

Monitoring of bacterial diseases of *Agaricus bisporus* in Serbia

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SUMMARY

Monitoring of button mushroom bacterial diseases was conducted to estimate the presence and identity of mycopathogenic bacteria and to determine the predominant bacterial pathogen in Serbia. Samples were collected from mushroom farms during 2006-2010 and also from fresh markets during 2014-2015. The collected samples showed either symptoms of brown blotch or different degrees of brown discoloration on caps and stalks of *Agaricus bisporus* resembling bacterial infection. The presence of bacterial droplets on gills was not recorded. The isolated bacteria were Gram-negative and fluorescent on King's medium B. In pathogenicity tests, most bacterial isolates induced superficial or sunken brown lesions with differences in the level of discoloration on *A. bisporus* tissue blocks after artificial inoculation. Based on LOPAT characteristics, the isolates were divided into two groups, showing characteristics of either the LOPAT group Va or group III. Based on these features and other differential biochemical characteristics, the presumptive *Pseudomonas tolaasii* isolates were confirmed by specific PCR. The other group of isolates was subjected to sequencing of the 16S rDNA. Based on these sequences most isolates were identified as *Pseudomonas agarici*, while two strains belonged to *Pseudomonas fluorescens*.

The survey resulted in detection and identification of *P. tolaasii* in 11 locations and *P. agarici* in 7 locations in Serbian mushroom farms. Most samples from fresh markets were infected with *P. tolaasii*, suggesting that this pathogen has been the predominant cause of bacterial diseases in Serbian mushroom-growing facilities over the past 10 years.

Keywords: Button mushroom; Mycopathogenic bacteria; *Pseudomonas*; Monitoring; Serbia

INTRODUCTION

Button mushroom (*Agaricus bisporus* (Lange) Imbach) is one of the most common cultivated edible mushrooms in Serbia with an annual yield of around 9,000 t. Its production is mostly intended for fresh market, but

although market demands are increasing, it is still at the level of small-scale growers (Milijašević-Marčić et al., 2012). A variety of harmful organisms may influence the yield and quality of this crop. Among them, mycopathogenic bacteria are considered less important in economic terms than fungi or viruses, but they are still

a concern because the means of their control are limited. There are several fluorescent pseudomonads with an ability to cause diseases of *A. bisporus* (Godfrey et al., 2001; Munsch et al., 2002; Iacobellis, 2011). Among them, *Pseudomonas tolaasii* (Paine, 1919; Tolaas, 1915) is the most common bacterial pathogen inducing dark-brown, sunken lesions on caps and stalks, and a resulting decrease in yield and quality, and shortened shelf life. Moreover, brown blotch symptoms on *A. bisporus* appearing as light-brown discoloration may also be caused by *P. reactans* (Wells et al., 1996; Iacobellis & Lo Cantore, 1997; 2003; Munch et al., 2002), *Pseudomonas* sp. strain NZ17 apparently related to *P. syringae* (Godfrey et al., 2001) and *P. costantinii* sp.nov. (Munch et al., 2002). The ginger blotch disease of *A. bisporus* is caused by *P. gingeri* (Wong & Preece, 1979) and the drippy gill of *A. bisporus* is caused by *P. agarici* (Young, 1970). In addition, Godfrey et al. (2001) and Iacobellis (2011) reported that a number of diverse pseudomonad species other than the known 'blotch-causing organisms' (*P. tolaasii*, *P. gingeri* and *P. reactans*) also have the ability to cause blotch diseases with various discolorations, and appear to participate in the expression of disease symptoms.

Unlike fungal pathogens, which have been thoroughly studied in Serbian mushroom farms (Potočnik et al. 2008, 2009, 2010, 2015; Kosanović et al., 2013), bacterial pathogens in *A. bisporus* had not been studied before 2006, when an infection with *Pseudomonas agarici* was reported for the first time in cultivated mushrooms in Serbia (Obradovic et al., 2006). Subsequently, symptoms resembling brown blotch were observed on several mushroom farms and *Pseudomonas tolaasii* was reported for the first time by Milijašević-Marčić et al. (2012). After these findings, an extensive survey of button mushroom bacterial diseases both in farms and on fresh markets was conducted to estimate the presence and identity of mycopathogenic bacteria and to identify the predominant bacterial pathogen in Serbia.

