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Kocuria sp. a potential antagonist of brown blotch caused by *Pseudomonas tolaasii*

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Key words: Biocontrol, Antagonist, Brown blotch, *Pseudomonas tolaasii*, *Agaricus bisporus*.

Abstract

Brown blotch disease, caused by *Pseudomonas tolaasii*, is a serious economic problem in the cultivation of the white button mushroom *Agaricus bisporus* in Iran. In this study, to screen antagonistic bacteria, 29 bacterial strains were isolated from wild mushrooms and 125 strains obtained from mushroom cultivation centers in Tehran province that were surveyed and samples were taken from button caps with or without visible symptoms, on the basis of their colony morphology. All isolates screened for biological control of Bacterial Blotch disease of cultivated mushroom caused by *P. tolaasii in vitro*. Among all tested bacteria, four isolates produced variable inhibition zones and reduced the symptom incidence on tissue blocks of *A. bisporus*. The polymerase chain reaction products of the bacterial strains were sequenced. Based on phenotypic, biochemical and molecular characteristics, the bacterial antagonists were identified as *Pseudomonas* spp. and *Kocuria* sp. The formation of blotches was entirely blocked by co-incubated antagonist and pathogen on mushroom sporocarps. To our knowledge, this is the first report of *kocuria* sp being a promising candidate as a biological control agent against *P. tolaasii*. Overall results of this study suggest that bacterial antagonists may be potential biocontrol agents for biological promotion of the health and growth of button mushroom.

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Introduction

Bacterial blotch is a Popular disease of the cultivated mushroom, *A. bisporus,* caused by the bacterium *P. tolaasii* (Paine, 1919). The disease was firstly found on *A. bisporus* nearly 100 years ago (Tolaas, 1915).This pathogen creates slightly sunken brown lesions on the surface of the maturing mushroom cap and stipe. Hypochlorite commonly recommended for controlling the disease. Although the pulverization with chlorine compounds suppresses the disease to some degree, it causes a yield decrease (Oliver and Gillaumes, 1981) and browning on the caps (Wong and Preece, 1985) and it is found to be risky on human health.

In recent years, biological control studies on the disease have been intensified (Munjal, Khanna and Garcha, 1989; Khanna *et al*., 1990). Antagonistic pseudomonades have been used with varying degrees of success in France (Samson *et al*., 1987), India (Khanna and Olivier, 1989; Khanna *et al*., 1990), Australia (Miller, Gillespie and Doyle, 1995) and Turkey (Bora and Özaktan, 2000). A formulation of such biological control agent containing *P. fluorescens* biotype G is now available in Australia under the trade name "Victus". This bacterium can be managed commercially by another bacterium, *P. fluorescens* biovar V, which establishes itself in the development of pathogen and competes to exclude the colonization and development of blotch population. Khanna *et al*. (1990) characterized 34 isolates of *P. fluorescens* and *P. putida* from French casing soil mixtures to identify one strain *P. fluorescens* biovar I as an antagonist of *P. tolaasii* in efficient control of blotch in *A. bisporus* mushroom beds. Spear and Miller (1995) reported large-scale commercial trials for the biological control of bacterial blotch disease. Two strains of P. fluorescens M4/2 and M5/3 and one strain *P. putida* 39.9 resulted in a reduction of disease caused by *P. tolaasii* by 87.34 - 90.5, 71.3 - 71.7 and 67.5 - 72.6 percent, respectively (Bora and Özaktan, 2000). The use of bacteriophages to control bacterial blotch of *A. bisporus* has also been shown to gain 70% of the crop loss under lab conditions (Munsch, Olivier and Houdeau, 1991). Bora and Özaktan (2000) evaluate that fluorescent pseudomonads (FP) are very effective antagonists for the biological control of the pathogens *Papulaspora byssina*, *Cladobotryum dendroides*, *Pseudomonas tolaasii* and *Mycogone perniciosa*.

