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Bacterial Brown Blotch and Marketing of Cultivated Table Mushroom. A Short Communication

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ABSTRACT

Cultivated table mushrooms are highly susceptible to a variety of microbial pathogens including bacteria. Consumers require high quality plates; completely white, a closed veil, short stalk and unclear gills. Bacterial browning in mushroom or what is known as mushroom blotch can affect outer surface of mushroom, either caps or stalks or both. This short article presents an outline of collected data, statistics and experimental analyses that cover the pattern and significance of bacterial brown blotch, the causal agent in relation to marketing of button mushrooms. Twelve bacterial isolates were isolated from superficial brown discolorations on the caps of cultivated *Agaricus bisporus*. Pathogenicity test of all the proposed isolates nearly induced the same virulence pattern on cubes of *A. bisporus* sliced from caps but with varied browning size and color. According to applied techniques used in identification, *P. tolaasii* was the major bacterial isolate associated with all plates containing blotched mushrooms collected during the study period. In addition, other isolates were identified as isolates of *P. fluorescens*, *P. Marginalis* and *P. "reactans"*.

1. Introduction

The worldwide net production value of mushrooms has reached over US $19.46 billion in 2016 [1], while the global mushroom market was evaluated at USD $ 45.8 billion in 2020 and expected to reach 63.24 billion by 2027 [2]. Table mushrooms, champignon, white button mushrooms (*Agaricus bisporus* J. E. Lange) accounted for 83% of the mushrooms sold as certified organic, while all specialty mushrooms made up the remainder. Mushrooms are present as fresh plates, canned, dried, frozen and even powdered. Fresh mushrooms have a short shelf life that lasts for 3–4 days, that's why many methods are used to extend their shelf life without compromising their nutritional quality. Button mushrooms have a mild accepted flavor, caps and stems are edible, and you can eat them cooked or even raw [3]. Diseases of different origins are very common during stages of mushroom cultivation, the bacterial ones such as stipe necrosis, drippy gills, and ginger and brown blotch diseases may lead to a huge loss in mushroom quality [4-5]. Bacterial brown blotch disease nearly target every species of mushroom, symptoms can arise at any stage and usually appear after a long, wet period caused by condensation from spraying.

"Blotch" refers to a group of diseases all caused by certain species of *Pseudomonads*. Blotch usually appears as small to medium irregular brown spots or patches when mushrooms are in their early button stage, either on harvested, refrigerated, or mushrooms sold in markets. Developed grey-brown lesions start on the cap and stalk, then may enlarge and coalesce, infected mushroom bodies became sticky [6]. Bacterial blotch may result in severe

outbreaks in mushroom houses which could lead to quick deterioration after harvest and losses in mushroom crop of up to 25% and hence market value [7]. *Pseudomonas tolaasii* is the main causative bacterial pathogen affecting cultivated button mushrooms. This pathogen induces dark-brown lesions, often sunken and little wet on mushroom caps and stalks, which in many cases render the crop unmarketable. Objectives of this short communication were mainly to highlight on the brown blotch in table mushroom local marketing in addition to record: incidence, isolation and identification of blotch causal agent(s).

2. Materials and Methods

2.1 Collection of samples

Fresh plates of table mushrooms were collected regularly from four hypermarkets in Cairo (Egypt) throughout a one-year study. Collection date, age, source, number/plate, brown blotched mushrooms were all documented Table 1. Data obtained during the screening were analyzed to evaluate the disease incidence and some selected samples were used to culture isolated bacteria before plating on King's B (KB) agar medium [8].

2.2 Isolation of bacteria from blotched mushroom tissues

Blotched sporophores were used to isolate *P. tolaasii* and other bacterial pathogens according to the method described by Lelliott and Stead [9]. Colored outer tissues especially from caps of mushrooms were squashed in sterilized tubes with sterile distilled water (2.5 mL), then diluted in series. 50 µL of appropriate dilutions were spread on KB agar medium [8] and 48-hours age bacterial colonies were purified by re-streaking over the same medium before maintaining on nutrient agar (with 2% glycerol) at 4°C.

2.3 In-vitro Pathogenicity

Pathogenicity screening of bacteria isolated from blotched mushrooms were tested according to **Abou-Zeid** [7]**.** Bioassay was run with two cubes of one cm^3 cut from one-day-old *A. bisporus* over filter paper moistened sterile water. Around 10 µL of each of the prepared bacterial suspension was spotted on one cube, while the other one was used as a negative control and spotted with sterile distilled water. Plates were observed for a minimum of two days for blotch progress and assay was repeated twice for each isolate.