MATERIALS AND METHODS

Samples of diseased button mushroom were collected from mushroom farms and fresh markets in 18 locations within a ten-year period (Table 1). Bacteria were isolated from *A. bisporus* fruiting bodies with bacterial blotch

Table 1. Results of monitoring of bacterial pathogens in *A. bisporus* in Serbia

Location	Strain designation	Year of isolation	Strain identity
1. Novi Slankamen	NSl6B6	2006	<i>P. tolaasii</i>
2. Čačak	Ča1B6	2006	<i>P. tolaasii</i>
3. Mol	Mol4B6	2006	<i>P. tolaasii</i>
4. Padina	Pad1B6	2006	<i>P. tolaasii</i>
5. Užice	Uz12B6	2006	<i>P. tolaasii</i>
6. Vinarce, Niš	Vin7B6; Vin31B6	2006	<i>P. tolaasii</i>
7. Borča, Beograd	Bo42B6	2006	<i>P. tolaasii</i>
8. Novi Sad	NS3B6; NS1B6	2007	<i>P. tolaasii</i>
9. Veliko Gradište	Vg1/1;Vg1/2 Vg3/1;Vg3/2	2010	<i>P. tolaasii</i>
10. Veliko Gradište (market)	S-54; S-55, S-56; S-57; S-58; S-76; S-77; S-78; S-79; S-91; S-92; S-93; S-94; S-95; S-96, S-97	2014/2015	<i>P. tolaasii</i>
11. Jošanica (market)	S-80; S-81; S-82; S-83; S-84; S-85	2015	<i>P. tolaasii</i>
12. Padinska Skela	OP-6	2006	<i>P. agarici</i>
13. Padina	Pad-4	2006	<i>P. agarici</i>
14. Požarevac	Poz 8/7	2006	<i>P. agarici</i>
15. Veliko Gradište	Veg 2	2006	<i>P. agarici</i>
16. Vračev Gaj	Vg 1	2006	<i>P. agarici</i>
17. Vinarce	Vin 6	2006	<i>P. agarici</i>
18. Jakovo	Jak 4	2006	<i>P. agarici</i>

symptoms or different degrees of brown discoloration on caps and stalks following the method of Lelliott and Stead (1987). Presumptive colonies which produced a green fluorescent pigment were further purified by subculturing. For short term use, all strains were maintained on King's medium B (KB) slopes at 4°C, and for long term storage they were preserved at –80°C in KB liquid medium with a final concentration of 20% (v/v) glycerol in a culture collection of the Institute of Pesticides and Environmental Protection, Belgrade.

Pathogenicity tests were carried out using healthy, one-day-old *A. bisporus* whole sporophores and cubes of cap tissue excised with a sterile scalpel. For both trials the tissue was placed into sterile Petri dishes containing filter paper dampened with sterile distilled water. Bacterial isolates and reference strains of *P. tolaasii* (CFBP 2068^T) and *P. agarici* (CFBP 2063^T) were cultured on solid KB medium for 24 hours and then suspended in sterile distilled water to reach the density of approximately 1×10^8 CFU ml⁻¹. Negative control sporophores/cubes were treated with sterile distilled water only (Munsch et al. 2000; Lo Cantore & Iacobellis, 2004). The inoculated mushroom tissue was incubated in a humid chamber at room temperature. Tissue degradation and discoloration of the cubes were recorded after 72 h. All bioassays were repeated in triplicate.

The bacterial isolates were evaluated by determining LOPAT characteristics and the following phenotypic properties: Gram reaction, glucose metabolism, fluorescence on KB medium, catalase activity, aesculin hydrolysis, Tween 80 hydrolysis, casein hydrolysis, gelatine hydrolysis, nitrate reduction, utilization of mannitol, sorbitol, erythritol, inositol, sucrose, trehalose, arabinose, and D(-) tartrate as carbon sources (Lelliott & Stead, 1987; Schaad et al. 2001).

