INTERNATİONAL JOURNAL OF AGRİCULTURE & BİOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18–0863/2018/20–12–2721–2730
DOI: 10.17957/IJAB/15.0819
http://www.fspublishers.org

Full Length Article

Characterization of *Pseudomonas tolaasii* Isolates, Causing Brown Blotch Disease of Mushroom (*Agaricus bisporus*)

Esin Basim[1*] and Hüseyin Basim[2]
[1]*Department of Organic Agriculture, Akdeniz University, Antalya, Turkey*
[2]*Plant Protection Department, Akdeniz University, Antalya, Turkey*
[*]For correspondence: esinbasim@akdeniz.edu.tr

Abstract

A total of 96 *Pseudomonas tolaasii* isolates were recovered from the suspected Brown blotch infected mushroom caps and cultured on King's medium B. The LOPAT test results showed Levan and pectinolytic activity, positive oxidase, arginine dihydrolase and hypersensitivity on tobacco. The isolates was identified by the conventional PCR using *Pt*-1A/*Pt*-1D1 primers and Real-time PCR. The BLAST analysis of the PCR products showed 98–100% similarities with the strains recovered from the NCBI GenBank database. All the isolates were pathogenic on *Agaricus bisporus* and slices of potatoes. The Koch's postulates were fulfilled. This is the first record on the characterization of *P. tolaasii* isolates in west Mediterranean region of Turkey as well as rapid detection of this pathogen directly from the bacterial cell without DNA extraction and from the diseased tissue, respectively by the Real-time PCR. © 2018 Friends Science Publishers

Keywords: *Agaricus bisporus*; Brown blotch disease; Conventional PCR; *Pseudomonas tolaasii*; Real-time PCR

Introduction

The most commonly grown mushroom worldwide is *Agaricus bisporus* (Soler-Rivas *et al.*, 1999). Mushrooms, in general, have been extensively used and consumed as foods to meet the daily nutritional and health needs (Cheung, 2010). The medicinal values of mushrooms encompass boosting the immune system, wound-healing, and impediments of tumor effects among many other health benefits (Dai *et al.*, 2009). The world total production of mushroom and truffles from 28,111 ha area was 10,378,163 tons. Out of this total, Turkey produced 38,767 tons in 2014 (FAO, 2014). The most common varieties of mushrooms cultivated in the world include *A. bisporus* (30%), *Pleurotus* spp. (27%) and *Lentinula* spp. (17%). In Turkey, Antalya province ranks first in mushroom production with the daily production of 83 tons, representing 74% of the cultivated mushroom in Turkey, Marmara and Ege regions ranked second and third, with the daily mushroom productions of 26 and 10 tons, respectively (Eren and Pekşen, 2016). The Korkuteli district of Antalya province is the main center for both compost and mushroom production, which produced 18,500 tons mushroom in 2013, representing 70% of the regional total, and this makes it the highest mushroom producing district in Turkey (Eren and Pekşen, 2016).

The cultivated mushrooms are threatened by numerous fungal and bacterial diseases that may lead to serious yield losses (Gill, 1995). Among such bacterial diseases of the commonly *A. bisporus* is the brown blotch (Tolaas, 1915) caused by the *Pseudomonas tolaasii* (Paine, 1919), which is one of the most important pathogens affecting millions of mushrooms worldwide including Turkey and causes substantial damage to yield quality (Soler-Rivas *et al.*, 1999). The brown blotch disease is characterized by slightly sunken brown lesions on the surface of the pileus (cap) and stipe and under favorable conditions, these lesions coalesce, covering a large part of the infected pileus, which may lead to tissue decay, thereby resulting in foul smell, and this condition can equally occur after mushroom harvest (Wells *et al*., 1996).

Several other fluorescent Pseudomonads pathogens are known to cause diseases in the mushrooms genus *Agaricus* and *Pleurotus* (Gill, 1995). These include a saprophytic bacterium called *P. reactans*, popularly established in the literature for its usefulness in performing "white line" test for precise *P. tolaasii* identification (Wong and Preece, 1979). This bacterium was established to contribute to the enhancement of the disease development in the commonly grown mushrooms such as *Pleurotus* spp. and *A. bisporus* as well as in *Pleurotus eryngii* mushrooms (Iacobellis and Lo Cantore, 1998). The two other species of bacteria found in connection with the cultivated mushrooms include *P. agarici* which causes drippy gill disease (Young, 1970) and *P. gingeri*, the causative agent of a ginger blotch of mushroom. Whereas the ginger blotch disease is distinguished by pale, yellowish brown small spots on the sporophores, which change to reddish-ginger as the mushroom grows with time. The *P. tolaasii* produces toxins called tolaasin, which the bacterium uses as a weapon to break up the mushroom

To cite this paper: Basim, E. and H. Basim, 2018. Characterization of *Pseudomonas tolaasii* ısolates, causing brown blotch disease of mushroom (*Agaricus bisporus*). *Int. J. Agric. Biol*., 20: 2721–2730

cellular membrane by creating pores or via the ion channel forming action (Rainey *et al.*, 1991). These toxins also play a major role in the formation of white lines when *P. tolaasii* is inoculated in the same petri plate with *P. reactans* which are white line reacting organisms (WLROs) (Wells *et al.*, 1996).

The identification of *P. tolaasii* was traditionally carried out based on the pathogenicity assay (Tolaas, 1915). The diagnosis of this pathogen also depends on the so-called "white-line-in-agar" (WLA) resulting from an interplay between saprophytic *P. reactans* and the pathogenic *P. tolaasii* which produce a white precipitate that is visible within 24 to 48 h incubation period at 25°C, when these two bacteria are streaked on agar few centimeters away from each other. Other molecular methods such as REP-PCR, ERIC and BOX-PCR technique have been applied for diversity studies among *P. tolaasii* pathogens (Shadi *et al.*, 2015). Conventional PCR using specific primer pairs (Milijasevic-Marcic *et al.*, 2012) was also used for the identification of *P. tolaasii*. Although there are reports on the presence of bacterial diseases associated with the cultivated mushrooms including brown blotch diseases caused by *P. tolaasii* in Turkey (Ozaktan *et al*., 2000), no much detail study has been conducted with regards to the characterization, identification and detection of this pathogen.

The objective of this study was to provide in-depth analysis in the characterization of *P. tolaasii* population in Turkey. This is the first work on the identification and detection of the pathogen from the direct bacterial cell and diseased mushroom tissue by Real-time PCR.

Materials and Methods

Sample Collection and İsolation

Samples of cultivated *A. bisporus* showing symptoms of brown blotch diseases (Fig. 1) were collected from 214 mushroom farms in Avdan, Kargalık, Gümüşlü, Söğütçük, Küçükköy, Yazır, Yelten, Yeşil Yayla, Alaaddin, Aşağı Pınar, Şemik, Sanayi all under Korkuteli district, during the surveys between 2015 and 2018. The putative bacteria were recovered from the brown blotch infected mushroom fruiting bodies. The fruiting bodies (caps) were cut, surface sterilized in 70% ethanol, after which the tissues were rinsed in three exchanges with sterilized distilled water. The dirty outermost layers of the caps were scraped off, and the internal necrotic tissues were carefully cut and put into Eppendorf tube containing 800 μL sterilized distilled water and grounded with sterilized plastic pestles until homogeneous contents were obtained. A loopful of the contents were streaked on the Nutrient Agar (NA) and King's B (KB) media (Wong and Preece, 1979). The petri dishes were incubated for 3 days at 25ºC. The bacterial colonies were further purified by re-streaking single desired colonies on the fresh KB media. The purified colonies were transferred in -80ºC storage media (Nutrient Broth with 30% glycerol) for further use.

