it rose to 3,206,738 tons according to FAO data in 2007, with China as the first world producer followed by United States of America and several European Countries. The Italian annual production of cultivated mushrooms is about 90.000 tons [2], and the most cultivated mushroom is the button mushroom *Agaricus bisporus* (Lange) Imbach followed by the oyster mushroom *Pleurotus ostreatus* (Jacq. ex Fr) Kum and a few other mushroom species including the king oyster mushroom (cardoncello) *P. eryngii* (DC ex Fr).

Cultivated mushrooms are attacked by a number of fungal and bacterial diseases that may cause significant production losses [3, 4, 5]. These occurrences are due to environmental conditions to which the mushroom cultivation is generally carried out. Though reliable data in this respect are not available, it is believed that the bacterial diseases are the main causes of product loss [3]. Several bacterial diseases of cultivated mushrooms of the genus *Agaricus* and *Pleurotus* are caused by fluorescent pseudomonads [5]. This is the case of brown blotch

disease of *A. bisporus* [6] and the yellowing of *P. ostreatus*, both caused by *P. tolaasii* [5]. Brown blotch symptoms on *A. bisporus* are also caused by *P. reactans* [7, 8, 9, 10], *Pseudomonas* sp. strain NZ17 apparently related to *P. syringae* [11] and *P. costantinii* sp.nov. [10]. The ginger blotch disease of *A. bisporus* is caused by *P. gingeri* [12], the mummy disease of *A. bisporus* is caused by *Pseudomonas* spp. [4, 13] and the drippy gill of *A. bisporus* [14] is caused by *P. agarici.* The latter pathogen was also reported as the causative agent of the yellowing of *P. ostreatus* observed for the first time in California [15] and, more recently, of the brown discoloration of mushrooms [8, 16, 17] observed in The Netherlands and Italy.

Also the yellowing of the king oyster mushroom (cardoncello), a mushroom whose cultivation was initially limited to some regions of southern Italy but nowadays cultivated worldwide, was reported to be caused by *P. tolaasii* [18, 19] or by *P. reactans* and fluorescent Pseudomonads [8, 9 ,20]. The etiology of this disease is not yet completely established.

Bacterial disease of cultivated mushrooms caused by bacteria other than fluorescent pseudomonads are the soft rot of *Agaricus* spp. caused by *Burkolderia gladioli* pv. *agaricicola* (ex *Pseudomonas gladioli* pv. *agaricicola*) and *Janthinobacterium agaricidannosum* sp. nov [21]. *B. g.* pv. *agaricicola* is also responsible for the cavity disease. *Ewingella americana* is responsible for the internal stipe necrosis of *A. bisporus* [22, 23].

The frequent occurrence of a variety of the mushroom alterations, probably caused by bacterial infections, in cultivation of *A. bisporus*, *P. ostreatus* and *P. eryngii* in several mushroom farms located in Apulia and Basilicata, Southern Italy, prompts a series of studies with the final aim to define the agent(s) responsible for the alterations observed, as well as to study the mechanisms of host pathogen interactions. Here, we present some results on the etiology of brown blotch disease of *A. bisporus* and yellowing of *P. ostreatus* and *P. eryngii* as well as some aspects of *P. tolaasii* and *P. reactans* interaction with the mushroom hosts.

ETIOLOGICAL ASPECTS

Brown blotch disease. The brown blotch disease of the button mushroom *Agaricus bisporus*, caused by the bacterium *Pseudomonas tolaasii* [5], was observed for the first time in some mushroom farms in the United States of America [24] and its etiology was defined a few years later [25]. In Italy, the disease has been reported for the first time in 1970 in a mushroom farm in Apuglia [26]. The disease is characterized by brown, irregular, sunken lesions on the pileus and/or stipe. Under favorable environmental conditions the lesions, initially small and separated, coalesce affecting large areas of the pileus which may gradually decay with the formation of a strong and disagreeable smell. This may occur also after harvest [7].

In our experience from *A. bisporus* sporophores showing brown blotch lesions, bacterial **c**olonies with different morphologies were ever obtained [8, 9]. In particular, from the altered pseudotissues were isolated bacteria that were positive in the white line assay (WLA) [12] when grown on agar media near to strain NCPPB1311 of *P. reactans*, and these isolates, as expected, showed the nutritional, biochemical, and pathogenicity characters of *P. tolaasii* strains [27, 28]. In the same isolation plates in addition to *P. tolaasii*, other bacteria with the biochemical characteristics of the fifth group of fluorescent pseudomonads [28] were ever obtained. Some of these isolates were identified as strains of *P. reactans* since they were positive in the WLA when grown on agar media near to type strain of *P. tolaasii* NCPPB2192 and showed the nutritional characteristics of *P. reactans* [27]. The simultaneous presence of *P. tolaasii* and *P. reactans* in altered mushroom tissues very often gave rise to white line precipitates between colonies of different morphologies. *P. reactans* strains caused the browning of *A. bisporus* tissue blocks, although at different levels depending on the strains, but the effect was lower when compared to *P. tolaasii*. The suspensions still capable to cause visible alterations of tissues was 10^6 c.f.u./ml or less in the case of P. t*olaasii* and 10^7-10^8 c.f.u./ml for *P. reactans* [8, 9]. Similar results were

independently obtained by other authors [7, 29].

