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RESEARCH PAPERS

Characterization of fluorescent pseudomonads responsible for the yellowing of oyster mushroom (*Pleurotus ostreatus*)

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Summary. Fluorescent pseudomonads isolated from different lesions on caps and/or stipes of cultivated *Pleurotus ostreatus* were identified as strains of *Pseudomonas tolaasii* or showed the White Line Assay (WLA) feature of *P. 'reactans'* or were WLA-negative fluorescent *Pseudomonas* spp. *Pseudomonas tolaasii* was consistently associated with brown-reddish blotches on *P. ostreatus* pseudo-tissues, and in the pathogenicity assays caused depressed dark brown lesions with deliquescence on *Agaricus bisporus* pseudo-tissues blocks and brown-reddish blotches and yellow discoloration on *P. ostreatus* sporocarps. *Pseudomonas 'reactans'* and the WLA-negative fluorescent *Pseudomonas* spp. were mostly associated with superficial yellow lesions on *P. ostreatus* sporocarps, and in pathogenicity assays caused light or dark brown discoloration, depending on the isolates, on *A. bisporus* pseudo-tissues blocks and the yellow discoloration of *P. ostreatus* sporocarps. The results of this study indicate that the aetiology of lesions on cultivated *P. ostreatus* involves a complex composed of interactions between *P. tolaasii*, *P. 'reactans'* and *Pseudomonas* spp., but that individually these bacteria cause different symptoms. This is the first report where the pathogenicity features of these pathogens has been clearly ascertained, and that has fully satisfied Koch's postulates for the bacteria on the host mushroom. On the basis of virulence, biochemical and physiological characters, the isolates of *P. 'reactans'* and *Pseudomonas* spp. responsible for yellowing of oyster mushroom belong to several species of *Pseudomonas*.

Key words: mushroom bacterial diseases, *Pseudomonas tolaasii*, *Pseudomonas 'reactans'*, *Pseudomonas* spp.

Introduction

World production of cultivated mushrooms is increasing, with almost six million tons having been produced in 2010 (FAOSTAT, 2012). China, the United States of America, and several European Countries are the major producers of these food crops. The annual Italian production of cultivated mushrooms is about 98,000 tons, mostly of *Agaricus bisporus* (Lange) Imbach (button mushroom) and *Pleurotus ostreatus* (Jacq. ex Fr) Kum (oyster mushroom) and a few other species including the king oyster mushroom *Pleurotus eryngii* (DC ex Fr) ('cardoncello'). Cultivated mushrooms are subject to a number of fungal, viral and bacterial diseases that can cause significant production losses (Fermor, 1987; Gill, 1995), with bacteria likely to be the main cause of product losses (Fermor, 1987). Several bacterial diseases of cultivated *Agaricus* spp. and *Pleurotus* spp. are caused by fluorescent pseudomonads (Gill, 1995), including brown blotch of *A. bisporus* (Paine, 1919) and yellowing of *P. ostreatus*, both of which are considered to be caused by *Pseudomonas tolaasii* (Ferri, 1985; Gill, 1995). Some authors have suggested that other undefined *Pseudomonas* spp. are involved in yellowing of *P. ostreatus* (Ferri, 1985; Gill, 1995), but only recently has more information on their roles become available (Lo Cantore, 2001; Munsch and Alatossava, 2002b; Sajben *et al*., 2011). Brown blotch symptoms on *A. bisporus* can also be caused by *P. costantinii* sp. nov. (Munsch *et al*., 2002), or other *Pseudomonas* spp. (Godfrey *et al*., 2001) including the so called *P. 'reactans'* (Wells *et al*.,

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1996; Iacobellis and Lo Cantore, 1997, 2003; Lo Cantore, 2001; Munsch *et al*., 2002). However, the role of *P. 'reactans'* in disease occurrence and symptom development has not been unequivocally demonstrated. *Pseudomonas 'reactans'* is a taxonomically invalid name given for the mushroom-associated bacteria producing the lipodepsipeptide, known as the White Line Inducing Principle (WLIP) (Mortishire-Smith *et al*., 1991; Lo Cantore *et al*., 2006). This interacts with tolaasins, a lipodepsipeptide family group (Basserello *et al*., 2004) produced by virulent strains of *P. tolaasii*, giving rise to a white precipitate the formation of which is used for the specific identification of *P. tolaasii* in the White Line Assay (WLA) (Wong and Preece, 1979). However, recent reports have indicated that the WLA is not as specific as previously claimed (Wong and Preece, 1979), because some other bacteria associated with mushrooms may give rise to the white precipitate in the assay (Munsch and Alatossava, 2002a). Furthermore, *A. bisporus* is also susceptible to *P. 'gingeri'* (which causes ginger blotch), a complex of *Pseudomonas* spp. (mummy disease) and *P. agarici* (drippy gill) (Gill, 1995), which was also reported as the cause of oyster mushroom yellow blotch when this disease was first observed in California (Bessette *et al*., 1985), and of brown discoloration of *A. bisporus* in the Netherlands and Italy (Gells *et al*., 1994; Iacobellis and Lo Cantore, 1997; Lo Cantore and Iacobellis, 2004). Yellowing of *P. eryngii* was reported to be caused by *P. tolaasii* (Ferri, 1985; Rodriguez Estrada and Royse, 2007) or *P. 'reactans'* and other fluorescent pseudomonads (Iacobellis and Lavermicocca, 1990; Iacobellis and Lo Cantore, 1997, 2003). However, the aetiology of this disease is not yet established.