The reference strains namely, *P. tolaasii* (NCPPB 2192), *P. reactans* (NCPPB 3149) and *P. gingeri* (NCPPB 3146) obtained from National Collection of Plant Pathogenic Bacteria (Fera-UK) were used as controls. The bacteria examined in this study are given in Table 1.

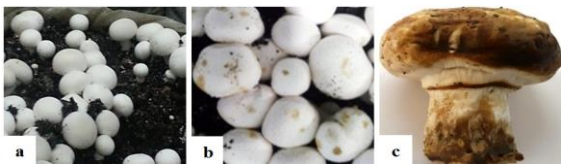


Fig. 1: Symptoms of brown blotch disease on the infected *Agaricus bisporus* sporophores (A) healthy mushroom, (B) initial symptoms (small brown spot) (C) severely infected brownish symptoms during the later stage of infection

Pathogenicity Test

All the isolates, including the reference strain, were tested for pathogenicity on healthy *A. bisporus* by using the mushroom tissue block test with little modification (Nair and Fahy, 1973). The tests were carried out by using mushroom caps (sporophores) cut with a flamed scalpel blade and placed on a 30 x 15 cm sterilized Whatman filter paper soaked with 1000 μL sterile distilled water. The excised caps were exposed to UV light for 20 min before inoculation was done. The caps were injected with the freshly cultured and adjusted (10^8 CFU/mL) bacterial suspension (10 μL) of *P. tolaasii* and *Pseudomonas "reactans"* by using a sterile injector with a 26 G-0.45×13 mm-gauge needle. The caps inoculated with sterile distilled water were used as a negative control. The tests were performed in four replicates for all the isolates using different tissue of *A. bisporus*. The inoculated caps were kept at 25°C and 90% relative humidity in an improvised moist chamber, which were later transferred to the controlled-room.

The putative pathogen was also inoculated on the potato slices placed on Whatman's fitler paper in the petri dishes by spreading 50 μL of approximately 10^8 CFU/mL of the bacterial suspension on the surfaces of the potato slices with the aid of the sterilized inoculation loop. The petri dishes were wrapped and incubated at 27°C for three days. Tissue degradation and other symptoms such as discoloration developed on the caps and potato slices were examined after 24 to 48 h of inoculation. The degree of disease severity on the mushroom tissue and potato slices were scored by using the empirical scale as described by Lo Cantore and Iacobellis (2014), where; 0, equivalent to control treatment (the distilled sterile water); 1, light brown discoloration; 2, marked browning discoloration; 3, marked sunken and watery browning discoloration.

Biochemical Properties of the Bacteria

The preliminary examination of the phenotypic characteristics of the putative pathogen was conducted by the

Table 1: Bacterial isolates used for the study

Bacterial isolates		Country	Year of isolation	WLA test	Pathogenicity	Virulence score			Classical PCR	Real-Time PCR
						1	2	3		
HBPt1	*P. tolaasii*	Turkey	2015	+	+			+	+	+
HBPt2	*P. tolaasii*	Turkey	2015	+	+		+		+	+
HBPt3	*P. tolaasii*	Turkey	2015	+	+		+		+	+
HBPt4	*P. tolaasii*	Turkey	2015	+	+		+		+	+
HBPt5	*P. tolaasii*	Turkey	2015	-	+		+		+	+
HBPt6	*P. tolaasii*	Turkey	2015	+	+	+			+	+
HBPt7	*P. tolaasii*	Turkey	2015	-	+		+		+	+
HBPt8	*P. tolaasii*	Turkey	2015	+	+			+	+	+
HBPt9	*P. tolaasii*	Turkey	2016	-	+			+	+	+
HBPt10	*P. tolaasii*	Turkey	2016	+	+			+	+	+
HBPt11	*P. tolaasii*	Turkey	2016	+	+			+	+	+
HBPt12	*P. tolaasii*	Turkey	2016	+	+			+	+	+
HBPt13	*P. tolaasii*	Turkey	2016	-	+			+	+	+
HBPt14	*P. tolaasii*	Turkey	2016	+	+			+	+	+
HBPt15	*P. tolaasii*	Turkey	2016	+	+			+	+	+
HBPt16	*P. tolaasii*	Turkey	2016	-	+			+	+	+
HBPt17	*P. tolaasii*	Turkey	2016	+	+			+	+	+
HBPt18	*P. tolaasii*	Turkey	2016	+	+		+		+	+
HBPt19	*P. tolaasii*	Turkey	2016	+	+		+		+	+
HBPt20	*P. tolaasii*	Turkey	2016	+	+			+	+	+
HBPt21	*P. tolaasii*	Turkey	2016	+	+			+	+	+
HBPt22	*P. tolaasii*	Turkey	2016	-	+			+	+	+
HBPt23	*P. tolaasii*	Turkey	2016	-	+			+	+	+
HBPt24	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt25	*P. tolaasii*	Turkey	2017	+	+		+		+	+
HBPt26	*P. tolaasii*	Turkey	2017	-	+	+			+	+
HBPt27	*P. tolaasii*	Turkey	2017	-	+		+		+	+
HBPt28	*P. tolaasii*	Turkey	2017	+	+		+		+	+
HBPt29	*P. tolaasii*	Turkey	2017	+	+	+			+	+
HBPt30	*P. tolaasii*	Turkey	2017	-	+	+			+	+
HBPt31	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt32	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt33	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt34	*P. tolaasii*	Turkey	2017	+	+	+			+	+
HBPt35	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt36	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt37	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt38	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt39	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt40	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt41	*P. tolaasii*	Turkey	2017	-	+			+	+	+
HBPt42	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt43	*P. tolaasii*	Turkey	2017	+	+	+			+	+
HBPt44	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt45	*P. tolaasii*	Turkey	2017	-	+			+	+	+
HBPt46	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt47	*P. tolaasii*	Turkey	2017	+	+		+		+	+
HBPt48	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt49	*P. tolaasii*	Turkey	2017	-	+		+		+	+
HBPt50	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt51	*P. tolaasii*	Turkey	2017	+	+			+	+	+
HBPt52	*P. tolaasii*	Turkey	2018	-	+	+			+	+
HBPt53	*P. tolaasii*	Turkey	2018	-	+			+	+	+
HBPt54	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt55	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt56	*P. tolaasii*	Turkey	2018	+	+	+			+	+
HBPt57	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt58	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt59	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt60	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt61	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt62	*P. tolaasii*	Turkey	2018	-	+			+	+	+
HBPt63	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt64	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt65	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt66	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt67	*P. tolaasii*	Turkey	2018	+	+	+			+	+
HBPt68	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt69	*P. tolaasii*	Turkey	2018	-	+			+	+	+
HBPt70	*P. tolaasii*	Turkey	2018	+	+			+	+	+