Other bacteria with the biochemical characteristics of the fifth group of fluorescent pseudomonads [28], not yet characterized but negative in the WLA, caused alterations of the tissue blocks in a comparable way to those of certain strains of *P. reactans* [8, 9].

Yellowing of oyster mushrooms. The yellowing of the oyster mushrooms *Pleurotus ostreatus*, a disease caused by *Pseudomonas tolaasii*, may interest all stages of development of the mushroom sporophores [3, 5, 18].When the disease occurs at the early differentiation stage the young sporophores turn to a yellowish-reddish color, show a slow development followed by a rapid wilting. The alteration can affect the whole bunch or parts of it. On developed sporophores depressed, yellowish-reddish lesions, sometimes surrounded by yellow-reddish halos may interest pilei and/or stipes. Under high temperature and humidity sporophores rapidly rot with the production of an unpleasant smell. Sometimes, yellow superficial discolored lesions may interest the whole sporophore or part of it. The disease has an unpredictable course. In the same cultivation all the substrate bags in production or only part of them may be interested by the above symptoms. In some bags the disease appears severe with a significant loss of production while in others only a change in color of sporophores is observed. The disease may affect the first flush and then disappear or vice versa; in other occasions the disease interest the cultivation throughout the period of production. Nothing is known about the factors that determine the strange course of the disease.

In our experience from *P. ostreatus* sporophores showing depressed, yellowish-reddish lesions bacterial **c**olonies with different morphologies were ever obtained. In particular, from the altered pseudotissues were isolated bacteria that were positive in the white line assay (WLA) [12] when grown on agar media near to strain NCPPB1311 of *P. reactans* and these isolates, as expected, showed the nutritional, biochemical, and pathogenicity characters of *P. tolaasii* strains [27, 28]. In addition to *P. tolaasii*, other bacteria with the biochemical characteristics of the fifth group of fluorescent pseudomonads [28] were obtained. Some of these isolates were identified as strains of *P. reactans* since they were positive in the WLA when grown on agar media near to type strain of *P. tolaasii* NCPPB2192 and, furthermore, they showed the biochemical and nutritional characteristics of *P. reactans* [27]. As expected *P. tolaasii* and *P. reactans* strains, in the pathogenicity assays on *A. bisporus* blocks, confirmed the higher virulence of the former when compared to the latter strains which in addition showed also a higher variability in this feature. Strains of *P. tolaasii* assayed for the virulence on *P. ostreatus* sporophores caused different symptoms depending on the inoculation procedure. In particular, they caused deep depressed lesions when their suspensions were injected by hypodermic syringes in the pileus flesh. In contrast, when drops of the same suspensions were deposited on the sporophore surface, only slight depressed yellow lesions were observed. When suspensions were sprayed on young not fully developed *P. ostreatus* sporophore bunches, they turned yellow, stunted and wilted. The same assay on more developed sporophore bunches caused the general yellowing followed by wilting. Only in some cases also depressed brownish-orange lesions were observed. It is not excluded these latter symptoms are consequence of bacteria penetration through micro wounds on the sporophore surface.

Strains of *P. reactans* in the above pathogenicity assays on *P. ostreatus* sporophores caused the bunches yellowing as well as sporophore treated tissues; they never caused depressed lesions. In general the virulence was lower when compared to the one caused by *P. tolaasii* strains.

Other fluorescent pseudomonads obtained in the same isolation plates beside *P. tolaasii* and *P. reactans* and negative in WLA showed a pathogenic feature similar to that of *P. reactans* strains and differed for some nutritional features from both *P. tolaasii* and *P. reactans.* They shared more nutritional characters with strains of *P. tolaasii* than with those of *P. reactans, P.*

agarici, P. gingeri and *Pseudomonas* spp. causal agents of the mummy disease used for comparison [30]. On the contrary, some of the above described fluorescent pseudomonads showed the nutritional feature of *P. tolaasii.* Apparently, the latter WLA negative isolates appeared to be hypovirulent strains of *P. tolaasii* which have lost production of the tolaasins. It is not clear if this happened being associated with the mushroom or during the isolation plate procedure.