In recent years in oyster cultivations bacterial disease symptoms possibly due to the yellowing and/or brown-reddish blotch (Ferri, 1985; Gill, 1995) caused by *P. tolaasii* have been observed in several mushroom farms in Apulia and Basilicata (southern Italy) (Lo Cantore and Iacobellis, 2002). The disease has been observed in all the phases of sporocarp development. In particular, developing mushroom bunches presented brown-reddish yellow-orange discolorations involving entire bunches or parts thereof, and affected mushrooms cease growth, wither and rot (Figure 1a). On developed sporocarps brown-reddish sunken blotches frequently surrounded by yellow halos were observed either on caps and/or stipes (Figure 1b and c). Furthermore, on developed sporocarps yellow superficial lesions affecting whole or part caps have also been observed (Figure 1d and e). In conditions of high temperature and humidity the affected sporocarps rapidly rotted and emanated unpleasant odour.

The losses of product and, in particular, the variety of the symptoms observed, prompted us to start studies directed at defining the agent/s responsible for the different diseases observed. This paper reports results from studies on the complex aetiology of yellowing of *P. ostreatus*.

Materials and methods

Isolation and maintenance of bacterial isolates

Bacteria were isolated from sporocarps of *P. ostreatus* mushrooms showing superficial yellowish lesions (seventeen specimens) or brown-reddish blotches (thirteen specimens), and grown on agar King's medium B (KB) (King *et al*., 1954). The diseased sporocarps were washed under running tap water and then the surfaces swabbed with cotton wool soaked with 75% ethyl alcohol. A fragment of pseudo-tissue was taken from each lesion and then triturated in a drop of sterile distilled water. Aliquots of the resulting bacterial suspensions were spread on KB. After 48 h incubation at 25°C, representative bacterial colonies with different morphologies were further purified on nutrient agar, and pure cultures were maintained at 4°C on 2% glycerine nutrient agar slants. Twenty-two isolates were obtained from brown-reddish blotches surrounded by yellow discoloration (Table 1), and 41 isolates were obtained from superficial yellow lesions (Table 2). All of the isolates were evaluated for fluorescence on KB (Lelliott and Stead, 1987), WLA (Wong and Preece, 1979), and virulence on *A. bisporus* pseudo-tissue blocks (Ercolani, 1970). Bacterial cultures were grown on KB for 48 h at 25°C unless otherwise stated.

Pathogenicity assays

The 63 bacterial isolates were assayed for pathogenicity on pseudo-tissue blocks of freshly harvested *A. bisporus* pseudo-tissues by inoculation with 20 μL of approx. 10^8 cfu mL^{-1} of each bacterial suspension (Ercolani, 1970). Sterile distilled water was used as a negative control in all assays. Each assay was re-

Table 1. Features of the bacterial isolates obtained from brown-reddish blotches surrounded by yellow discoloration on *Pleurotus ostreatus* sporocarps.

No. isolates[a]	Fluorescence		WLA[b]		Virulence score[c]		
	YG	B	P. t.	P. r.	1	2	3
12	+	-	-	+			+
4	+	-	+	-		+	
4	+	-	-	-		+	
1	+	-	-	-	+		
1	-	-	-	-	-	-	-

[a] Bacterial isolates representative of the isolation from thirteen specimens.
[b] White Line Assay performed following the method of Wong and Preece (1979).
[c] Pathogenicity assays were performed on *Agaricus bisporus* pseudo-tissue blocks (Ercolani, 1970).
YG, Production of yellow-green fluorescent pigment on KB.
B, Production of blue fluorescent pigment on KB.
P. t., *Pseudomonas tolaasii* type strain NCPPB2192.
P. r., *Pseudomonas 'reactans'* NCPPB1311.
+, Presence of the character; -, absence of the character.

Table 2. Features of the bacterial isolates obtained from superficial yellowish lesions present on *Pleurotus ostreatus* sporocarps.

No. isolates[a]	Fluorescence		WLA[b]		Virulence score[c]		
	YG	B	P. t.	P. r.	1	2	3
6	+	-	-	+			+
4	+	-	+	-		+	
13	+	-	-	-		+	
3	+	-	-	-	+		
2	+	-	-	-	-	-	-
4	-	+	-	-		+	
2	-	-	-	-		+	
1	-	-	-	-	+		
6	-	-	-	-	-	-	-

For all footnotes See Table 1.

peated at least twice with two replicates. The degree of disease on *A. bisporus* pseudo-tissue blocks was scored using the following empirical scale: 0, equivalent to the distilled sterile water control treatment; 1, light brown discoloration; 2, marked browning discoloration; 3, marked sunken browning discoloration with deliquescence.

Selected representative bacterial isolates which produced fluorescent pigments when grown on KB, showed different degrees of virulence in the *A. bisporus* pseudo-tissue assay and with positive (putative strains of *P. tolaasii* and *P. 'reactans'*) and negative WLA reactions were further evaluated for pathogenicity on whole *P. ostreatus* sporocarps. Compost

Table 3. Biochemical and nutritional characters and virulence of selected bacterial isolates obtained from lesions on *Pleurotus ostreatus* sporocarps.