Table 1: Continued

Table 1: Continued

Bacterial isolates		Country	Year of isolation	WLA test	Pathogenicity	Virulence score			Classical PCR	Real-time PCR
						1	2	3		
HBPt71	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt72	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt73	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt74	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt75	*P. tolaasii*	Turkey	2018	+	+		+		+	+
HBPt76	*P. tolaasii*	Turkey	2018	+	+	+			+	+
HBPt77	*P. tolaasii*	Turkey	2018	+	+		+		+	+
HBPt78	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt79	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt80	*P. tolaasii*	Turkey	2018	+	+		+		+	+
HBPt81	*P. tolaasii*	Turkey	2018	+	+		+		+	+
HBPt82	*P. tolaasii*	Turkey	2018	-	+		+		+	+
HBPt83	*P. tolaasii*	Turkey	2018	-	+		+		+	+
HBPt84	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt85	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt86	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt87	*P. tolaasii*	Turkey	2018	+	+	+			+	+
HBPt88	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt89	*P. tolaasii*	Turkey	2018	-	+			+	+	+
HBPt90	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt91	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt92	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt93	*P. tolaasii*	Turkey	2018	-	+			+	+	+
HBPt94	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt95	*P. tolaasii*	Turkey	2018	+	+			+	+	+
HBPt96	*P. tolaasii*	Turkey	2018	+	+			+	+	+
NCPPB2192	*P. tolaasii*	UK	1969	+	+			+	+	+
NCPPB3149	*P. reactans*	UK	1981	-	-	-	-	-	-	-
NCPPB3146	*P. gingeri*	UK	1983	-	-	-	-	-	-	-
DSM-50259	*P. cichorii*	Germany	1980	-	-	-	-	-	-	-
HBPc1	*P. corrugata*	Turkey	2008	-	-	-	-	-	-	-
9.1.6	*P. fluorescens*	Makedonya	2004	-	-	-	-	-	-	-
DSM-16733	*P. mediterranea*	Germany	2002	-	-	-	-	-	-	-
LMG 2353	*P. viridiflava*	Belgian	2001	-	-	-	-	-	-	-
DSM-4610	*Dickeya chrysanthemi*	Germany	1998	-	-	-	-	-	-	-
DSM-30168	*P. c.* subspp. *carotovorum*	Germany	1999	-	-	-	-	-	-	-
NCPPB2979	*C. m.* subspp. *michiganensis*	Hungary	1987	-	-	-	-	-	-	-
HBXav2	*X. a.* pv. *vesicatoria*	Turkey	2006	-	-	-	-	-	-	-
HBPst12	*P. syringae* pv. *tomato*	Turkey	2007	-	-	-	-	-	-	-

following tests: Gram reaction, fluorescent pigment production, catalase activity, gelatine hydrolysis, starch production, casein hydrolysis, and sucrose utilization as described by Lelliott and Stead (1987). The grown bacterial cultures on the KB media were examined under UV light for the presence of fluorescent pigment. The *P. tolaasii* reference strain (NCPPB 2192) was used as a positive control.

The bacterial isolates characteristics were examined for levan, oxidase and arginine dihydrolase production; pectinolytic activity; and hypersensitivity on tobacco (LOPAT) according to Lelliott and Stead (1987).

Tolaasin Assay

The tolaasin activity of *P. tolaasii* which is a extracellular toxin, and the main elicitor of disease was examined in this study by inoculating surface sterilized slices of potato tubers. The tolaasin toxins were extracted and purified by passing the overnight grown bacteria in a nutrient broth through cold sterilization filter. The inoculation was done by using different levels of diluted toxin concentrations that, included stock, 10^{-1} and 10^{-2}. The inoculated potatoes were monitored for tissues blackening according to Shirata *et al.* (1995).

White Line Assay (WLA)

The WLA test was carried out on the KB medium as described by Wong and Preece (1979). The *P. tolaasii* isolates recovered from *A. bisporus* from 214 mushroom farms in the Korkuteli district were streaked about one centimeter on each side of the reference *P. reactans* (NCPPB 3149) placed in the middle of the petri dish. The *P. tolaasii* type strain (NCPPB 2192) and *P. gingeri* (NCPPB 3146) were used as positive and negative controls, respectively. The petri dishes were incubated at 25°C for at least two to three days. The occurrence of a white line precipitate between the two bacteria was regarded as a positive reaction.

PCR Assays

The tolaasin gene from the isolates were amplified by PCR using primers, Pt-1A (5'-ATCCCTTCGGCGTTTACCTG-3') and Pt-1D1 (5'- CAAAGTAACCCTGCTTCTGC-3'), (Milijasevic-Marcic *et al*., 2012) from direct bacterial suspension without DNA extraction. Each PCR reaction contains a 50 μL - final volume including 5.0 μL 10x Taq buffer with KCl (Thermo Scientific, Massachusetts, USA),

3.0 μL of 2.5 m*M* $MgCl_2$, 8.0 μL of 100 m*M* dNTPs, 1 μL (320 pmol) of each primer, 0.25 μL of 5U/Ml Taq DNA polymerase (Thermo Scientific, Massachusetts, USA), 27.8 μL of sterile distilled water, and 4.0 μL of bacterial suspension. The *P. tolaasii* strain (NCPPB 2192) (Paine, 1919) was included as a positive control. The PCR programme included initial step of 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 52°C, and 2 min at 72°C and a final step of 5 min at 72°C. The PCR products (10 μL) and 100 bp DNA Ladder (Thermo Scientific, Massachusetts, USA) were run by gel electrophoresis in 1.5% agarose gel in 0.5x TAE (Tris-Base 4,84 g, Acetic Acid [Glacial] 1,02 mL, 0,5 M EDTA [pH: 8,0] 2 mL, ddH_2O 1000 mL) buffer at 75 V/cm for 1 h 30 min. The agarose gel was stained with ethidium bromide (0,5 μg/mL) for 10 min., and photographed using imaging system (Viber Lourmat SR 12575 UV transilluminator, France). The PCR tests were carried out using all the ninety six isolates.

Real-time PCR using both direct bacterial cell suspension and macerated infected *A. bisporus* tissues were conducted in a Cepheid Smart Cycler II (USA). The negative controls used included bacterial suspension template from *P. gingeri* (NCPPB 3146), and *P. "reactans"* (NCPPB 3149).

Each PCR reaction contains a 29.6 μL - final volume including 3.0 μL 10x Taq buffer with KCl (Thermo Scientific, Massachusetts, USA), 7.2 μL of 2.5 m*M* $MgCl_2$, 4.8 μL of 100 m*M* dNTPs, 1.2 μL (320 pmol) of each primer, 0.36 μL of 5U/Ml Taq DNA polymerase (Thermo Scientific, Massachusetts, USA), 0,3 μL SYBR Green I (Roche Diagnostics Corporation, USA), 9.54 μL of sterile distilled water, and 2.0 μL of bacterial suspension. The *P. tolaasii* strain (NCPPB 2192) (Paine, 1919) was included as a positive control. The PCR programme included initial step of 2 min at 94°C, followed by 35 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C and a final step of 2 min at 72°C. The single amplification product was confirmed by melting-curve temperature analysis using 60°C to 95°C at 0.2°C/s ramp rate.