2.4 Characterization of isolated bacteria

Characterization of the selected isolates was conducted by examining colony characteristics followed by tests for production of fluorescent pigments (under UV at a wave length of 366 nm) using reference strains as positive and negative controls [9-10].

2.5 Biochemical Tests "LOPAT" profile

LOPAT profile is proved very useful for the identification of *Pseudomonads* sp. according to Lelliott and Stead [9]. Fluorescent bacteria were subjected to biochemical tests. The "LOPAT" pattern is a series of determinative tests: L, levan production; O, oxidase production; P, pectinolitic activity; A, arginine dihydrolase production; and T, tobacco hypersensibility is a series of determinative tests: L: levan production; O: oxidase production; P: pectinolitic activity; A: arginine dihydrolase production; and T, tobacco hypersensibility.

Levan production from sucrose (L): After incubation, morphology of colonies developing on nutrient agar plates (Difco, USA) enrich with 5% sucrose was examined. Oxidase test (O): Oxidase identification sticks (Difco, Sparks, MD, USA) were used to test isolates grown for 24 h on nutrient agar.

Pectinolytic activity on potato tubers (P): Thick slices of sterilized peeled potato were placed on a moist sterile filter paper (3 slices/plate) and were inoculated with 15-20 µL of each bacterial suspension (10^8 CFU/mL) in comparison to control.

Arginine dihydrolase production (A): Screw-capped test tubes containing 3 mL arginine dihydrolase medium (a semi-liquid medium) before autoclaving for 20 min. Each tube was inoculated by a bacterial isolate, was covered with a thin layer of sterile liquid paraffin oil, tightly capped, and incubated at 25 °C for 4 days.

Tobacco hypersensitivity (T): Bacterial suspension (10^8 CFU/mL) was injected as thick of bacteria grown for on into the mesophyll of a healthy fully grown tobacco leaf (two leaves of different plants were used for each isolate). Plants were grown in pots and necrosis in injected areas within 24 h was considered a positive result.

2.6 Statistical analysis

IBM SPSS statistics (V. 26.0, IBM Corp., USA, 2019) was applied for data analysis. All data were expressed as Mean ± SD for quantitative parametric measures. The comparison between two independent mean groups for parametric data was determined using the Student t-test. While, comparison between more than two groups for parametric data was carried out using Analysis of Variance (ANOVA).

Table 1 Collection of mushroom samples

Seasons	Source	*Age of product (day)	No. of tested plates	No. of sporophores/ plate**	% of blotching/ plate**	Spot count /individual sporophore**
Winter	I	1	6	13±0.5	28.3±14.62	2.33±1.24
	II	1	7	16±1	19.16±19.66	4±1.6
	III	7	13	12±1	41.9±27.8	2.6±1.24
	IV	4	15	12.5±0.5	29±15.6	3.6±1.69
Spring	I	1	10	11.5±1.5	33±10.77	3±1.63
	II	2	6	15±1	40±8.16	2.6±1.24
	III	4	5	14±1	80±14.14	3±1.6
	IV	1	35	16±1	45±11.18	2.6±1.24
Summer	I	1	6	15.5±1.5	15±5	2.3±1.24
	II	3	5	11±1	30±10.95	2.6±1.24
	III	2	6	11±1	16.6±13.43	2.3±1.24
	IV	1	10	15.5±0.5	13±4.58	2.5±0.5
Autumn	I	1	12	14.5±0.5	8.63±8.8	2.6±1.24
	II	2	11	12±1	31.8±5.74	3±1.6
	III	4	7	12±1	34.28±23.05	4.6±1.24
	IV	2	12	10.5±0.5	16.25±11.92	2±0.81

*= Age of product (from production date to collection date), **= data are given as mean value ± standard deviation

3. Results

3.1 Incidence of brown blotch in commercial mushroom

This study was conducted over the course of one year (2018) including the four seasons on mushroom plates collected from four different major retail stores (hypermarkets) in Cairo, Egypt. A statistical analysis was designed to prove possible significant correlations between different criteria of the data gained along the survey on the bacterial blotch incidence calculating blotch % per plate and blotch spot count in each individual mushroom. The first correlation was made for blotch incidence and the four seasons at which samples were collected during the year. Mushrooms collected during spring had the higher blotching percentage ($P < 0.05$) followed by winter. Another significant correlation was established for retail stores of fresh mushroom plates, plates collected from two stores (II) and (III) had a higher percentage of blotch throughout the study time (with P value <0.001) Fig. 1 and 2.