Strains	Fluorescence	[a]WLA		[b]Virulence score	[c]Growth on:								Biolog			
		P. t.	P. r.		Erytritol, sorbitol	L-arabinose	L-rhamnose	L-arabitol	2-ketogluconate	n-valerate	D-tartrate	Histamine	Identification	Probability (%)	Similarity index value	Distance
[d]NCPPB2192	YG	-	+	3	+	-	-	+	+	+	-	-	*P. tolaasii*	100	0.93	1.00
NCPPB2325	YG	-	+	3	+	-	-	+	+	+	-	-	"	90	0.61	4.86
USB1	YG	-	+	3	+	-	-	+	+	+	-	-	"	100	0.85	2.19
USB17	YG	-	+	3	+	-	-	+	+	+	-	-	"	100	0.95	0.70
USB22	YG	-	+	3	+	-	-	+	+	+	-	-	"	99	0.89	1.63
USB26	YG	-	+	3	+	-	-	+	+	+	-	-	"	100	0.93	1.01
USB32	YG	-	+	3	+	-	-	+	+	+	-	-	"	100	0.85	2.19
USB42	YG	-	+	3	+	-	-	+	+	+	-	-	"	100	0.94	0.89
USB57	YG	-	+	3	+	-	-	+	+	+	-	-	"	100	0.96	0.51
USB105	YG	-	+	3	+	-	-	+	+	+	-	-	"	100	0.86	2.00
USB107	YG	-	+	3	+	-	-	+	+	+	-	-	"	100	0.89	1.58
[e]NCPPB2289	B	-	-	2	-	-	-	-	-	-	-	-	*P. agarici*	100	0.78	3.24
NCPPB2472	B	-	-	2	-	-	-	-	-	-	-	-	"	99	0.51	7.90
NCPPB2874	YG	-	-	1	-	+	-	-	+	+	+	-	NI	-	-	-
NCPPB2875	YG	-	-	1	-	+	-	-	+	+	+	-	"	-	-	-
NCPPB3146	YG	+	-	3	+	-	-	-	-	+	-	+	*Pseudomonas* spp.	-	[f]0.19	[f]10.7
NCPPB1311	YG	+	-	2	+	+	+	+	+	+	-	-	*P. synxantha*	86	0.62	4.15
USB8	YG	+	-	2	+	+	+	+	+	+	-	-	*P. fluorescens* biotype A	99	0.59	6.30
USB13	YG	+	-	2	+	+	+	+	+	+	-	-	"	87	0.61	4.56
USB23	YG	+	-	2	+	+	+	+	+	+	-	-	"	98	0.75	3.55
USB16	YG	+	-	2	+	+	+	+	+	+	-	-	*P. marginalis*	100	0.86	2.05
USB20	YG	+	-	2	+	+	+	+	+	+	-	-	"	64	0.53	2.59
USB49	YG	+	-	2	+	+	+	+	+	+	-	-	"	89	0.70	3.15
USB6	YG	+	-	2	+	+	+	+	+	+	-	-	*P. fluorescens*	79	0.63	3.03
USB12	YG	+	-	2	+	+	+	+	+	+	-	-	*Pseudomonas* spp.	-	[f]0.32	[f]7.86
USB33	YG	-	-	2	+	-	-	+	+	+	-	+	"	-	[g]0.38	[g]4.88
USB36	YG	-	-	2	+	-	-	+	+	+	-	-	*P. tolaasii*	100	0.88	1.78

(Continued)

Table 3. (Continued)

Strains	Fluorescence	[a]WLA		[b]Virulence score	[c]Growth on:								Biolog			
		P. t.	P. r.		Erytritol, sorbitol	L-arabinose	L-rhamnose	L-arabitol	2-ketogluconate	n-valerate	D-tartrate	Histamine	Identification	Probability (%)	Similarity index value	Distance
USB34	YG	-	-	2	+	-	-	+	+	+	-	+	*P. fluorescens*	99	0.79	3.05
USB55	B	-	-	2	+	-	-	+	+	+	-	+	"	91	0.67	4.03
USB56	B	-	-	2	+	-	-	+	+	+	-	+	"	94	0.69	3.99

[a] White Line Assay performed following the method of Wong and Preece (1979); [b]Pathogenicity assays were performed on *Agaricus bisporus* pseudo-tissue blocks (Ercolani, 1970); [c]Nutritional assays performed following the method of Goor *et al*. (1986); [d]Type strain of *Pseudomonas tolaasii*; [e]Type strain of *P. agarici*; [f]Values in comparison to *P. fluorescens* which resulted the bacterial species nearest to the analysed strain; [g]Values in comparison to *P. fluorescens* biotype A which resulted the bacterial species nearest to the analysed strain; P. t., *P. tolaasii* type strain NCPPB2192; P. r., *P. 'reactans'* NCPPB1311; YG: Production of yellow-green fluorescent pigment on KB; B: Production of blue fluorescent pigment on KB; NI, not identified; ICMP, International Collection of Micro-organisms from Plants, New Zealand; NCPPB, National Collection Plant Pathogenic Bacteria, UK; USB, Collection of Plant Associated Bacteria, Università degli Studi della Basilicata, Italy.

bags colonised by a commercially available *P. ostreatus* strain were maintained in a growth chamber at 14–16°C and about 98% relative humidity for sporocarp production. Developed sporocarps, were inoculated *in situ* using bacterial masses or suspensions of the respective bacterial isolates.

Bacterial masses were taken with sterile toothpicks and then sporocarps caps were prick-inoculated. Bacterial masses were also inoculated onto sporocarps that were not wounded.

Ten μL drops of bacterial suspensions containing approx. 10^8 cfu mL^{-1} were deposited on the cap surface of *P. ostreatus* sporocarps, or aliquots of bacterial suspensions were injected with a sterile syringe under the cuticle layer of caps and at the base of stipes of developing sporocarp bunches. The same bacterial suspensions were also sprayed on developing sporocarp bunches. After inoculation, sporocarps were covered with plastic bags and maintained at approx. 20°C and approx. 98% relative humidity. Plastic bags were removed after 24 h incubation. *Pseudomonas tolaasii* type strain NCPPB2192 and *P. 'reactans'* isolate NCPPB1311 were used for comparison. At least three individual sporocarps or developing sporocarp bunches were used for each assay and the assays were repeated at least twice for each isolate.

White Line Assay

Bacterial isolates were evaluated in the WLA for the ability to form white precipitate in KB medium when grown adjacent to *P. tolaasii* and/or *P. 'reactans'* strains. Type strain NCPPB2192 of *P. tolaasii* and isolate NCPPB1311 of *P. 'reactans'* were used. The method reported by Wong and Preece (1979), with minor modification, was followed.