The sensitivity of detection of *P. tolaasii* was determined by serial dilution assay. Tenfold serial dilution (10^{-1} to 10^{-9}) of the bacterial suspensions were carried out, and 2 μL (1×10^8 CFU) each of the suspensions was used for the Real-time PCR and the same time spread on the nutrient agar and incubated at 25°C for three days, after which the total number of cells (CFU) was counted to assess the detection limit of the bacteria. The number of cells corresponding to related Ct values was estimated in Real-time PCR.

Other sisters outgroup of bacteria, namely *P. cichorii*, *P. fluorescens*, *P. mediterranea*, *P. viridiflava*, *P. corrugata*, *P. syringae* pv. *tomato*, *Pectobacterium carotovorum* subspp. *carotovorum*, *Dickeya chrysanthemi*, *Clavibacter michiganensis* subspp. *michiganensis* and *Xanthomonas axonopodis* pv. *vesicatoria* were also tested with the primer specific to tolaasin producing gene in the primer specificity and the selectivity test.

Phylogenetic Analysis

The sequenced PCR products were edited, and then aligned using Bio-Edit Version 7.2.5 (Hall, 1999). The PCR products were sequenced by BM Labosis-Macro Gene (Çankaya, Ankara) (www.bmlabosis.com).The homology of the obtained sequences were search with other *P. tolaasii* and related species in the NCBI GenBank database (Table 2) with using the program BLAST 2.2.1 (Altschul *et al*., 1990). Evolutionary and phylogenetic relationship of the species was analysed by MEGA 5.1 (Tamura *et al.*, 2011), using the Maximum Likelihood method based on the Kimura 2-parameter models (Kimura, 1980). The bootstrap was carried out with 1000 replicates.

Results

Sample Collection and Isolation

A total of 176 suspected brown blotch infected *A. bisporus* mushroom samples showing symptoms that included wet, sunken brown lesions on the surface of the pileus and stipe (Fig. 1) were obtained at the end of the surveys, from 214 mushroom farms in the various villages and towns of Korkuteli district. In 2015 survey, 8 isolates were detected as *P. tolaasii*, in the 2016 survey, 15 isolates were detected as *P. tolaasii*, in 2017 and 2018 surveys, 28 and 45 isolates were detected as *P. tolaasii*, respectively (Table 1). The putative bacteria produced a circular light yellowish to creamy colonies on both nutrient agar and King's B media within 24 h.

Pathogenicity Test

The putative pathogen caused irregular sunken, wet brown lesions on the sporophores of *A. bisporus* after 48 h of inoculation (Fig. 2 and Table 1). When the inoculated mushrooms were further dissected, brown to chocolate colors were observed in the internal tissue of the caps. The symptoms produced by the inoculated isolates in this study were consistent with those caused by the *P. tolaasii* type strain (NCPPB 2192) used as a positive control. All the isolates also caused varied tissue degradation and discoloration (blackening) on the potato slices and were categorized into three groups based on the degree of their virulence (Fig. 2 and Table 1). The isolates showed different virulence levels on potato slices, but no differences were observed on sporophores of *A. bisporus*. Tissues inoculated with sterile distilled water and *P. reactans* did not show any symptoms of degradation and browning. The bacteria were constantly re-isolated from the diseased mushroom and potato tissues. The Koch's postulates were fulfilled.

Biochemical Properties of the Bacteria

The results of the phenotypic characterization based on the

Table 2: GenBank nucleotide homology of *P. tolaasii* with Turkey isolates

Strain/Isolates	Gene	Genbank Accession No
P. tolaasii HBPt9-2016-Turkey	*tolaasin*	MH427009
P. tolaasii HBPt30-2016-Turkey	*tolaasin*	MH427010
P. tolaasii K59	*tolaasin*	EF061509.1
P. tolaasii K40	*tolaasin*	EF061508.1
P. tolaasii K22	*tolaasin*	EF061510.1
P. tolaasii S4	*tolaasin*	EF061511.1
P. tolaasii C42	*tolaasin*	EF061506.1
P. tolaasii C23	*tolaasin*	EF061507.1
P. tolaasii	*tolaasin*	AF291753.1
P. azotoformans S4	*tolaasin*	CP014546.1
P. fluorescens LBUM636	*tolaasin*	CP012400.1
P. yamanorum strain LMG 27247	*tolaasin*	LT629793.1

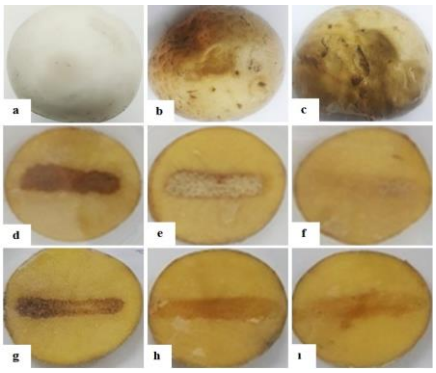


Fig. 2: The pathogenicity test showing symptoms on *Agaricus bisporus* sporophores and potato slices (A) healthy cap treated with sterile distilled water (B) irregular dark-brown, sunken lesions on the cap (C) advanced stage of infection showing wet and sunken symptoms (D) potato tissue maceration by most virulent isolates (E) moderate virulent isolates (F) less virulent isolates. Tolaasin activity using serial dilutions of tolaasin (G) stock tolaasin (H) 10^{-1} dilution (I) 10^{-2} dilution

biochemical characters of the putative pathogen carried out following Lelliott and Stead (1987), showed both positive (+) and negative (-) reactions as follows: Gram reaction (-), catalase activity (+), gelatine hydrolysis (+), starch production (-), casein hydrolysis (+), sucrose utilization (-) and fluorescent pigment (+). The LOPAT test also showed Levan (-), pectinolitic activity (-), oxidase (+), arginine dihydrolase (+) and positive hypersensitivity on tobacco plant. The included reference *P. tolaasii* type strain (NCPPB 2192) also exhibited the same characteristics as our isolates.

Tolaasin Assay

The tolaasin activity on the inoculated potatoes also showed positively by causing tissue degradation and discoloration. The tolaasin toxin is the main disease elicitor of *P. tolaasii* responsible for causing diseases. The potatoes inoculated with the stock toxin concentration showed more blackening symptoms than those inoculated with 10^{-1} and 10^{-2} dilutions (Fig. 2).