The objective of this study is to identify effective antagonist for biological control of this disease. Chemical control of *P. tolaasii* by sodium hypochlorite solution has limitations (Wong and Preece, 1985). It was thought, therefore, worthwhile to investigate the possibility of biological control of this disease. It led to the isolation of bacteria antagonistic from wild mushrooms sporocarps that are the isolate condition to *P. tolaasii;* and this communication reports their isolation and their antagonism to *P. tolaasii*.

Materials and methods

Isolation and maintenance of bacterial isolates

The pathogenic bacteria strain used in this experiment isolated from an infected (which showing superficial brown blotches) *A.bisporus* basidiocarp following Koch's postulates. The infected mushrooms obtained from a mushroom farm located in Tehran province. Other bacteria isolate obtained from wild mushroom from northern forests of Iran, Gilan province and mushroom farm's Tehran province. Bacteria isolated from sporocarps of wild mushroom and *A.bisporus* mushrooms. The sporocarps washed under running tap water and then the surfaces swabbed with cotton wool soaked with 75% ethanol and grown on Nutrient Agar (NA). After 48h incubation at 25°C, representative bacterial colonies with different morphologies were further purified on Nutrient Agar, King's B medium (KB) (King *et al*., 1954). Pure cultures maintained at 4°C on 2%glycerine nutrient agar slants. The isolated bacteria stored at -80°C in containing sterile water 20% (v/v) glycerol until use. *P. tolaasii* was isolated and identified by the White Line Assay (WLA) using the method described by (Wong and Preece, 1979) pitting test on the mushroom tissue (Kim, Kim and

Kang 1994) and pathogenicity by the mushroom tissue block test (Gandy, 1968). All of the isolates evaluated for fluorescence on KB (Lelliott and Stead, 1987), WLA (Wong and Preece, 1979), and virulence on *A. bisporus* pseudo-tissue blocks (Ercolani, 1970).

Tolaasin Assay

Tolaasin activity was examined by inoculating cells on potato tuber slices, which showed blackening in the presence of the toxic effect (Shirata *et al*., 1995). The level of tolaasin activity in culture supernatants were semiquantitatively determined by the extent of blackening of serially diluted samples (Shirata *et al*., 1995; Murata and Yumi, 1996). When 1.0×10^7cells of *P. tolaasii* were inoculated blackening developed at the inoculation site. A slice inoculated solely with *P. tolaasii* strain to expand marked blackening.

LOPAT

Bacterial isolates were evaluated for LOPAT (where LOPAT is a series of determinative tests: levan production; oxidase production; pectinolitic activity; arginine dihydrolase production and tobacco hypersensibility) characteristics (Lelliott and Stead, 1987) and for the differential nutritional characteristics of bacterial pathogens associated with cultivated mushrooms (Goor *et al*., 1986).

Biochemical characteristics

Pathogen characterization was carried out by determining the following set of phenotypic properties: Gram reaction, glucose metabolism, levan formation, Kovac's oxidase test, potato rot, arginine dihydrolase activity, tobacco hypersensitivity, fluorescence on KB medium, catalase activity, Tween 80 hydrolysis, casein hydrolysis, gelatine hydrolysis, nitrate reduction (Lelliott and Stead, 1987; Schaad and Jones, 2001).

Preparation of mixture of different bacteria

To test the PCR method developed in this study, cultures were grown on KB medium for 48 h and the mixtures were prepared with a bacterial suspension of *P. tolaasii* and other bacteria with ratios10^6 cells/ml, the bacterial suspension were determined by the T80 UV/VIS Spectrometer OD_{450}:1/6. Total genomic DNA of bacterial strains was isolated by the TIANamp bacteria DNA kit (Tiangen).

Bacterial Biocontrol Agents (Antibiosis)

29 bacteria, isolated from wild mushrooms were tested for biological control of Blotch disease caused by *P.tolaasii*. Single colonies of *P.tolaasii* were grown on king'B medium; incubate for 48h at 25°C. A suspension of each bacterium (10^8cfu) was spread on pathogen colonies. After 48 h incubation at 25°C, representative of the inhabitation zone were measured in millimeters. Plates were sealed with Para film and incubated at 24°C. The bacterial colony diameter and the diameter of the zone in which growth of *P.tolaasii* was inhibited were determined after 3 days.