Characterization of bacterial isolates

Seventeen WLA-positive isolates with the features of putative strains of either *P. tolaasii* or *P. 'reactans'*, as well as five WLA-negative isolates (Table 3), were evaluated for LOPAT characters (Lelliott and Stead, 1987), the differential nutritional features of bacteria associated to mushrooms (Goor *et al*., 1986) and for metabolic feature on 95 carbon sources on GN2 microplates (Biolog Inc.). *Pseudomonas tolaasii* strains NCPPB2192 and NCPPB2325, *P. 'reactans'* strain NCPPB1311, *P. agarici* strains NCPPB2472 and NCPPB2289, *P. 'gingeri'* strain NCPPB3146, and *Pseudomonas* spp. strains NCPPB2874 and NCPPB2875, responsible for the mummy disease, obtained from the National Collection of Plant Pathogenic Bacteria (York, UK), were used for comparison.

Results

Bacterial isolation

Isolation of bacteria from thirteen mushroom specimens showing brown-reddish blotches surrounded by yellow halos on caps and/or stipes of *P. ostreatus* sporocarps (Figure 1b and c) gave rise to colonies with different morphologies. Twenty-two bacterial isolates, representative of the colony morphologies present in the different isolation plates, were selected for further characterization. Twenty-one of these isolates produced fluorescent yellow-green pigments on KB, and sixteen were positive in the WLA. Among the WLA-positive isolates, twelve formed white precipitate when grown near *P. 'reactans'* strain NCPPB1311, and thus are likely to be *P. tolaasii.* Four formed white precipitate with *P. tolaasii* type strain NCPPB2192 and are thus likely to be *P. 'reactans'* (Wong and Preece, 1979). The remaining five fluorescent isolates were negative in the WLA (Table 1).

The bacteria isolated from seventeen specimens showing superficial yellow discoloured lesions either on developed or on young developing sporocarp bunches (Figure 1a, d and e) gave rise to bacterial colonies with different morphologies. Among the 41 selected bacterial isolates, 32 produced fluorescent pigments. Twenty-eight of these produced yellow-green fluorescent pigments, and four produced blue fluorescent pigments. Only ten of the 28 yellow-green fluorescent isolates were positive in the WLA, and of these six formed white precipitate when grown near strain NCPPB1311 of *P. 'reactans'* and were considered putative *P. tolaasii* strains. Four isolates formed white precipitate when grown near type strain NCPPB2192 of *P. tolaasii* and were considered to be putative *P. 'reactans'* strains (Table 2). None of the four isolate producing blue fluorescent pigments were positive in the WLA.

Pathogenicity assays on *Agaricus bisporus* pseudo-tissue blocks

The 18 putative *P. tolaasii* strains, on the basis of the WLA feature, caused depressed brown discoloration lesions on *A. bisporus* pseudo-tissues after 24 h, with plectenchyma deliquescence after 48 h and gave virulence scores of 3 (Tables 1, 2 and 3; Figure 2a). In the same assay the eight putative *P. 'reactans'* isolates, on the basis of the WLA feature, caused light brown lesions within the plectenchyma after 24 h incubation and marked brown discoloration after 48 h giving an average virulence score of 2. Variability in virulence was observed among the above isolates but deliquescence of *A. bisporus* pseudo-tissues blocks was never observed (Tables 1, 2 and 3; Figure 2b). The majority of the 24 fluorescent isolates that were negative in WLA caused brown discoloration (virulence score 2), some caused light discoloration (virulence score 1), and others did not have any visible effect after 48 h incubation (Tables 1 and 2; Figure 2c–l). In the same pathogenicity assay, only three out the thirteen non-fluorescent isolates caused visible symptoms with virulence scores of 1 or 2 (Tables 1 and 2).

Pathogenicity assays on *Pleurotus ostreatus* sporocarps

The selected bacterial isolates, obtained from the different symptoms observed on *Pleurotus ostreatus* sporocarps and representative of the different phenotypes previously described, produced disease symptoms on *P. ostreatus* sporocarps, though the type and intensity of the symptoms differed among isolates, and also depended on the inoculation method. Putative *P. tolaasii* isolates USB1 and USB22, which caused sunken brown discolorations with deliquescence on *A. bisporus* pseudo-tissue blocks and gave virulence scores of 3 (Table 3), produced slightly sunken yellow-orange lesions surrounded by yellow halos 24 h after bacterial mass inoculation on the surface of *P. ostreatus* sporocarps. After 72 h the sunken lesions were more pronounced, and the yellow-orange discoloration extended around the inoculation points. Mechanical wound or injection inoculations resulted in more rapid symptom development, but lesions were essentially the same as in bacterial mass inoculations 72 h after inoculation. Symptom progression was essentially the same as with *P. tolaasii* strain NCPPB2192 (Figures 3a, b, 4a, b). Putative *P. 'reactans'* isolates USB6 and USB20, which caused brown discoloration of *A. bisporus* pseudo-tissues with virulence scores of 2 (Table 3), produced yellowing of the inoculated pseudo-tissues but symptoms did not appear on sporocarps until after about 48 h incubation. Wounding or injection inoculation advanced yellowing to 24 h after inoculation with more discoloration at 72 h, but no sunken lesions were observed with either inoculation methods, either with USB6 and USB20 or strain NCPPB1311 of *P. 'reactans'* used for