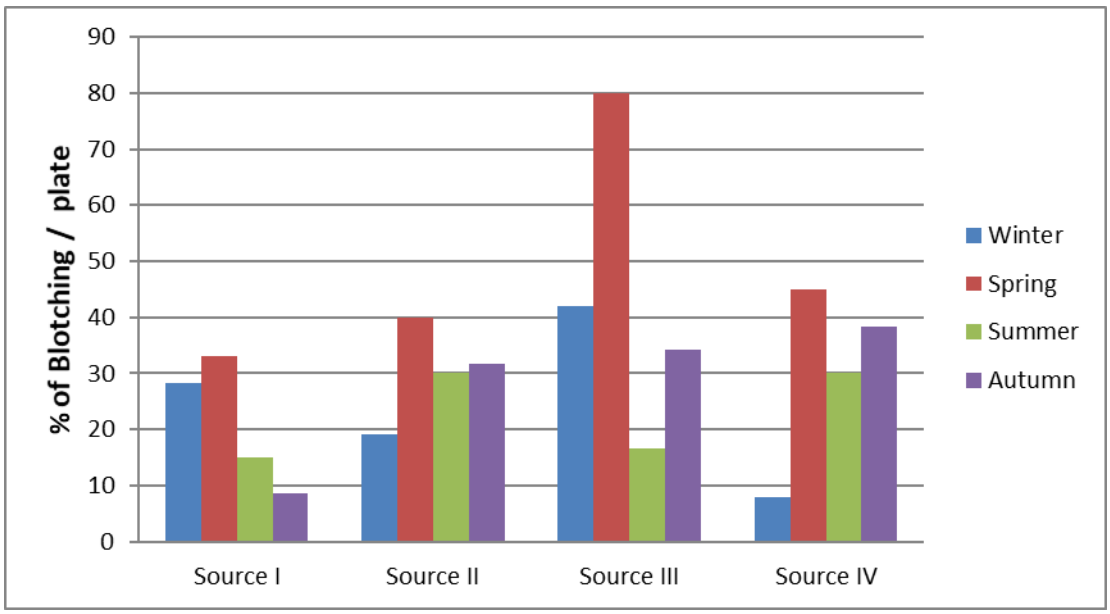

Fig. 1 The correlation between the percentage of blotch per plate and the source from which the samples were collected during the four seasons of the study period

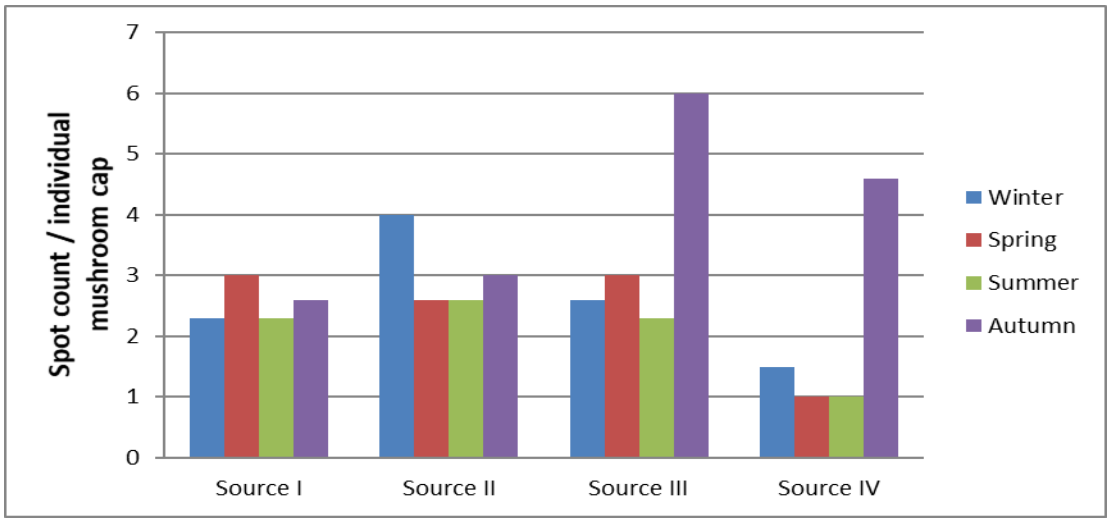

Fig. 2 The correlation between the spot count per individual mushroom cap and the source from which the samples were collected during the four seasons of the study period

3.2 Identification of the etiological agent

Bacterial isolates

A total of 12 bacterial isolates were recovered from *A. bisporus* showing browning symptoms Fig. 3. Isolation from blotched areas was always successful and grown colonies had typical morphology of *Pseudomonas* on nutritive agar after incubation.