Screening Bacteria for Antagonism (Rapid Screening)

The blocks, together with pathogen and antagonist, pathogen only and antagonist only inoculated blocks as controls were incubated at 25°C in plates lined with moistened filter paper to maintain a high humidity.

After incubation for 24 and 48h the inoculated blocks were assessed for the pitting and browning symptoms associated with *P. tolaasii* attack. Isolates that did not cause blotching were tested for antagonism against *P. tolaasii* by mixing Cell-free extracts of isolates in sterile distilled water suspensions with *P. tolaasii* and inoculating them as turbid, distilled water suspensions on mushroom blocks. Blocks were incubated at 25°C (Khanna and Olivier, 1989). 29 bacteria were tested in this way and isolates were selected for detailed study. This test was performed on excised tissue from fresh mushroom caps. *P.tolaasii* and the antagonist bacteria to be tested were grown separately in King's B liquid medium. Suspensions of the bacteria (approximately 10^8cfu/ml) were mixed in the ratio three, two and one parts antagonist: one part *P. tolaasii* and inoculated

(0.1ml) on the surface of the mushroom tissue blocks.

Determination of optimum concentration for antagonist

In the following tests were performed on excised tissue from fresh mushroom caps. *P. tolaasii* and the antagonist bacteria to be tested were grown separately in King's B liquid medium. Suspensions of the bacteria (approximately 10^8cfu/ml) were mixed in the ratio three, two and one parts antagonist: one part *P. tolaasii* and inoculated (0.1 ml) on the surface of the mushroom tissue blocks. Suspensions of *P.tolaasii and antagonist bacteria* (0.5Macfarland=1×10^8cfu/ml) (10^8cells/ml: antagonist and OD_{450}:1/6=10^6cells/ml: pathogen) at ratios of 1:1, 2:1 and 3:1, incubated three times 6h at 25°C and then determine whether biocontrol had occurred.

Screening procedure for siderophore production in solid media

Isolates that did not cause blotching may inhibit growth and/or tolaasin production of *P. tolaasii* strain or, otherwise, that it may inactivate tolaasins, according to the pervious tests, were examined as a biocontrol agents based on siderophore and antibiotic production (Henry, Lynch and Fermor, 1991).

Siderophore production

Assayed by Plate containing 80 µM $FeCl_3$/L added to KB medium. The antagonists were inoculated three times at equidistant points on each of three dishes, with and without iron supplementation, and incubated at 25°C for 24 h. These agar plates were over sprayed with a suspension (10^8cfu/ml) of *P. tolaasii* and incubated for a further 24h. Colony diameters and the diameter of any clearing zones surrounding the colonies were then measured. If no inhibition zone developed between the colonies of antagonists and *P. tolaasii* within 5 days, it was considered that the antagonism may be due to the production of siderophores. The experiment was repeated and an analysis of variance was performed on the results.

Antibiotic production

Antibiotic production by the antagonists was assayed by inoculating plates of Potato Dextrose Agar (PDA) with bacteria grown on KMB medium at 25°C for 24 h. The plates were incubated at 25°C for 96h and than they were lightly over sprayed with a suspension of *P. tolaasii* as described above. Colony diameters and clearing zone diameters were measured after 48 h (Henry, Lynch and Fermor, 1991).