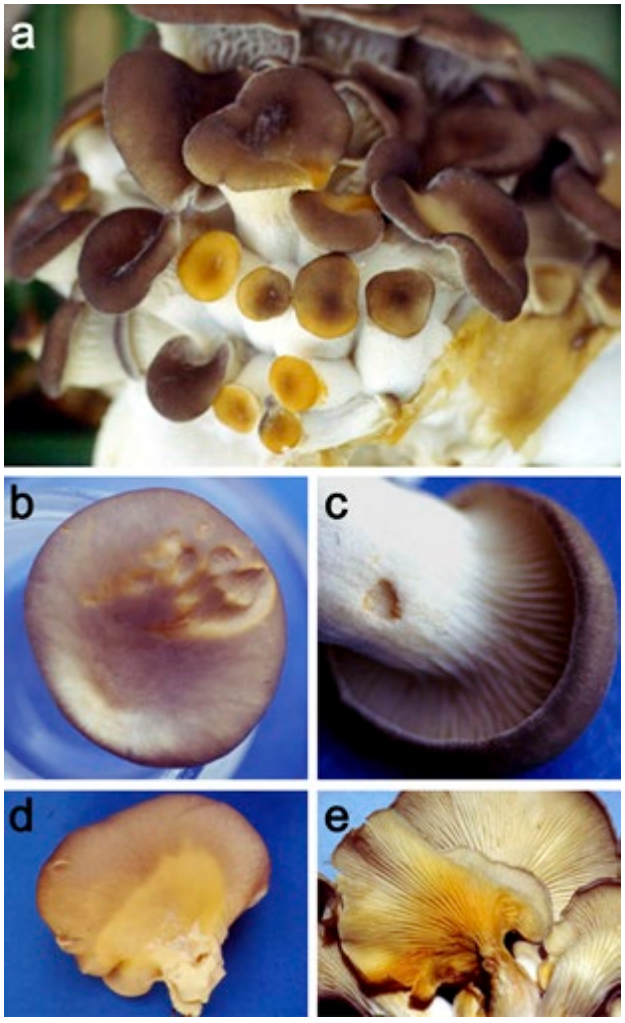

Figure 1. Symptoms of yellowing and/or brown-reddish blotch on *Pleurotus ostreatus* sporocarps. Yellow-orange superficial discoloration on developing sporocarp bunches were followed by cessation of growth, withering and tissue rot (a), brown-reddish blotches surrounded by yellow discoloration on cap (b) and stipe (c), yellow superficial lesions on cap (d) and gill (e).

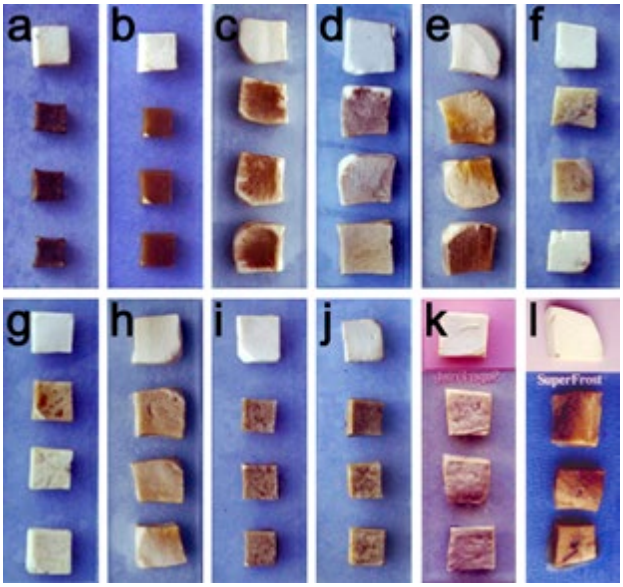

Figure 2. Pathogenicity assays on *Agaricus bisporus* pseudo-tissues. Depressed brown discoloration and deliquescence caused by *Pseudomonas tolaasii* strain NCPPB2192 (a), marked brown discoloration without deliquescence caused by *P. 'reactans'* strain NCPPB1311 (b) and browning or light discoloration caused by fluorescent *Pseudomonas* spp. (c, USB34; d, USB36; e, USB44; f, USB45; g, USB52; h, USB53; I, USB55; j, USB56; k, USB111; l, USB113) on *A. bisporus* pseudo-tissues blocks. The pseudo-tissues block at the top of each pseudo-tissue block set was treated with sterile distilled water as negative control.

comparison (Figures 3c and 4c). WLA-negative isolates USB33, USB34 and USB36 (yellow-green fluorescence), USB55 and USB56 (blue fluorescence), all causing brown discoloration of *A. bisporus* pseudo-tissue blocks and with virulence scores of 2 (Table 3), caused symptoms similar to isolates USB6, USB20 and NCPPB1311 of *P. 'reactans'*. Also in this case no sunken lesions were observed (Figure 3d).

The nebulisation of putative *P. tolaasii* strains USB1, USB22 and the type strain NCPPB2192 on young developing sporocarp bunches caused yellowing after 24 h incubation, followed by severe yellow discoloration (Figure 5a), yellow-to-reddish blotches, and cessation of sporocarp growth (Figure 5b). Spray inoculation of putative *P. 'reactans'* isolates USB6, USB20 and NCPPB1311 on developing sporocarps bunches caused yellow discoloration and decreased growth rate after 48 h incubation, but no sunken lesions (Figure 5c). Similar results were obtained with injection inoculations (Figures 5d, e and f).