3.3 Pathogenicity

The used scale of browning varied during the incubation period (3 days at 25°C was sufficient to recognize development of blotch) in *Agaricus* cubes treated with 10 µL of the test isolates (10^8 CFU mL^{-1}). Fig. 4 shows the pathogenicity test of two of the tested isolates along three successive days.

3.4 Production of fluorescence and Colonies' morphology

Yellowish-green pigments were recognized for test isolates cultured on KB media Fig. 5. These isolates showed identical "LOPAT" profile which is characteristic for the group V of fluorescent Pseudomonads Table 2.

Observations at different times for colonies of the two bacterial isolates KZ III 1 of *Pseudomonas tolaasii* and MIII 1 of *P. "reactans"*, thioglycolate supplemented media revealed slower growth rate compared with colonies of the same bacterial isolates which were grown in the absence of sodium thioglycolate. Colonies have displayed a distinct morphology and were actually smaller in diameter. Fig. 5

Fig. 3 "BROWN BLOTCH" on the capes of blotched sporophores of *A. bisporus* collected during the study

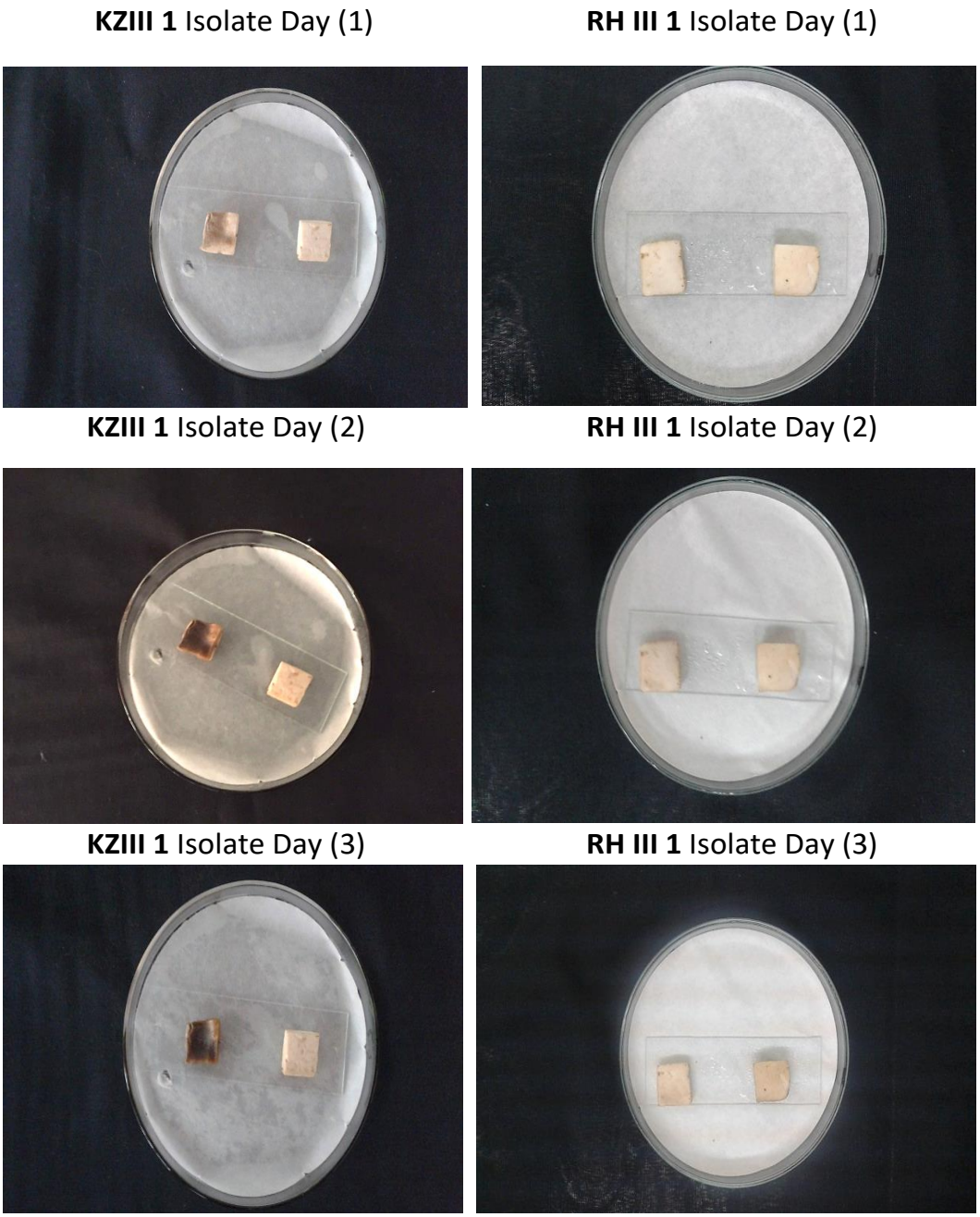

Fig. 4 Cube pathogenicity bioassay of the bacterial isolate KZIII1 (showed positive pathogenicity results) and RHIII1 (showed negative pathogenicity results) along three successive days, each treatment was represented with two cubes, the right side cube in all Petri dishes was inoculated with distilled H_2O as control and the left side cube was inoculated with the bacterial suspension

3.5 Profile of the examined Pseudomonas isolates using Biolog Database

Test isolates of *Pseudomonas* were able to differently use 23 carbon sources which allowed to group them together. The difference in utilizing such carbon sources by them using the Biolog computerized system were examined Table 3. Verified isolates of *P. tolaasii* showed a variable phenotype as they proved different in behavior in using thirteen carbon using thirteen carbon sources out of the ninty-five used. In addition, other isolates were identified as isolates of *P. fluorescens*, and of *P. marginalis*. Isolates proposed to be *P. "reactans"*, have been identified differently from reference strain since this bacterial species is not in Biolog Inc.'s database, so have been identified differently Table 4.