Molecular identification was conducted to confirm the identification of *P. tolaasii* isolates according to a PCR protocol described by Lee et al. (2002) using the primer pair Pt-1A, Pt-1D1 specific for its detection. The reference strain of *P. tolaasii* CFBP 2068^T was used as positive control. Another group of isolates was identified based on analysing the 16S rDNA sequence. A PCR assay with the universal primers 27F and 1541R was performed for amplification of the bacterial 16S rDNA (Edwards et al., 1989; Lane, 1991). Positive amplicons were sequenced in forward direction with the 27F primer. Sequencing was performed by a commercial service (Macrogen Inc., S. Korea). The obtained sequences were assembled using Pregap4 from the Staden program package (Staden et al., 2000).

RESULTS AND DISCUSSION

Our monitoring of bacterial diseases in mushroom farms and fresh markets in Serbia revealed the presence of symptoms of brown blotch or different degrees of brown discoloration on fruiting bodies of *A. bisporus* (Figures 1 and 2). Over a hundred bacterial isolates were obtained after isolation from symptomatic samples. Forty-five green fluorescent, Gram negative isolates from different sources and locations were selected for pathogenicity tests. The bacterial isolates showed different degrees of discoloration and tissue degradation on the mushroom cubes, varying from light to dark brown (Figure 3). Thirty-six bacterial isolates caused sunken brown lesions on *A. bisporus* cap tissue blocks after 72 h, consistent with those caused by the reference strain of *P. tolaasii*. The remaining isolates showed light brown superficial discoloration both on tissue blocks and sporophores similar to the *P. agarici* reference strain.



Figure 1. Sunken brown lesions on *A. bisporus* caps induced by *P. tolaasii*



Figure 2. Superficial brown lesions on *A. bisporus* caps induced by *P. agarici*



Figure 3. Superficial and sunken brown lesions with differences in the level of discoloration on *A. bisporus* tissue blocks after artificial inoculation-pathogenicity tests

Based on the studied biochemical properties, all tested bacterial isolates could also be divided into two groups. Those that showed LOPAT characteristics of the group Va also had the following biochemical properties: catalase positive, aesculin hydrolysis negative, Tween 80 hydrolysis positive, casein hydrolysis positive; gelatine hydrolysis and nitrate reduction negative; they used mannitol, erythritol, sorbitol, inositol and trehalose as carbon sources, but did not use sucrose, arabinose or D(-)tartrate, showing characteristics of *P. tolaasii* (Table 2). Their identity was confirmed in PCR tests. The other group of isolates (7), as well as the reference

strain of *P. agarici*, showed LOPAT characteristics of the group III, and did not utilize sorbitol, erythritol, L-arabinose, L-rhamnose, L-arabitol, 2-ketogluconate or D-tartrate (Table 2). The identity of these seven isolates was confirmed based on analyses of the 16S rDNA sequence as *P. agarici* (deposited in the NCBI GenBank under the accession numbers: KX261220; KX261221; KX261222; KX261223; KX261224; KX261225 and KX261226 for strains Poz 8/7; Jak 4; OP-6; Pad-4; Veg2; Vg 1 and Vin 6, respectively). However, the remaining two bacterial isolates showed 99 or 100% similarity with *P. fluorescens*.

Table 2. Biochemical characteristics of bacterial strains

	<i>P. tolaasii</i> strains (36) and *CFBP 2068 ^T	<i>P. agarici</i> strains (7) and *CFBP 2063 ^T
Gram stain	-	-
Glucose (O/F) ¹ metabolism	O	O
Levan production	-	-
Oxidase activity	+	+
Potato rot	-	-
Arginine dehydrolase	-	-
Tobacco hypersensitivity	-	+
Fluorescence on KB medium	+	+
Catalase activity	+	nt
Aesculin hydrolysis	-	nt
Tween 80 hydrolysis	+	nt
Casein hydrolyses	+	nt
Gelatine hydrolyses	-	nt
Nitrate reduction	-	-
Utilization of:		
mannitol	+	+
erythritol	+	-
sorbitol	+	-
inositol	+	nt
sucrose	-	-
trehalose	+	-
L-arabinose	-	-
L-arabitol	nt	-
D(-)tartrate	-	-
L-rhamnose	nt	-

Legend: O/F test - oxidative metabolism of glucose; + = positive result, - negative result; nt – not tested.