White Line Assay

All the bacteria isolates were evaluated in white line test according to the procedure of Wong and Preece (1979), and the results showed that most of the Turkey's isolates reacted positively with the *P. reactans* (NCPPB 3149), which produced a white line precipitate in agar. Few of the isolates failed to produce a white line precipitate, but they were found to be pathogenic when inoculated on the mushroom and potatoes, as well as tested positive for PCR test. The reference type strain, *P.tolaasii* (NCPPB 2192), used as a positive control also reacted positively with the *P. reactans*, which resulted in the formation of white line precipitate between these two bacteria. The negative controls, *P. gingeri* (NCPPB 3146) and *P. fluorescens* (9.1.6) did not produce any white line precipitate (Fig. 3 and Table 1).

PCR Assays

The identification of all the isolates recovered from *A. bisporus* from various mushroom farms in the Korkuteli district, including the reference strain was achieved with the conventional PCR using primer pairs that were specific to tolaasin producing gene of *P. tolaasii*, which yielded amplification fragments of approximately 449 bp (Fig. 4). Only 30 isolates were presented in the conventional PCR result and the rest shown in Table 1.

In the real-time PCR assays, *P. tolaasii* isolates recovered from the *A. bisporus*, and the *P. tolaasii* reference strain included as a positive control, were detected within a Ct range of 12.63 to 17.72. The negative controls using bacterial suspension template from *P. gingeri* (NCPPB 3146), *P. "reactans"* (NCPPB 3149) as well as sterile distilled water failed to produce any peak, and thereby, recorded 0.0 values (Fig. 5A).

The melting-curve analysis showed that the isolates recovered from the *A. bisporus* in this study and the reference *P. tolaasii* positive control yielded various single melt peaks at different temperatures. The negative controls failed to rise at any given temperature (Fig. 5B).

The total number of cells/CFU counted after the tenfold serial dilutions streaked on the nutrient agar showed uncountable numbers of bacterial colonies from the stock, 10^{-1}, 10^{-2} and 10^{-3} serial dilution suspensions, whereas 1126, 120, 14 and 2 colonies were counted from 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} serial dilution suspensions, respectively. The serial dilution of 10^{-8} and 10^{-9} suspensions recorded 0.0 bacteria cells. The SYBR Green dye showed a sensitivity and detection limit of ca. 10^{7} serial dilutions corresponding to 2 CFU/2 μL. The C_t values yielded by different serial dilutions are also shown (Fig. 6).

The other sister outgroup of bacteria such as *P.*

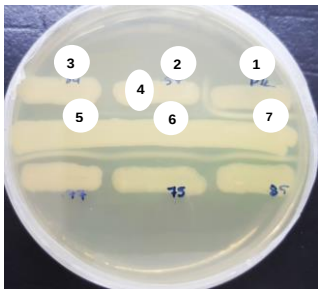


Fig. 3: White line test, on King's B medium. 1=*P.tolaasii* (NCPPB 2192), 2= *P. Gingeri* (NCPPB 3146), 3= *P. fluorescens* (9.1.6), 4= *Pseudomonas "reactans"* (NCPPB 3149), 5= *P. tolaasii* (HBPt1), 6= *P. tolaasii* (HBPt2), 7= *P. tolaasii* (HBPt3)

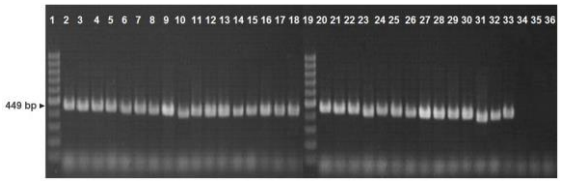


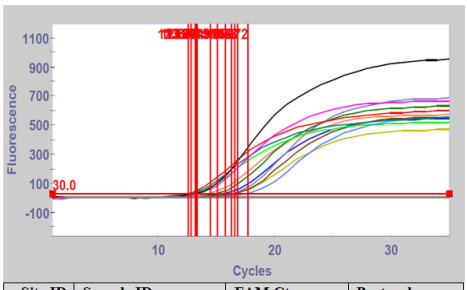
Fig. 4: Agarose gel of PCR products of *Pseudomonas tolaasii* isolates using Pt-1A/Pt-1D primer pairs. Lanes 1 and 19 = 100 bp DNA ladder; lanes 2-32= isolates recovered from *Agaricus bisporus* in this study, lane 33 = *Pseudomonas tolaasii* positive control (NCPPB 2192), lane 34 = negative control (*Pseudomonas gingeri*, NCPPB 3146), lane 35 = negative control (*Pseudomonas "reactans"* NCPPB 3149), lane 36 = negative control with water

cichorii, *P. corrugata*, *P. fluorescens*, *P. mediterranea*,*P. viridiflava*, *P. syringae* pv. *tomato*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Dickeya chrysanthemi*, *Clavibacter michiganensis* subspp. *michiganensis* and *Xanthomonas axonopodis* pv. *vesicatoria* tested in the sensitivity and the selectivity test showed that, all the tested strains failed to produce amplification signals and thereby recorded 0.0 mean Ct values. The *P. tolaasii* used as a positive control yielded a mean Ct value of 25.97 (Fig. 7).

The *P. tolaasii* pathogen was also detected directly from macerated *A. bisporus* tissue without bacterial isolation in the Real-time PCR, which yielded mean Ct values of 15.74 and 21.14 for the reference strain and the Turkey's isolate, respectively (Fig. 8).

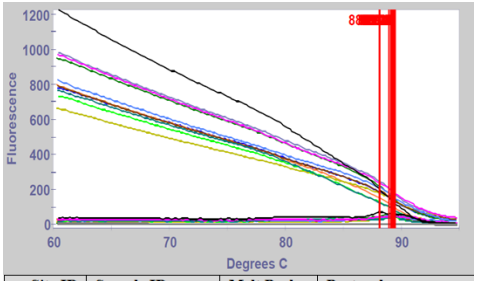
Phylogenetic Analysis

The BLAST analysis of the sequenced PCR products of *P. tolaasii* isolates showed high homology, which ranged from 98 to 100% with those recovered from the NCBI GenBank. The hierarchical binary cluster dendrogram grouped the *P. tolaasii* isolates HBPt30 and HBPt9 recovered from *A. bisporus* in Turkey, and those strains retrieved from NCBI GenBank (Table 2) into various clusters, which showed



Site ID	Sample ID	FAM Ct	Protocol
A1	HBPt3	15.85	Pt-SYBR
A2	HBPt9	16.36	Pt-SYBR
A3	HBPt16	14.49	Pt-SYBR
A4	HBPt22	15.11	Pt-SYBR
A5	HBPt30	16.88	Pt-SYBR
A6	HBPt41	16.60	Pt-SYBR
A7	HBPt52	12.63	Pt-SYBR
A8	HBPt63	13.24	Pt-SYBR
A9	HBPt81	17.72	Pt-SYBR
A10	HBPt83	12.85	Pt-SYBR
A11	HBPt92	13.42	Pt-SYBR
A12	NCPPB2192	13.29	Pt-SYBR
A13	Negative Control	0.00	Pt-SYBR

Fig. 5A: Real-time PCR analysis of some *Pseudomonas tolaasii* isolates with SYBR Green dye