These results indicate that all the above described bacteria appear to contribute, though at different degrees, to the development of symptoms of the yellowing of *P. ostreatus*. In particular, *P. tolaasii* cause depressed yellowish-reddish lesions. On the contrary *P. reactans* and the other fluorescent pseudomonads appear mainly responsible for the yellowish discoloration of whole or part of the sporophores. This evaluation is supported by the fact that bacteria with the feature of *P. reactans* and/or the negative WLA above described fluorescent pseudomonads were mainly obtained from superficial yellow discolored lesion on full developed sporophores or from yellow and mummified young sporophores.

Yellowing of cardoncello. The yellowing of the king oyster mushrooms (cardoncello) *Peurotus eryngii* is a disease whose etiology was not well defined for many years. Initially it was attributed to fluorescent pseudomonads related to *P. tolaasii* [18], but other studies indicated that fluorescent pseudomonads different from *P. tolaasii* were associated to the altered mushrooms [20]. The first symptoms of the disease are light brown discoloration of the pilei which then turn into reddish-brown. Stem symptoms are represented by hydropic areas, often elongated, which may coalesce, interesting the whole stems. Symptoms of the disease may interest primordia still in the casing soil layer or on fully developed sporophores. Mushroom sporophores turn stunted and wilted and then, under high temperature and humidity condition, bacterial exudates form on the sporophores which rapidly rot with the production of an unpleasant smell. The disease shows very unique features since it may occur in a disruptive way in the first flush and then it disappears resulting in normal production and vice versa; in other cases the disease may lead to the entire production loss. The disease initially can be localized to a substrate bags in production possibly interesting only a part of a mushroom bunch and then shortly the whole cultivation.

In our experience the isolation from the king oyster mushrooms sporophores showing the yellowing symptoms gave rise to different fluorescent pseudomonads. Most of them were positive in the *A. bisporus* tissue block assay [26] though the intensity of the alteration was lower than the one caused by strains of *P. tolaasii* used for comparison. Some of these bacterial isolates, obtained repeatedly in different isolations, were positive in the WLA when grown near type strain NCPPB2192 of *P. tolaasii,* belonged to the group V of fluorescent pseudomonads and showed the biochemical and the differential nutritional characters of *P. reactans* [27].

Pathogenicity assays *in vivo* on bags of *P. eryngii* commercial substrate by adding in the casing soil suspensions of strain P. NCPPB1311 of *P. reactans* naturally resistant to rifampicin allowed to demonstrate, at least in part, the Koch's postulates. From sporophores showing the classical yellowing symptoms bacteria resistant to rifampicin with the same characteristics of the *P. reactans* strain used in artificial inoculations and other not rifampicin resistant bacteria with the features of *P. reactans* as well as other bacteria belonging to group V of fluorescent pseudomonads were obtained [9,31]. In some occasion, the artificial inoculation with *P. reactans* strains was unsuccessful for so far unknown reasons.

These results indicate that *P. reactans* can be considered responsible for yellowing of cardoncello. The fact that bacteria with the feature of *P. reactans* were obtained from a wild cardoncello sporophore showing a large area of yellowing on the pileus reinforces this possibility (unpublished results). It is not excluded, however, that also the fluorescent pseudomonads, isolated together with strains of *P. reactans* may be responsible and/or contribute to the development of' the yellowing of cardoncello. In our experience from the cardoncello

sporophores with symptoms of yellowing, bacteria with the feature of *P. tolaasii* have never been obtained. In this regard other authors reported the latter pathogen as the causal agent of the "batteriosi del cardoncello" [18] or bacterial blotch of *P. eryngii* [19]. However, these authors refer to yellowish depressed blotch as the main symptom. Although further evidences are necessary it is not excluded that, as in the case of *P. ostreatus*, the different symptoms are related to the different pathogens involved. In fact, in pathogenicity assays when drops of strains of *P. tolaasii* suspensions were deposited or injected on *P. eryngii* sporophores dark brown-reddish depressed lesions were observed. In the same assays strains of *P. reactans* caused brown-reddish and superficial lesions. The latter symptoms resemble the natural ones caused by *P. tolaasii* and *P. reactans*, respectively.

ASPECT OF MUSHROOM-PATHOGENS INTERACTIONS

***Pseudomonas tolaasii* lipodepsipeptides.** *P. tolaasii* is a common pathogen of cultivated mushrooms such as *Agaricus bisporus*, *Pleurotus ostreatus* [5] and apparently *P. eryngii* [18, 19]. Virulent strains of *P. tolaasii* produce the lipodepsipeptides (LDPs) tolaasins which appear to be responsible for symptom development [32, 33].