Polymerase Chain Reaction (PCR) tests

16SrRNA Gene Amplification and Sequencing: The primers used for amplification of full length 16SrRNA gene were universal primer P1 (forward primer, 5` - CGGGATCCAGAGTTTGATCCTGGTCAGAACGAACG CT - 3`) and P6 (reverse primer, 5`- CGGGATCCTACGGCTACCTTGTTACGACTTCACCCC - 3), which correspond to *Escherichia coli* positions 8-37 and 1479-1506, respectively and amplifies 1500bp fragment (Tan *et al.*, 1997)(Tariq *et al.*, 2010). Each 25µL of reaction mixture contained 1U of *Taq* Polymerase, 2.5µL 10x PCR buffer, 2µL MgCl2, and 1 µL dNTPs (2.5mM), 1µL of each primer (100 ng µL-1) and 1µL template DNA (12.5 ngµL-1). Reaction mixture (25 µL), prepared for 16S rRNA gene amplification was initially denatured at 94°C for 2 min followed by 25 cycles consisting of denaturation at 94°C for 60sec, primer annealing at 52°C for 60sec and primer extension at 72°C for 3 min and finally extension at 72°C for 20min in a thermal cycler (T100 thermal cycler Bio-Rad). The amplification products were separated by electrophoresis (1% agarose gel in Tris—Borate—EDTA buffer, containing ethidium bromide) and photographed under UV light (Fig.1).

Detection of Pseudomonas tolaasii

To confirm the identity of the isolated strains, a PCR test was conducted on each isolate and on the culture of the reference strain according to the PCR protcol given by (Lee, Jeong and Cha, 2002). Total genomic DNA of bacterial strain *P.tolaasii* was isolated by the TIANamp bacteria DNA kit (Tiangen) with slight modifications. PCR was conducted using primer pair Pt-1A, Pt-1D1 (metabion, Germany), specific for

detection of *P. tolaasii* where expected PCR products are 449 base pairs (bp). Presence of the tolaasin gene at each strain was revealed by the specific primers Pt-1A (forward primer, 5`-ATCCCTTCGGCGTTTACCTG-3`) and Pt-1D1 (reverse primer, 5`-CAAAGTAACCCTGCTTCTGC-3`)(Wells *et al.*, 1996; Lee, Jeong and Cha, 2002; Sajben *et al.*, 2011; Milijašević-Marčić *et al.*, 2012; Zhang, Hu and Gu, 2013). Each 25 µL of reaction mixture contained 1 U of *Taq* Polymerase, 2.5 µL 10x PCR buffer, 2 µL MgCl2, and 1 µL dNTPs (2.5 mM), 1 µL of each primer (100 ng µL-1) and 1µL template DNA (12.5 ng µL-1). Reaction mixture (25 µL), prepared for gene amplification was initially denatured at 94°C for 2 min followed by 25 cycles consisting of denaturation at 94°C for 60sec, primer annealing at 52°C for 60sec and primer extension at 72°C for 3 min and finally extension at 72°C for 20min in a thermal cycler (T100 thermal cycler Bio-Rad).The amplification products were separated by electrophoresis (1% agarose gel in Tris—Borate—EDTA buffer, containing ethidium bromide) and photographed under Ultraviolet (UV) light. Cloned PCR products were sequenced by Bioneer (Fig. 2).

Results and Discussion

Biochemical characteristics pathogenic bacteria isolates showed the LOPAT characteristics of the group Va (Lelliott and Stead, 1987), being negative for levan production and the ability to produce a hypersensitivity reaction in tobacco, oxidase and arginine dihydrolase positive. The results from other physiological and biochemical tests of the bacterium were as follows: oxidative metabolism of glucose; catalase activity positive; Tween 80 hydrolysis positive; gelatine hydrolysis Positive and nitrate reduction negative. Based on these features, bacterial strains isolated from mushroom caps were identified as *P. tolaasii*.

The results for antagonistic bacteria *kocuria sp*. shows positive Gram stain, lack of Fluorescence on King's B medium, all LOPAT characteristics negative, oxidative metabolism of glucose; Tween 80 hydrolysis and gelatine hydrolysis negative, catalase and nitrate reduction positive.