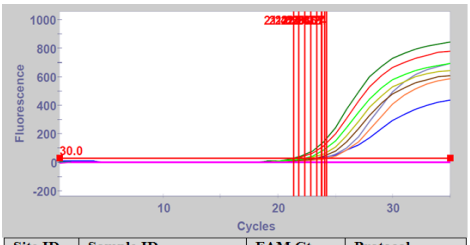
Site ID	Sample ID	Melt Peak	Protocol
A1	HBPt3	89.08	Pt-SYBR-melt
A2	HBPt9	89.33	Pt-SYBR-melt
A3	HBPt16	89.17	Pt-SYBR-melt
A4	HBPt22	88.94	Pt-SYBR-melt
A5	HBPt30	89.26	Pt-SYBR-melt
A6	HBPt41	89.21	Pt-SYBR-melt
A7	HBPt52	89.46	Pt-SYBR-melt
A8	HBPt63	89.32	Pt-SYBR-melt
A9	HBPt81	89.36	Pt-SYBR-melt
A10	HBPt83	89.34	Pt-SYBR-melt
A11	HBPt92	89.20	Pt-SYBR-melt
A12	NCPPB2192	88.13	Pt-SYBR-melt
A13	Negative Control		Pt-SYBR-melt

Fig. 5B: Post-amplification melting-curve analysis of the isolates

100% homology, implying a closer genetic similarity or relationship among them. The Turkey's isolates as mentioned above, however, showed low similarity with the out group, implying a distance genetic relationship between them (Fig. 9). The phylogenetic analysis was constructed by using the nucleotide sequences of the tolaasin producing gene. The deleted and obscure regions within the sequences were excluded for the comparison of the analyzed sequences.

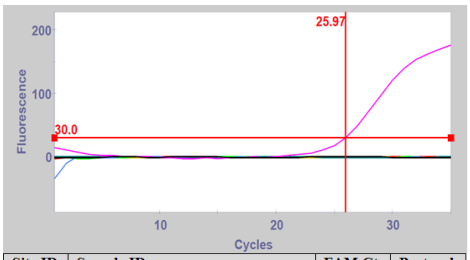
Discussion

The cultivation of mushroom has a long history in Turkey's agricultural sector as it contributes immensely to economic and nutritional needs of the people. Although there are few



Site ID	Sample ID	FAM Ct	Protocol
A1	Pt Stock Solution	23.79	Pt-SYBR
A2	Pt 10^{-1}	24.07	Pt-SYBR
A3	Pt 10^{-2}	24.24	Pt-SYBR
A5	Pt 10^{-3}	21.41	Pt-SYBR
A6	Pt 10^{-4}	22.83	Pt-SYBR
A7	Pt 10^{-5}	23.36	Pt-SYBR
A8	Pt 10^{-6}	21.82	Pt-SYBR
A9	Pt 10^{-7}	22.35	Pt-SYBR
A10	Pt 10^{-8}	00.00	Pt-SYBR
A11	Pt 10^{-9}	00.00	Pt-SYBR
A12	Negative Control	00.00	Pt-SYBR

Fig. 6: Sensitivity of the SYBR Green dye and Pt-1A/Pt-1D1primer in detecting *P. tolaasii* inserially diluted bacterial suspension

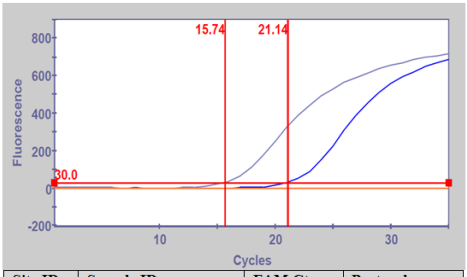


Site ID	Sample ID	FAM Ct	Protocol
A1	*P. cichorii*	00.00	Pt-SYBR
A2	*P. corrugata*	00.00	Pt-SYBR
A3	*P. fluorescens*	00.00	Pt-SYBR
A5	*P. mediterranea*	00.00	Pt-SYBR
A6	*P. viridiflava*	00.00	Pt-SYBR
A7	*P. tomato* pv. *tomato*	00.00	Pt-SYBR
A8	*D. chrysanthemi*	00.00	Pt-SYBR
A9	*P. carotovorum* subsp. *carotovorum*	00.00	Pt-SYBR
A10	*X. axonopodis* pv. *vesicatoria*	00.00	Pt-SYBR
A11	*C. michiganensis* subsp. *michiganensis*	00.00	Pt-SYBR
A12	Positive Control	25.97	Pt-SYBR
A13	Negative Control	00.00	Pt-SYBR

Fig. 7: Determination of the specificity of Pt-1A/Pt-1D1 primer against out-group bacteria in Real-time PCR test

reports of brown blotch disease caused by *P. tolaasii* on mushrooms in Turkey (Ozaktan *et al*., 2000), not much serious and detail research has been conducted in that regard, the goal of this study was to provide for the first time much in-depth knowledge on the correct detection and identification of this serious threat to mushroom production in the major mushroom production centers of Korkuteli, which produces about 70% of mushroom in Turkey.

The mushroom brown blotch disease has a worldwide distribution in all areas where mushroom production is part of an agricultural history (Tolaas, 1915; Wong and Preece, 1979). The *P. tolaasii* is considered to be an endemic pathogen of casing soil and compost used for mushroom production and forms part of its flora where it was found to be associated with the mushroom hyphae. The compost and casing soil were reported by Samson *et al.* (1987) to be the primary source of inoculum for *P. tolaasii* causing brown blotch diseases of mushrooms. Our findings are in agreement with the above results since all the isolates recovered from



Site ID	Sample ID	FAM Ct	Protocol
A1	Diseased plant tissue	21.14	Pt-SYBR
A2	Positive Control	15.74	Pt-SYBR
A3	Negative Control	00.00	Pt-SYBR

Fig. 8: Real-time PCR detection of the pathogen from macerated *A. bisporus* tissue and peaks produced from amplified disease tissue

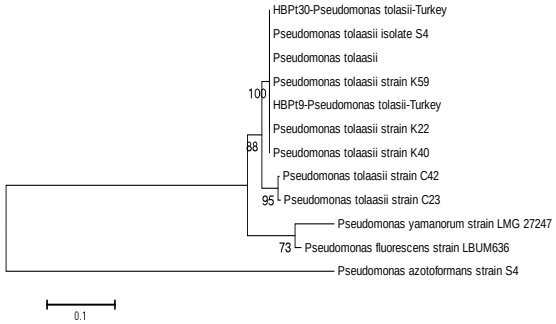


Fig. 9: The Maximum likelihood tree showing the phylogenetic relationship between Turkey's isolate and other strains from NCBI GenBank. Bootstrap values (expressed as percentages of 1000 replications) are shown at the node

the *A. bisporus* samples were collected from mushrooms grown on compost media.

The outcome of our survey revealed that *P. tolaasii* was the major bacterial pathogen associated with mushrooms, which caused brown blotch disease on *A. bisporus* collected from the 214 mushroom farms across the Korkuteli district as confirmed by different identification and detection techniques used in this study. The biochemical tests carried out on all the isolates showed that all the phenotypic attributes of *P. tolaasii* necessary for the identification of this pathogen were strongly in affirmative, and our results based on these assays have been supported by the findings reported by Milijasevic-Marcic *et al.* (2012).