Two components of the toxin preparations, designed tolaasin I (Tol I) and tolaasin II (Tol II), were first identified [34]. The primary structure of Tol I bears a β-OH octanoic acid blocking group at the N-terminus, a sequence of seven successive D-amino acids at the N-terminal region of the peptide (Pro 2 –Val 8), with a Ser-Leu-Val repeat, and then alternate L- and D-amino acids. It also contains a 2,3-dehydro-2-aminobutyric acid □(ΔBut) residue at positions 1 and 13, a D-homoserine (Hse 16) and a D-2,4-diaminobutyric acid (D-Dab 17). Finally, a lactone ring is formed between the hydroxyl of D-Thr 14 and the C-terminal L-Lys 18. Tol II differs from Tol I for the amino acid in position 16th in the peptide moyety: homoserine in Tol I and glycine in Tol II. More recently five other tolaasin analogs, called tolaasins A–E, have been isolated from the culture filtrate of type strain NCPPB2192 of *P. tolaasii* [35]. These tolaasin analogs showed chemical modifications in the fatty acid residue or in the lactone macrocycle. In particular, Tol A, D and E as well as tol II showed a modification in one or two aminoacidic residues at position 15 and/or 16 in respect of Tol I while tolaasin B exhibited a different fatty acid residue linked to the N-terminal residue. Tol C, showing the opening of the macrocyclic lactone ring, could be an artifact hydrolysis product of Tol I. However, this hypothesis is not confirmed, as the corresponding linear product originated from the other tolaasins was not found. The antimicrobial activity of HPLC grade tolaasins A-E, assayed in comparison with tolaasin I and II showed the gram-positive bacterium *Bacillus megaterium* as the most sensible test micro-organism used in the study [35]. In fact, all the analogs, but not tolaasin C, inhibited the grown of *B. megaterium* though differences among their specific activities were observed. The most active analogs appear to be tolaasin D followed by tolaasin I and II with a minimal inhibitory quantity of 0.16, 0.32 and 0.64 μg. Tolaasins A, B, and E resulted less active, with a minimal inhibitory quantity of 1.28 and 2.56, respectively. The antimicrobial activity of tolaasins appeared to be modulated by the structural modifications present in the different natural analogs of either Tol I or II. In fact, the opening of the lactone ring occurring in the case of Tol C eliminated the antibacterial activity [35].

Tolaasin appeared to be synthesized, as several other microbial LDPs, by a peptide synthetase complex [36] and its production and efficiency is mediated by an extragenomic factor [37].

Tolaasins disrupt the *A. bisporus* plasma membrane and vacuole membranes through the ion channel forming activity [32, 38] and the biosurfactant activity [39]. Two types of ion channels both inhibited by Zn^{2+} were identified by the incorporation of tolaasin into lipid bilayer [40]; Ni^{2+} inhibited the pore activity of Tol I but the mechanism is not yet determined [41].

Using model membranes of different lipid compositions a barrel-stave mechanism of action of Tol I, based on a valuable increment in the helical content of the LDP which was inserted in the membrane core and oriented parallel to the lipid acyl chain, was proposed [42]. Tol I and Tol II showed similar activities. The other tolaasin analogues were instead remarkably less active (up to 15 times when compared to tolaasin I) both on red blood cell and model membranes [43].

***Pseudomonas reactans* lipodepsipeptide.** *P. reactans*, beside *P. tolaasii*, is responsible for the brown blotch of *Agaricus bisporus*, the yellowing of *Pleurotus ostreatus* [5] and *P. eryngii* [8, 9]. Virulent strains of *P. reactans* produce an extracellular substance called the white line-inducing principle (WLIP) a LDP composed by the N-terminal β-hydroxydecanoic acid and a peptide moiety composed of nine aminoacids, six of which are in the D-form. The molecule contains a lactone ring between D-allo-threonine and N-terminal L-isoleucine [44]. Comparative antimicrobial assays of WLIP, produced by *P. reactans* strain NCPPB1311, and tolaasin I, produced by *P. tolaasii* type strain NCPPB2192, showed that both LDPs inhibited the growth of fungi - including the cultivated mushrooms *A. bisporus, Lentinula edodes* and *Pleurotus* spp. - chromista and Gram-positive bacteria. Assays of the two LDPs on blocks of *A. bisporus* showed their capacity to alter the pseudo-tissues of the mushrooms though WLIP was less active than Tol I. Nevertheless, *P. reactans* NCPPB1311, at least *in vitro*, produced more WLIP than the quantity of Tol I produced by type strain NCPPB2192 of *P. tolaasii*, suggesting that the lower antimicrobial activity of WLIP was compensated by the higher quantity produced in culture [45]. Contrary to previous studies, Tol I was found to inhibit the growth of Gram-negative bacteria belonging to the genera *Escherichia*, *Erwinia*, *Agrobacterium*, *Pseudomonas* and *Xanthomonas*. The only Gram-negative bacterium affected by WLIP was *E. carotovora* subsp. *carotovora*.