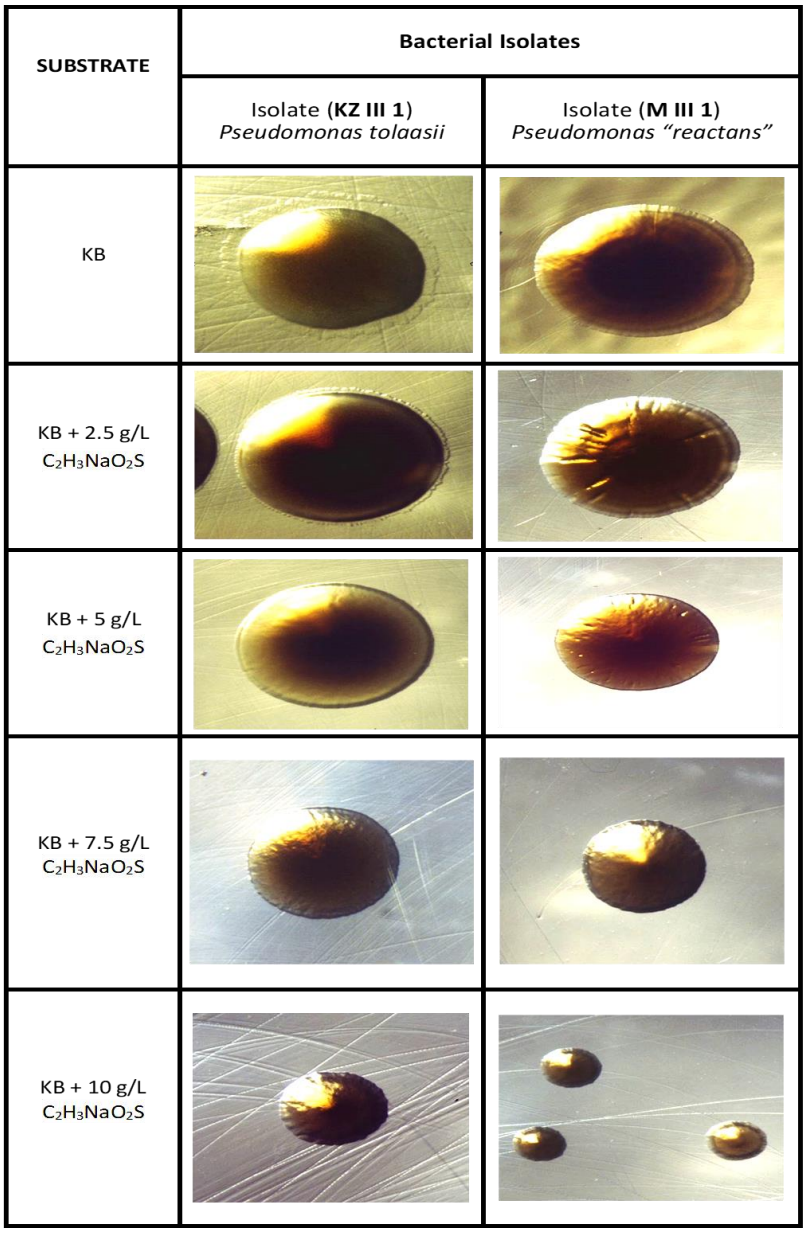

Fig. 5 Colonies of the *Pseudomonas tolaasii* isolate (KZ III) and the isolate (M III 1) of *Pseudomonas reactans* grown on KB and on the same substrate with 2.5 and 5 and 7.5 and 10 g/L of $C_2H_3NaO_2S$ observed after 9 days. KB = KB Substrate [8]

Table 2 Major phenotypic characteristics of the examined Pseudomonas isolates

Isolate code	Fluorescent Pigment Production	"LOPAT" profile				
		L	O	P	A	T
P. tolaasii ICMP 6551	YG	-	+	-	+	-
P. "reactans" ICMP 6956	YG	-	+	-	+	-
P. fluorescence ICMP 5334	Y	-	+	-	+	-
P. marginalis NCPPB2325	Y	-	+	-	+	-
P. agarici NCPPB2289	Y	-	+	-	+	-
AR II	YG	-	+	-	+	-
KZ III 1	YG	-	+	-	+	-
KZ III	YG	-	+	-	+	-
M II	YG	-	+	-	+	-
M III 1	Y	-	+	-	+	-
RH III 2	Y	-	+	-	+	-

L=Levan production, O=Oxidase reaction, P=Pectinolitic activity, A=Arginine dihydrolase production, T=Tobacco hypersensitivity; NCPPB = National Collection Plant Pathogenic Bacteria, (UK); ICMP = International Collection of Microorganisms from Plants (Auckland, New Zealand); YG=Yellowish-green pigment, Y=Yellow pigment; - = absence of character whereas + = presence of the character

Table 3 Differences in the nutritional profile of the tested isolates using the Biolog identification system