The identification and detection of *P. tolaasii* have previously been reported to be based on two main features that included pathogenicity test and the formation of white line precipitates when the *P. tolaasii* pathogen is streaked several millimeters away from the nonpathogenic *P. reactans* (Wong and Preece, 1979; Rainey *et al*., 1991).

The pathogenicity test of all the isolates using bacterial suspension as well as the extracted tolaasin toxins induced the same virulence symptoms on the caps of inoculated *A. bisporus* and slices of potatoes with varied degradation and

discoloration.

The pathogenicity of *P. tolaasii* on mushrooms have been confirmed by Paine (1919), as well as reported in several other countries such as Japan, The Netherlands, Spain, USA, Korea and Serbia (Tolaas, 1915; Milijasevic-Marcic *et al*., 2012). The ability of *P. tolaasii* to cause brown blotch diseases on several other mushroom species such as *A. bisporus*, *Flammulina velutipes*, *Lentinula edodes* and *Pleurotus ostreatus* have been documented (Rainey *et al.*, 1991). Moquet *et al.* (1996) carried out pathogenicity tests on a large number of wild and hybrid *A. bisporus* mushrooms by using two strains of *P. tolaasii* and their toxins. They found a huge varied symptom intensity among the strains.

The isolates also produced varied symptoms, ranging from blackening to light dark brown discoloration on the inoculated potatoes. All the *P. tolaasii* isolates showed clearly different virulence scores on the inoculated potato slices, but the virulence of the isolates could not be differentiated by using *A. bisporus* sphorophores. The potato slices can, therefore, be used efficiently to determine the virulence of *P. tolaasii* isolates.

The tolaasin activity on the inoculated slices of potato which showed blackening symptoms as a result of the effects of this toxin has been reported (Shirata *et al*., 1995). The tolaasin toxin produced by *P. tolaasii* is considered to be the virulence factor responsible for causing brown blotch diseases of mushrooms as well as plays a major role in the development of a white line precipitate in agar test (Rainey *et al.*, 1991). The treatment of mushroom caps with 10 μg mL^{-1} tolaasin toxins resulted in pitting (Hutchison and Johnstone, 1993), and 30 μg mL^{-1} of the toxin led to the formation of both browning and pitting symptoms after 16 h of incubation as a result of direct effects of the tolaasin activity on the hyphal membranes. The toxin has also been reported to cause disruption and collapse of the vacuole and plasma membranes of mushroom cells (Rainey *et al.*, 1991). The above findings tallied with the results of this study in which varied degree of pitting and browning discolorations have been observed after 12 to 48 h of inoculation.

Another most commonly used method for the detection of *P. tolaasii* is white line-in-agar test. Most of the isolates recovered from the *A. bisporus* in this study yielded a white line precipitates when *P. tolaasii* isolates were streaked several millimeters away from the *P. reactans* isolates. The white line precipitate formed as a result of specific interactions between water soluble tolaasin and diffusible components yielded by *P. tolaasii* and *P. reactans*, respectively. Wong and Preece (1979) recommended the use of the white line assay for the detection of *P. tolaasii*. Several other works have confirmed and proved the specificity of the white line test involving interaction between *P. tolaasii* and *P. reactans* strains (Rainey *et al*., 1991; Soler-Rivas *et al*., 1999). Interestingly, some few other isolates in this study were white line negative, but they were pathogenic when inoculated on the *A. bisporus* caps as well as produced positive results with the PCR tests. Heeb and Haas (2001), also showed that some fluorescens *Pseudomonas* isolates were hypovirulent strains of *P. tolaasii* which have lost their pathogenic status due to harsh environmental conditions. It was not surprising when some of our isolates failed to produce a white line precipitate in the agar. It should, however, be noted that failure to produce a white line precipitate in agar, does not necessarily mean that these isolates are nonpathogenic as it was clearly discovered in this study. We found out that, the isolates which were white line negative were positive for other tests including pathogenicity test. Cutri *et al.* (1984) in their work, reported that only *P. tolaasii* which are pathogenic can react with *P.reactans* and produce a white line precipitate, but it was later found out by Munsch and Alatossava (2002) who showed that, the specificity and reliability of this test was questionable since other fluorescens *Pseudomonas* could also produce this precipitate when they react with *P. reactans*. The exact mechanism of the white line test is not clearly understood and we recommend that further studies need to be carried out on the white line negative isolates of *P. tolaasii* pathogens.

The advent of molecular techniques in the detection and identification of *Pseudomonas* species has revolutionized the taxonomic hierarchy of this pathogen and made the identification of *P. tolaasii* more reliable. The identification of *P. tolaasii* and other related *Pseudomonas* can be achieved molecularly, by using tolaasin gene specific primers, protein genes and sequence analysis of *rpoB* and 16S rRNA genes (Tayeb *et al.*, 2005). To confirm the true identity of the bacterial isolates, all the isolates were subjected to conventional PCR yielded the expected fragment sizes. Our result is in agreement with (Wells *et al.*, 1996; Milijasevic-Marcic *et al.*, 2012) who also obtained the same fragment sizes using the same primer pairs. To ensure quick detection of *P. tolaasii* from both isolated bacteria and macerated *A. bisporus* tissue without bacteria and genomic DNA isolation from infected samples, real-time PCR was produced different mean Ct values due to the amount of starting genomic DNA target in the reaction. The detection of *P. tolaasii* directly from macerated *A. bisporus* tissue without bacteria isolation, implies that Real-time PCR could be used for quick detection of this pathogen from large number of samples within a relatively short period of time (20–25 min), which could save time and cost involved in the bacterial isolation from the infected mushrooms. Real-time PCR using the tolaasin producing gene primers were tested against other sisters outgroup of bacteria strains, but all the tested outgroups could not be amplified by these primers except *P. tolaasii* isolates. The sensitivity of the Real-time PCR was about 10^{-7} serial dilution suspension, corresponding to the detection limit of 1 CFU/μL.

The confirmation of amplified single gene sequence of *P. tolaasii* was done by using the post-amplification melting-curve analysis, which yielded the single melt peaks. The various single peaks produced implies that the amplification was from only a single specific gene of *P. tolaasii*, signifying the success of the reaction assays.

The BLAST analysis of the amplified PCR products of *P. tolaasii* after sequence showed 98–100% similarities with the isolates retrieved from the NCBI GenBank database. The high similarities of the Turkey's isolate to those of the GenBank also confirmed the true identity of the isolates used in this study. The phylogenic cluster analysis also grouped the Turkey's isolate together with other isolates recovered from the GenBank nucleotide database into various clusters, which showed various homologies. This implies that the Turkey's isolates shared similar evolutionary history to some of the strains recovered from the GenBank.

Conclusion

The *P. tolaasii* population in the west Mediterranean region of Turkey were for the first time, successfully characterized as well as detected directly from the bacterial cell without DNA extraction and from the diseased tissue, respectively by Real-time PCR. Interestingly, some white line negative isolates were found to be pathogenic as well as tested positive for PCR tests, and to the best of our knowledge, this is the first report on the white line negative isolates causing disease on the inoculated mushrooms as well as tested positive for PCR test in Turkey.