*Results of biochemical tests for reference strains (Schaad et al., 2001; Lo Cantore and Iacobellis, 2004).

Generally, the results of this survey showed that *P. tolaasii* was the predominant bacterium associated with brown discoloration symptoms on *A. bisporus*, which is not unexpected considering that *P. tolaasii* has been recognized as the most common button mushroom pathogen inducing considerable losses in the cultivation process. However, recent studies have pointed out that brown blotch of *A. bisporus*, caused by *P. tolaasii*, is a complex disease since some other pseudomonad species also have the ability to cause blotch symptoms with various levels of discolorations (Godfrey et al., 2001; Iacobellis, 2011). Moreover, the pathogenicity tests in our study also showed differences in the level of discoloration of the inoculated mushroom cap tissue, implying that some other fluorescent pseudomonads participated in the expression of disease symptoms. On the other hand, *P. agarici* was detected and identified in seven different locations from 2006-2010. Further monitoring in fresh markets did not show repeated isolation of this bacterium. It is also noteworthy that typical drippy gill symptoms were not observed in samples infected

with *P. agarici*. The same observation had been made by Geels et al. (1994) and Lo Cantore and Iacobellis (2004), suggesting a pathogen ability to induce different symptoms in various environmental conditions.

In regard to the economic impact of *P. tolaasii* as the predominant bacterial pathogen in Serbian mushroom farms, it mostly depends on the phytosanitary measures conducted in growing facilities. Although small growers are still prevailing, they are willing to introduce and apply strict sanitary measures and novelties in mushroom growing techniques.

CONCLUSIONS

The survey resulted in the detection and identification of *P. tolaasii* in 11 locations and *P. agarici* in seven locations of Serbian mushroom farms. Most samples from fresh markets were infected with *P. tolaasii*, suggesting that this pathogen has been the predominant cause of bacterial diseases in Serbian mushroom-growing facilities over the past 10 years.

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Monitoring bakterioza šampinjona u Srbiji

REZIME

Monitoring bakterioza šampinjona sproveden je s ciljem da se proceni prisustvo i identitet mikopatogenih bakterija i utvrdi dominantna bakterija na šampinjonima u Srbiji. Uzorci su prikupljeni iz gajilišta šampinjona u periodu 2006-2010. godine i sa zelenih pijaca tokom 2014-2015. godine. Na prikupljenim uzorcima registrovani su simptomi smeđe pegavosti i promene boje tkiva različitog stepena na šeširima i drškama *Agaricus bisporus* koji su nalikovali bakterijskoj infekciji. Prisustvo bakterijskog eksudata na listićima sporofora nije zabeleženo. Izolati bakterija su bili gramnegativni i stvarali su fluorescentni pigment na King-ovoj podlozi B. U testovima patogenosti, većina izolata izazivala je površinske ili udubljene braon lezije sa različitim stepenom promene boje na isečcima tkiva *A. bisporus* nakon veštačke inokulacije. Na osnovu LOPAT testova, izolati su podeljeni u dve grupe, jednu sa karakteristikama LOPAT Va grupe i drugu sa karakteristikama LOPAT grupe III. Na osnovu ovih osobina i drugih diferencijalnih biohemijskih karakteristika, identitet izolata za koje se pretpostavilo da pripadaju *Pseudomonas tolaasii* potvrđen je specifičnom PCR reakcijom. Kod druge grupe izolata sekvencioniran je 16S rDNK. Na osnovu ovih sekvenci većina izolata je identifikovana kao *Pseudomonas agarici*, dok su dva soja pripadnici *Pseudomonas fluorescens*.

Kao rezultat istraživanja *P. tolaasii* detektovana je i identifikovana na 11 lokaliteta, a *P. agarici* na sedam lokaliteta u gajilištima šampinjona u Srbiji. Većina uzoraka sa zelenih pijaca bili su zaraženi bakterijom *P. tolaasii*, ukazujući na to da je ovaj patogen bio dominantan uzročnik bakterioza na šampinjonima u gajilištima u Srbiji u prethodnih 10 godina.

Ključne reči: Šampinjon; Mikopatogene bakterije; *Pseudomonas*; Monitoring; Srbija