Isolates	Tween 80	D-fructose	D- mannose	L- rhamnose	D-sorbitol	saccharose	xylitol	Mono-methyl succinate	Lactone D-galactonic acid	D-galacturonic acid	D-glucosaminic acid	D-glucuronic acid	α-hydroxybutyric acid	p-hydroxyphenylacetic acid	Propionic acid	Glucuronamide	Alaninamide	Hydroxy L-proline	L-threonine	D, L-carnitine	Uridine	2-amino ethanol	D,L-α glycerol phosphate
BIOLOG*	+ -	+	+ -	+	-	-	+ -	+	+ -	+	+	+	+ -	-	+	+	+	+	+ -	+ -	+	+	+
P. marginalis NCPPB2325	+ -	+	+ -	+	+ -	-	+ -	+	+	+	+	+	-	-	+	+ -	+-	+	+ -	+ -	+-	+-	+ -
P. "reactans" ICMP 6956	+ -	+	+	+	+ -	-	+	+	+	-	+	-	-	-	+	-	+-	+	+	+ -	+	+-	+
P. tolaasii ICMP 6551	+ -	+-	+ -	+	+ -	-	+ -	+	+	+	+	+	-	+-	+ -	+	+-	+	+	+ -	+	+-	+ -
P.fluorescence ICMP 5334	+ -	+	+	+	+ -	-	+	+	+	+	+	+	+ -	-	+	+	+-	+	+	+ -	+-	+	+ -
MIII1	+ -	+	+	-	+	-	+ -	+	+	+	+	+	-	+ -	+	+ -	+-	+	+ -	+ -	+	+ -	+ -
RHIII2	+ -	+	+	+	+ -	-	+	+	+	+	+	+	+ -	-	+	+	+-	+	+	-	+ -	+	+ -
ARII	-	+	+	+	+ -	-	+ -	+	+	+	+	+	-	+	-	+	+-	+	-	-	+ -	+ -	+
KZIII1	+ -	+	+	-	+	-	+ -	+	+	-	+	+	+ -	+ -	+ -	+ -	+	+	+	+ -	+	+	+ -
MII	+ -	+ -	+ -	+	+ -	-	-	+	+	+	+	+	-	+ -	+ -	+ -	-	+	+ -	+ -	+ -	+ -	-
KZII1	+	+	+	+	+ -	-	-	+	+	+	+	+	-	-	+ -	+	-	+	+ -	-	+ -	-	+ -