Acknowledgements

The authors gratefully acknowledge financial support by *the Scientific Research Projects (BAP) of Akdeniz University, Antalya, Turkey (Project Number: FBA-2018-3038)*

References

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic local alignment search tool. *J. Mol. Biol*., 215: 403–410

Cheung, P.C.K., 2010. The nutritional and health benefits of mushrooms. *Nutr. Bull.*, 35: 292–299

Cutri, S.S., B.J. Macauley and W.P. Roberts, 1984. Characteristics of pathogenic non fluorescent (smooth) and nonpathogenic fluorescent (rough) forms of *Pseudomonas tolaasii* and *Pseudomonas gingeri*. *J. Appl. Bacteriol*., 57: 291–298

Dai, Y.C., Z.L. Yang, B.K. Cui, C.J. Yu and L.W. Zhou, 2009. Species diversity and utilization of medicinal mushrooms and fungi in China (Review). *Int. J. Med. Mushr.*, 11: 287–302

Eren, E. and A. Pekşen, 2016. Türkiye'de kültür mantarı sektörünün durumu ve geleceğine bakış (Status and Future Outlook of Cultivated Mushroom). *Turk. J. Agric. Food Sci. Technol.*, 4: 189–196

FAO, 2014. Food and Agriculture Organization of the United Nations. Available at: *http://faostat.fao.org* (Accessed: 23 March 2017)

Gill, W.M., 1995. Bacterial diseases of *Agaricus* mushrooms. *Rep. Tott. Mycol. Inst.*, 33: 34–55

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98NT. *Nucl. Acids Symp. Series*, 41: 95–98

Heeb, S. and D. Haas, 2001. Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. *Mol. Plant-Microb. Interact.*, 14: 1351–1363

Hutchison, M.L. and K. Johnstone, 1993. Evidence for the involvement of the surface active properties of the extracellular toxin tolaasin in the manifestation of brown blotch disease symptoms by *Pseudomonas tolaasii* on *Agaricus bisporus*. *Physiol. Mol. Plant Pathol.*, 42: 373–384

Iacobellis, N.S. and P. Lo Cantore, 1998. Recenti acquisizioni sul determinismo della batteriosi del carboncello (*Pleurotus eryngii*). *Agricol. Ricerca*, 176: 51–54

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol*., 16: 111–120

Lelliott, R.A. and D.E. Stead, 1987. Methods for the diagnosis of bacterial diseases of plants. *In: Methods in Plant Pathology*, Vol. 2, p: 216. Preece, T.F. (ed.). Blackwell Scientific Publications, Oxford, UK

Lo Cantore, P. and N.S. Iacobellis, 2014. Characterization of fluorescent pseudomonads responsible for the yellowing of oyster mushroom (*Pleurotus ostreatus). Phytopathol. Mediterr*., 53: 54–65

Milijasevic-Marcic, S., B. Todorovic, I. Potocnik, M. Stepanovic and E. Rekanovic, 2012. First report of *Pseudomonas tolaasii* on *Agaricus bisporus* in Serbia. *Phytoparasitica*, 40: 299–303

Moquet, F., M. Mamoun and J.M. Olivier, 1996. *Pseudomonas tolaasii* and tolaasin: Comparison of symptom induction on a wide range of *Agaricus bisporus* strains. *FEMS Microbiol. Lett.*, 142: 99–103

Munsch, P. and T. Alatossava, 2002. The white-line-in-agar test is not specific for two cultivated mushroom associated pseudomonads, *Pseudomonas tolaasii* and *Pseudomonas* "*reactans*". *Microbiol. Res.*, 157: 7–11

Nair, N.G. and P.C. Fahy, 1973. Toxin production by *Pseudomonas tolaasii* Paine. *Aust. J. Biol. Sci.*, 26: 509–512

Ozaktan, H., T. Bora and U. Fidan, 2000. Recent observations on the diseases of cultivated mushroom (*Agaricus bisporus*) in Turkey. *In: XVth International Congress on the Science and Cultivation of Edible Fungi*, p: 28. Maastricht, The Netherlands

Paine, S.G., 1919. Studies in bacteriosis. 11. A brown blotch disease of cultivated mushrooms. *Ann. Appl. Biol*., 5: 206–219

Rainey, P.B., C.L. Brodey and K. Johnstone, 1991. Biological properties and spectrum of activity of tolaasin, a lipodepsipeptide toxin produced by the mushroom pathogen *Pseudomonas tolaasii*. *Physiol. Mol. Plant Pathol*., 39: 57–70

Samson, R., G. Houdeau, P. Khanna, J. Guillaumes and J.M. Olivier, 1987. Variability of fluorescent *Pseudomonas* populations in composts and casing soils used for mushroom cultures. *Dev. Crop Sci.*, 10: 19–25

Shadi, T., N. Hassanzadeh, A. Heydari and H.K. Jolfaee, 2015. Study on genetic diversity of *Pseudomonas tolaasii* and *Pseudomonas reactans* bacteria associated with mushroom brown blotch disease employing ERIC and BOX-PCR techniques. *Int. J. Agrıc. Crop Sci.*, 8: 398–405

Shirata, A., K. Sugaya, M. Takasugi and K. Monde, 1995. Isolation and biological activity of toxins produced by a Japanese strain of *Pseudomonas tolaasii*, the pathogen of bacterial rot of cultivated oyster mushroom. *Ann. Phytopathol. Soc. Jap*., 61: 493–502

Soler-Rivas, C., S. Jolivet, N. Arpin, J.M. Olivier and H.J. Wichers, 1999. Biochemical and physiological aspects of brown blotch disease of *Agaricus bisporus*. *FEMS Microbiol. Rev.*, 23: 591–614

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, 2011. MEGA5: molecular evolutionary genetic analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.*, 28: 2731–2739

Tayeb, L.A., E. Ageron, F. Grimont and P.A.D. Grimont, 2005. Molecular phylogeny of the genus *Pseudomonas* based on *rpoB* sequences and application for the identification of isolates. *Res. Microbiol*., 156: 763–773

Tolaas, A.G., 1915. A bacterial disease of cultivated mushrooms. *Phytopathology*, 5: 51–54

Wells, J.M., G.M. Sapers, W.F. Fett, J.E. Butterfield, J.B. Jones, H. Bouzar and F.C. Miller, 1996. Postharvest discoloration of the cultivated mushroom *Agaricus bisporus* caused by *Pseudomonas tolaasii*, *P. "reactans"*, and *P. "gingeri". Phytopathology*, 86: 1098–1104

Wong, W.C. and T.F. Preece, 1979. Identification of *Pseudomonas tolaasii*: the white line in agar and mushroom tissue block rapid pitting tests. *J. Appl. Microbiol*., 47: 401–407

Young, J.M., 1970. Drippy gill: A bacterial disease of cultivated mushrooms caused by *Pseudomonas agarici* n.spp. *N.Z. J. Agric. Res*., 13: 977–990

(Received 20 June 2018; Accepted 02 July 2018)