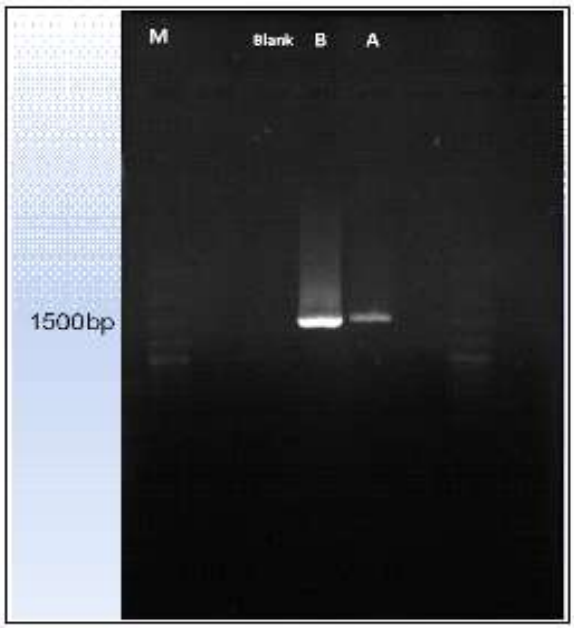

Fig. 1. PCR products, which were carried out with DNA from *Kucuria sp*. and *pseudomonas spp*. from mushrooms and primer set P1-P6 were separated by agarose gel electrophoresis. Lane A: *kucuria sp*.Lane B: *pseudomonas spp*.With the 1500-bp band.

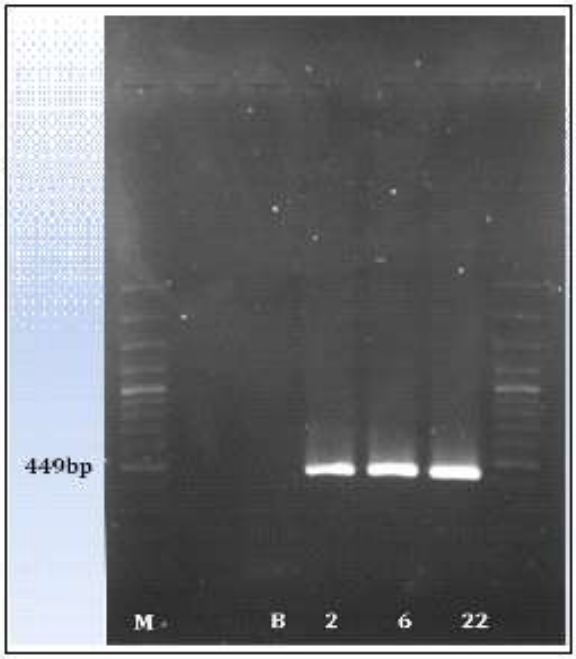

Fig. 2. PCR products, which were carried out with DNA from Pseudomonas tolaasii and other bacterial isolates from mushrooms and primer set Pt-1A / Pt-1D1 were separated by agarose gel electrophoresis. Lanes 2, 6 and 22: *P. tolaasi* .The 449-bp band.

Bacteria strains *kocuria sp*. and *pseudomonas spp*., sprayed on freshly harvested mushroom caps, blocked the Bacterial Blotch incidence Efficacy of antagonistic *kocuria sp*. on the development of bacterial blotch disease, applied to the mushrooms three times in 4 repetitions. For effective biological control of *P. tolaasii*, application of *Pseudomonas* spp. and *kocuria* sp. suspensions with pathogen, simultaneously, will provide adequate control (Fig.3).

Fig. 3. Screening Bacteria for Antagonism on mushroom fresh caps. NC: Negative Control, PC: Positive Control (inoculated with p.tolaasii only), A-D selected antagonists.

P. tolaasii is one of the pathogen which is difficult to control and sometimes cause blotch epidemics in mushroom farms. Due to the ineffectiveness of chemical control agents and breeding resistant varieties (Geels *et al*., 1991; Royse and Wuest, 1980), biological control studies become more important. Since these antagonistic bacteria belong to the same taxonomical group as the pathogen, it is predicted that the pseudomonads will have a superior effect in biological control as compared to other bacterial spp. (Baker and Scher, 1987). This also explains why pseudomonads strains are inhibitory for *P. tolaasii* (Miller and Spear, 1995). At the moment, there is no report of *kocuria* sp. as a potential biocontrol agent of *P. tolaasii*.

Many problems must be solved for the practical use of the bacterium for controlling the disease. Further work is needed in the development of this research to evaluate strains under mushroom growing house conditions or farms.

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