***** = Nutritional Profile of Pseudomonasaccording to the database of *"*Biolog Inc".; **NCPPB** = National Collection Plant Pathogenic Bacteria, (UK); **ICMP =** International Collection of Microorganisms from Plants (Auckland,New Zealand); **+** = presence of the character; **+-** = intermediate character; **-** = absence of the character

Table 4 Identification of Pseudomonas isolates and their respective morphological variants through the computerized Biolog System

Isolate code	Identification	Probability (%)	Similitude	Distance
ICMP 6956	P. "*reactans*"	86	0,834	0,43
ICMP 5334	*P. fluorescens*	98	0,718	4,07
NCPPB2325	*P. marginalis*	74	0,507	2,29
RHIII2	*P. marginalis*	86	0,624	4,15
ICMP 6551	*P. tolaasii*	96	0,519	7,09
ARII	*P. tolaasii*	89	0,324	6,24
KZIII	*P. tolaasii*	97	0,885	1,27
MII	*P.tolaasii*	91	0,837	1,13
MIII1	*P. "reactans"*	97	0,740	3,55
KZII1	*P.tolaasii*	69	0,527	2,89

NCPPB: National Collection Plant Pathogenic Bacteria, UK; **ICMP**: International Collection of Microorganisms from Plants (Auckland, New Zealand)

4. Discussion

Mushroom marketing sector in Egypt has a moderate economic and nutritional contribution. Few reports are available for brown blotch disease caused by Pseudomonas species on mushrooms in Egypt [7-11]. Blotch in cultivated table mushrooms is a production threat in several farms and in markets. Browning in mushrooms is a diffused worldwide bacterial disease caused mainly by Pseudomonas especially (*P. tolaasii*) which is becoming endemic bacteria in many mushroom farms. Identification of Pseudomonas is important, KB medium has been used for specific isolation of different fluorescent *Pseudomonas* species causing the bacterial blotch on mushroom tissue. *P. tolaasii* was the major bacterial pathogen associated with all plates containing blotched mushrooms in the four covered hypermarkets according to applied techniques used in identification. Sodium thioglycolate broth is a multipurpose, enriched, differential medium used to isolate anaerobes and organisms that may be present in low numbers in a specimen. Biochemical tests for all pseudomonas isolates showed that 4 attributes to *P. tolaasii*, results of assays have been supported by the findings reported by **Abou-Zeid** [7] and **Milijasevic-Marcic *et al.*** [12]. Confirmation of *P. tolaasii* is mainly based on pathogenicity test in addition to white line assay with *P. reactans* [13]. Pathogenicity test of all the proposed isolates (6) using bacterial suspension nearly induced the same virulence pattern on cubes of *A. bisporus* sliced from caps but with varied browning size. Virulence of *P. tolaasii* to button mushrooms has been confirmed in other countries like Japan, Korea, USA, Spain, and Serbia [12]. Another confirmatory method for the detection of *P. tolaasii* is the white line in agar test, many *P. tolaasii* isolates recovered in this study yielded a white line precipitates when were streaked away from *P. reactans* even if few other isolates in this study were white line negative even if they proved positive in pathogenicity test (Unpublished Data). It has reported by **Munsch and Alatossava** [14] that both specificity and reliability of this test may be questionable because some fluorescence Pseudomonads could also produce this white precipitate when they react with *P. ''reactans''*. In conclusion, loss in mushroom crops from bacterial blotch with other mushroom diseases is attributed to the reduction in product quality; moreover, browning significantly affect the marketability of mushrooms for color change and texture . Remains, Chemical control remains the most generally used method to enhance mushroom production, marketing and to limit brown blotch of *A. bisporus* which still an important disease for many growers.

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