Characterization of bacterial isolates

Seventeen selected virulent, WLA-positive fluorescent isolates had LOPAT (-+-+-) and confirmatory (++--) phenotypes characteristic of the V_a group of fluorescent pseudomonads (Lelliott and Stead, 1987). Isolates USB1 (deposited with the International Collection of Micro-organisms from Plants

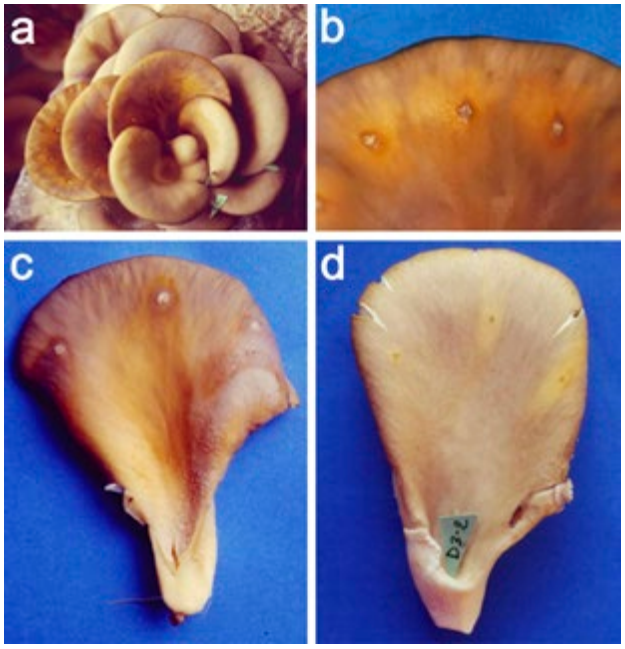

Figure 3. Pathogenicity assays on *Pleurotus ostreatus* whole sporocarps inoculated with bacterial masses. Sunken yellow-orange lesions surrounded by yellow halos caused by strains USB1, USB22 and NCPPB2192 of *Pseudomonas tolaasii* (a and b), and yellow-orange lesions caused by strains USB6, USB20 and NCPPB1311 of *P. 'reactans'* (c) and strains USB33, USB34, USB36, USB55 and USB56 of fluorescent *Pseudomonas* spp. (d) on *P. ostreatus* sporocarps.

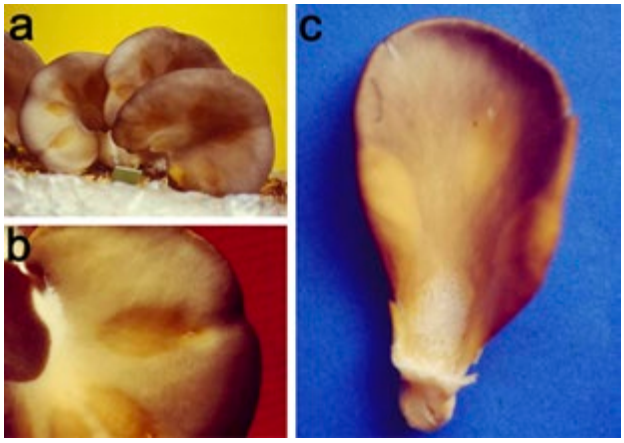

Figure 4. Pathogenicity assays on *Pleurotus ostreatus* whole sporocarps syringe-injected with bacterial suspensions. Yellow-brown sunken lesions surrounded by yellowish areas (a and b) and yellow superficial lesions (c) on caps of *P. ostreatus* sporocarps syringe-injected with 10^8 cfu mL^{-1} bacterial suspensions of strains USB1, USB22 and NCPPB2192 of *Pseudomonas tolaasii* (a and b) and of strains USB6, USB20 and NCPPB1311 of *P. 'reactans'* (c).

(ICMP), Auckland, New Zealand, accession number ICMP13791), USB17, USB22, USB26, USB32, USB42, USB57, USB105 and USB107, evaluated for the differential metabolic characters of bacteria associated with cultivated mushrooms (Goor *et al*., 1986), were indistinguishable from *P. tolaasii* type strain NCPPB2192 (Table 3). The Biolog system confirmed the above identification. Isolates USB6, USB8, USB12, USB13, USB16, USB20 (deposited at ICMP, accession number ICMP13793), USB23 and USB49 had biochemical and nutritional characters and virulence features that were indistinguishable from those of *P. 'reactans'* strain NCPPB1311 (Table 3). However, the Biolog system identified putative *P. 'reactans'* isolates USB8, USB13 and USB23 as strains of *P. fluorescens* biotype A, USB16, USB20 (ICMP13793) and USB49 as strains of *P. marginalis*, USB6 as a strain of *P. fluorescens*, NCPPB1311 as a strain of *P. synxantha*, and USB12 as *Pseudomonas* spp. (Table 3). The WLA-negative and virulent *Pseudomonas* spp. isolates USB33, USB34, USB36, USB55 and USB56 had slightly different biochemical and nutritional profiles. In particular, isolate USB36 showed the typical profile of *P. tolaasii*, whereas isolates USB34 and USB56 differed from *P. tolaasii* only in the ability to grow on minimal medium containing histamine (Table 3). Furthermore, isolates USB33 and USB55 differed from *P. tolaasii* strains, for the absence of lecitinase production (isolate USB33) and for the ability to produce acid from sucrose (isolate USB55), and both strains for the ability to grow on minimal medium containing histamine (Table 3). Biolog identified isolates USB34, USB55 and USB56 as strains of *P. fluorescens*, whereas isolates USB33 was identified as *Pseudomonas* spp. and USB36 as a *P. tolaasii* strain (Table 3).

The fluorescent pseudomonads obtained from the symptomatic *P. ostreatus* sporocarps after the pathogenicity assays showed the same WLA, virulence on *A. bisporus* pseudo-tissue blocks and nutritional features as the *P. tolaasii*, *P. 'reactans'* and WLA negative *Pseudomonas* spp. isolates used in the pathogenicity assays.