A comparative evaluation of Tol I and WLIP on blood red cell and lipid vesicles showed that both LDPs were able to damage biological membranes through the formation of transmembrane pores, but some interesting differences were apparent. The conformation of WLIP changed slightly when it passed from the buffer solution to the lipid environment. The LDP had an insufficient length to pass through the entire membrane and exhibited a permeabilizing activity in the same range of that of detergents, suggesting a detergent-like activity for WLIP [42].

Further, though preliminary, experiments on lipid vesicles using solutions of Tol I and WLIP in different ratio provide evidences of an interaction of the two LDPs leading to an apparent antagonistic action [43].

The pathogenesis of *P. reactans* on the host mushrooms (*A. bisporus*, *P. ostreatus* and *P. eryngii*) is far to be understood but the antifungal activity of WLIP together with the finding that avirulent morphological variants of *P. reactans* lack WLIP production [9] suggest that WLIP may play an important role in the interaction of the producing bacterium *P. reactans* and cultivated mushrooms [45].

***Pseudomonas tolaasii* volatile substances.** Strains of *P. tolaasii*, besides tolaasins, produce *in vitro* other biological active substances which appear to be important in the biology of the pathogen and in the *Pseudomonas tolaasii*/mushrooms interactions. In fact, it was demonstrated that virulent strains of *P. tolaasii* produce *in vitro* an array of volatile substances (VOCs) which inhibit the growth of *Agaricus bisporus* and *Pleurotus ostreatus* mycelia as well as they cause the brown discoloration of *A. bisporus* and *P. ostreatus* pseudotissues blocks. Furthermore, this pool of volatile substances inhibited the seed germination and/or seedling growth of several cultivated herbaceous plants (lettuce, broccoli) and several word-wide diffused weeds (unpublished results). GS-MS analysis of the VOCs showed the complexity of the volatile mixture. On average dimethyl sulfide and methanethiol were found the main compounds of the mixture. These pure substances in a concentration/effect figure, though with at a different

concentration range, reproduced the above toxic effects on either mycelia, pseudotissue blocks of *A. bisporus* and *P. ostreatus* as well as on the seed germination and/or seedling growth of several cultivated herbaceous plants. The antifungal activity of these substances clearly suggested a potential role of these substances in the *P. tolaasii* mushrooms interactions in different phases of *A. bisporus* and *P. ostreatus* cultivation. Furthermore, the possible technological exploitation of this feature is not excluded. However, other studies appear necessary.

CONCLUSION

The results here reported clearly indicate that brown blotch of *Agaricus bisporus* and yellowing of *Pleurotus ostreatus*, reported to be caused by *P. tolaasii*, appear to be complex diseases caused by several bacteria. In particular, *P. tolaasii*, to be considered the main and more aggressive pathogen, as well as *P. reactans* and other not yet characterized fluorescent pseudomonads, appear to contribute, though at different degrees, to the expression of the above disease symptoms. So far, it is not yet clear whether the two pathogens are the causal agents of the yellowing of *P. eryngii*. It is not excluded that in the different cultivation conditions (mushroom strain, cultivation procedure, substrate composition, environmental condition, etc) one of the two pathogens may be prevalent and responsible for the yellowing caused by *P. reactans* and blotch disease caused by *P. tolaasii*. As a matter of the fact in our experience never *P. tolaasii* was isolated from *P. eryngii* specimens showing the symptoms of the yellowing. Of interest is the definitive characterization of *P. reactans*, only in part addressed in the past, which appears, on the basis of preliminary RE-PCR analysis, not to be a homogeneous genetic group. Further biochemical, nutritional and genetic characterization of a collection of *P. reactans* strains in comparison to *P. tolaasii* are in progress. Efforts leading to the pathogen classification appear necessary too.

The different level of virulence in *P. tolaasii* and *P. reactans* strains seems to parallel the different level of biological activity of tolaasins and WLIP which, though with a different mechanism, have cell membranes as target for their toxicity.

Of interest is the evaluation of the role of volatile substances produced by strains of *P. tolaasii* in the blotch symptom expression and in the pathogen-mushrooms interactions.

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