Discussion

It is well known that *P. tolaasii* is the causal agent of yellowing of the *P. ostreatus*, and that some unde-

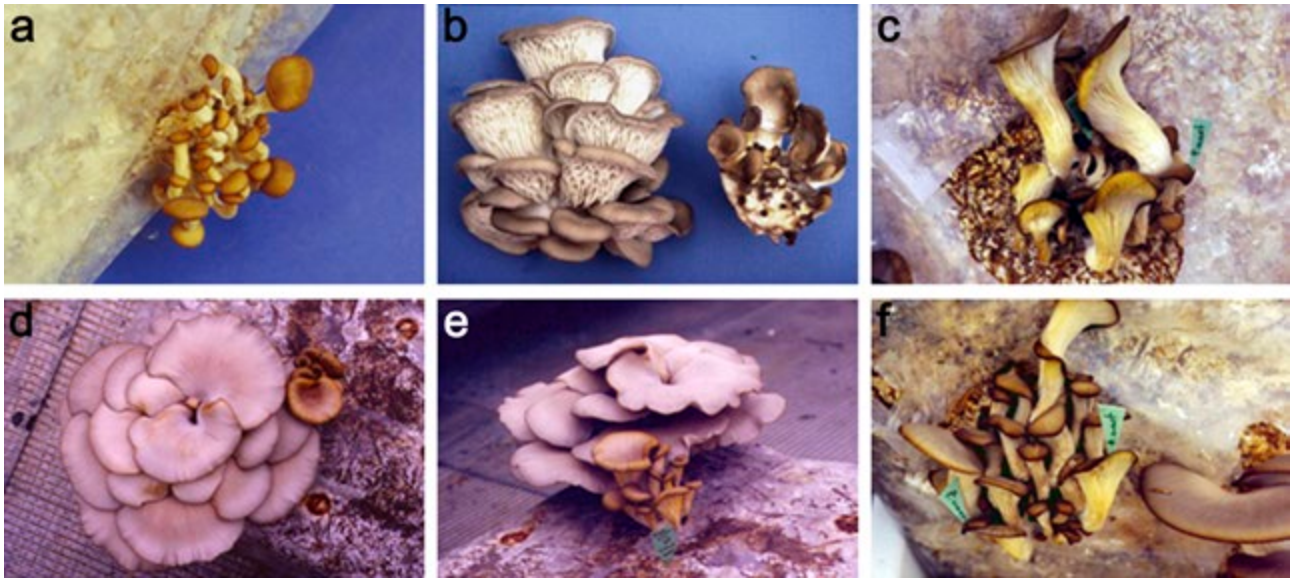

Figure 5. Pathogenicity assays on *Pleurotus ostreatus* whole sporocarps sprayed or injected into the stipe tissues with bacterial suspensions. Yellow-orange discolouration (a, c, d, e and f) or yellow-orange blotches (b) on sporocarp bunches of *P. ostreatus* and growth cessation after spray inoculation (a, b and c) or injection into stipe pseudo-tissues (d, e and f) with 10^8 cfu mL^{-1} bacterial suspensions of strains USB1, USB22 and NCPPB2192 of *Pseudomonas tolaasii* (a, b, d and e) and of strains USB6, USB20 and NCPPB1311 of *P. 'reactans'* (c and f).

fined *Pseudomonas* spp. are also possibly involved in the disease (Ferri, 1985; Gill, 1995). However, no firm evidence on the virulence of these *Pseudomonas* spp. has been published to date (Goor *et al*., 1986; Munsch and Alatossava, 2002a: 2002b). The occurrence of yellowing in oyster mushroom cultivation possibly caused by *P. tolaasii* (Ferri, 1985; Gill, 1995), and the variety of symptoms at all phases of *P. ostreatus* sporocarp development prompted the present study, aiming to isolate bacteria from the different types of lesions. The final aim was to define the agent/s responsible of the diseases observed. The results here reported clearly show that the yellowing of *P. ostreatus* has complex aetiology, and that beside *P. tolaasii,* other *Pseudomonas* spp. are responsible for some of the observed symptoms, confirming previous indications (Ferri, 1985; Gill, 1995).

In particular, from depressed brown-reddish lesions surrounded by yellow discoloration either on caps and/or stipes, fluorescent pseudomonads with different colony morphologies and virulence have been obtained. Only isolates identified as strains of *P. tolaasii* - on the basis of the WLA feature (Wong and Preece, 1979), biochemical and physiological characters (Lelliott and Stead, 1987), nutritional features obtained by the Biolog computer assisted system and following the identification scheme of Goor *et al*. (1986) - caused marked sunken brown discoloration with deliquescence of *A. bisporus* pseudo-tissue blocks. In contrast, isolates belonging to *P. 'reactans'* [as determined from the WLA feature (Wong and Preece, 1979), the nutritional features following the identification scheme of Goor *et al*. (1986)], and being *Pseudomonas* spp. negative in the WLA caused light brown discoloration or marked brown discoloration of *A. bisporus* pseudo-tissue blocks depending on the isolate.

Fluorescent pseudomonads were also obtained from superficial yellow lesions on mature *P. ostreatus* sporocarps and on developing sporocarp bunches, but only a few of these isolates had the typical features of *P. tolaasii*. The majority of the fluorescent pseudomonads obtained from these host specimens, either WLA negative or with the WLA feature of *P. 'reactans',* showed reduced virulence on *A. bisporus* pseudo-tissue blocks when compared with *P. tolaasii* strains.

From this evidence, we suggest that the yellowing of *P. ostreatus* by strains of *P. tolaasii* can be expected

to cause characteristic depressed brown lesions and yellowing of infected pseudo-tissues, whereas strains of *P. 'reactans'* and the WLA-negative fluorescent *Pseudomonas* spp. isolates were responsible for yellow discoloration *P. ostreatus* sporocarps.

The ability of *P. tolaasii* to cause depressed sunken lesions may depend, on differences in the toxicity of tolaasin and of the so called WLIP (Mortishire-Smith *et al*., 1991; Lo Cantore *et al*., 2006), and possibly on other, yet to be defined, virulence factors. No information is available on the virulence factors in the WLA-negative fluorescent *Pseudomonas* spp. isolates, though recent results indicate the importance of extracellular enzymes in the decaying process of *P. ostreatus* sporocarps (Sajben *et al*., 2011).

There have been some reports of undefined *Pseudomonas* spp. associated with yellowing of *P. ostreatus* (Ferri, 1985; Goor *et al*., 1986; Gill, 1995; Munsch and Alatossava, 2002a, 2002b), but to our knowledge this is the first report of a complex aetiology involving *P. tolaasii*, *P. 'reactans'* and *Pseudomonas* spp. in the yellowing of *P. ostreatus*. In particular, this is the first report where the pathogenicity feature of the these pathogens was clearly ascertained in *in situ* pathogenicity assays, and where Koch's postulates on the host mushroom have been fully satisfied. Recently, similar results have been reported but the pathogenicity of the *Pseudomonas* spp., mostly identified as belonging to *P. fluorescens* biovar V, was determined with a novel *in vitro* necrosis test (Sajben *et al*., 2011).

A similar complex aetiological picture has been ascertained for *A. bisporus*. Several studies on brown blotch of button mushroom have shown that, beside *P. tolaasii*, other *Pseudomonas* spp., including *P. costantinii* sp. nov. (Munsch *et al*., 2002), *Pseudomonas* spp. (Godfrey *et al*., 2001), and isolates of *P. 'reactans'* (Goor *et al*., 1986; Wells *et al*., 1996; Iacobellis and Lo Cantore, 1997, 2003; Godfrey *et al*., 2001; Lo Cantore, 2001; Munsch and Alatossava, 2002b) are responsible for symptom development (Tolaas, 1915; Paine, 1919). The virulence of *P. 'reactans'* on *A. bisporus* pseudo-tissue blocks with visible pseudo-tissues damage is generally less than that of *P. tolaasii*, with as few as 10^6 cfu mL^{-1} of *P. tolaasii* and 10^7-10^8 cfu mL^{-1} for *P. 'reactans'* (Wells *et al*., 1996; Iacobellis and Lo Cantore, 1997, 2003; Munsch *et al*., 2000; Lo Cantore, 2001). However, it is important to note that the minimal quantity of bacteria necessary to cause visible alteration of host pseudo-tissues may be variable between pathogenicity assays, and this mainly depends on the assay conditions with the quality and freshness of the *A. bisporus* sporocarps as an important factor. Variability in virulence of *P. 'reactans'* isolates was also observed (Goor *et al*., 1986; Wells *et al*., 1996; Iacobellis and Lo Cantore, 1997, 2003; Godfrey *et al*., 2001; Lo Cantore, 2001; Munsch and Alatossava, 2002b). This feature was attributed to the formation of morphological variants in *P. 'reactans'* cultures which were negative in the WLA, due to the loss of WLIP production, and were avirulent (Lo Cantore, 2001; Lo Cantore and Iacobellis, 2002; Iacobellis and Lo Cantore, 2003; Lo Cantore *et al*., 2006). Consequently, the WLIP produced by strain NCPPB1311 that, like tolaasin, alters membrane function, was considered as a virulence factor of *P. 'reactans'* (Mortishire-Smith *et al*., 1991; Lo Cantore, 2001; Iacobellis and Lo Cantore, 2003; Coraiola *et al*., 2006; Lo Cantore *et al*., 2006). However, the variability in virulence of *P. 'reactans'* may be due to the fact that *P. 'reactans'* is a heterogeneous group of bacteria, as supported by siderotyping data (Munsch *et al*., 2000), molecular characterization currently available (Godfrey *et al*., 2001) and results here reported. The so-called *P. 'reactans'* isolates appear to be strains of different entities such as *P. fluorescens* biotype A, *P. marginalis*, *P. fluorescens, P. synxantha* or other *Pseudomonas* spp., which may produce lipodepsipeptide analogues interacting with tolaasin in the WLA but with toxicities different from the WLIP. Research in this respect may provide better understanding of the virulence and identification of the so-called *P. 'reactans'*. Our preliminary studies, still underway, on the phenotypic and molecular characterization of a collection of *P. 'reactans'* isolates (20 isolates), some of which were used in the present study, indicate different rep-PCR (Louws *et al*., 1994) amplicon patterns among the isolates, that have been identified, however, on the basis of the 16S rDNA sequences as strains of *P. fluorescens* (unpublished results). The results apparently confirm the low resolution of the 16S rDNA sequence analysis at the intrageneric level. Consequently we have decided to perform Multi Locus Sequence Analysis, which should permit the correct phylogenic relationship among the isolates and possibly for their accurate identification (Mulet *et al*., 2010).

Some WLA-negative fluorescent *Pseudomonas* spp. had metabolic phenotypes in common with *P. tolaasii* whereas others did not. In general, however they were more similar to *P. tolaasii* than with *P. 're-*

actans', *P. agarici*, *P. 'gingeri'* or *Pseudomonas* spp. associated with mummy disease of *A. bisporus* (Goor *et al*., 1986; Lelliott and Stead, 1987). Some of these isolates may therefore be hypovirulent strains of *P. tolaasii* which have lost the ability to produce tolaasin in response to environmental stimuli (Grewal *et al*., 1995; Heeb and Haas, 2001).

In conclusion, the fluorescent *Pseudomonas* spp. here described contributed, though in different ways, to the development of the symptoms of yellowing of *P. ostreatus*. In particular, *P. tolaasii* strains are capable of causing depressed lesions and yellowing of infected host pseudo-tissues, whereas both strains of *P. 'reactans'* and the WLA negative fluorescent *Pseudomonas* sp. may be responsible only for yellowing of *P. ostreatus* sporocarps.

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