Biological Control 119 (2018) 20–26

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Screening of endofungal bacteria isolated from wild growing mushrooms as potential biological control agents against brown blotch and internal stipe necrosis diseases of *Agaricus bisporus*

Mohammad Ali Aslani, Behrouz Harighi*, Jafar Abdollahzadeh

Department of Plant Protection, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran

ARTICLE INFO

Keywords:
Agaricus bisporus
Biocontrol
Endofungal bacteria
Ewingella americana
Pseudomonas tolaasii

ABSTRACT

Brown blotch and internal stipe necrosis caused by *Pseudomonas tolaasii* and *Ewingella americana*, respectively are two important diseases of cultivated mushroom. The aim of the present study was to evaluate antagonistic activity of endofungal bacteria isolated from wild growing mushrooms against *Pseudomonas tolaasii* and *Ewingella americana*. Both pathogens were isolated from infected cultivable button mushrooms and characterized by phylogenetic analysis. A total of sixty-six endofungal bacteria were isolated from wild growing mushrooms at various locations of Kurdistan Province. Based on preliminary phenotypic properties, eleven selected isolates were further characterized. *16 s rRNA* gene-based phylogenetic analysis revealed that these isolates had 99–100% similarity to *Pseudomonas, Bacillus, Serretia, Stenotrophomonas* and *Brochothrix* genera. The results of protease, lipase and siderophore production were varied amongst isolates tested. Under *in vivo* conditions, Bi1 strain with 96.7% reduction of brown blotch symptoms and De4 strain with 72.25% reduction of internal stipe necrosis symptom had the highest antagonistic activity against *P. tolaasii* and *E. americana*, respectively. To the best of our knowledge, this is the first report of the isolation of endofungal bacteria from wild-growing mushrooms with biological control activity.

1. Introduction

The amount of cultivated mushroom production exceeds 1.03×10^7 tons per year worldwide and Iran with a production of approximately 8.6×10^4 tons per year is in the top ten of mushroom producing countries (Anonymous, 2014). Cultivated mushrooms are infected by a number of bacteria that cause noticeable production losses (Iacobellis, 2011). The most common disease that can occur worldwide is brown blotch. Several species belonging to the genus *Pseudomonas* exhibit brown blotch disease (Munsch et al., 2002). Amongst them, *Pseudomonas tolaasii* has been reported as the most important pathogen (Soler-Rivas et al., 1999a,b). *P. tolaasii* is a soil-habiting bacterial pathogen known as the cause of brown blotch disease in almost all species of mushrooms. The disease is characterized by surface discoloration on caps and stipes, which develops into dark brown, water-soaked and/or pitted lesions (Rainey et al., 1992). The disease has worldwide distribution and is found in several countries with high economic importance (Saxon et al., 2014). Brown blotch disease of the button mushroom was firstly reported from the northern part of Iran (Rahimian et al., 1995). Since then, the disease has been documented from the cultivated mushroom growing centers in various locations in Iran (Tajalipour et al., 2014).

Internal stipe necrosis disease of mushrooms caused by *Ewingella americana* was first described in the United Kingdom (Inglis et al., 1996). Since then, the disease has been reported in New Zealand, Korea and Spain (Reyes et al., 2004; Chowdhury et al., 2007; Lee et al., 2009; Gonzalez et al., 2012). The symptoms emerge as the internal stipe browning and collapse of infected tissues. In the majority of cases, symptoms appear only during harvest.

Every effective method for controlling bacterial infection of cultivated mushroom should be safe because of human consumption. Furthermore, some bacteria have beneficial effects on the healthy development of mushrooms (Rainey, 1991; Cho et al., 2003). Therefore, complete exclusion of all bacterial populations would be detrimental. Widely used disease preventative methods include addition of chlorinated compounds to mushroom beds (Royse and Wuest, 1980; Geels et al., 1991), and control of environmental conditions such as moisture and temperature before and/or after harvest (Singh et al., 2010). Alternative disease prevention methods such as using *Bdellovibrio bacteriovorus* (Saxon et al., 2014), *Streptomyces* species (Sahin, 2005), *P.*

* Corresponding author.
E-mail address: bharighi@uok.ac.ir (B. Harighi).

https://doi.org/10.1016/j.biocontrol.2018.01.006
Received 17 July 2017; Received in revised form 14 January 2018; Accepted 16 January 2018

fluorescens and *P. reactants* (Soler-Rivas et al., 1999a,b) as antagonistic bacteria and biocontrol agents (Sajben-Nagy et al., 2012) have also been investigated.

In many environments, bacteria have various interactions with fungi that are important for both partners. Both gram-positive and -negative bacteria belonging to *Alpha-*, *Beta-*, *Gammaproteobacteria* and *Firmicutes* from fungi living in the soil, rhizosphere and above ground part of the plants have been reported (Hoffman and Arnold, 2010; Kobayashi and Crouch, 2009). Mushrooms with above ground fruitbodies harboring highly diverse bacteria. Bacterial communities may have various interactions with mushrooms, such as inhibiting pathogens, providing growth hormones, fix atmospheric nitrogen and trigger/inhibited fruitbody formation (Pent et al., 2017). The exploration of such bacteria may be useful for improving cultivated mushrooms (Cho et al., 2003) and/or identifying bacterial antagonists against various pathogens (Tsukamoto et al., 2002).

The objectives of the present study were to identify and characterize the causal agents of mushroom brown blotch and internal stipe necrosis diseases, to identify and characterize endophytic bacteria from wild growing mushrooms and to assess their biological control efficiency against bacterial pathogens.

2. Materials and methods

2.1. Isolation, characterization and identification of bacterial pathogens

Cultivated button mushrooms (*Agaricus bisporus*) with symptoms similar to brown blotch and internal stipe necrosis were collected from three mushroom growing centers in Kurdistan Province, western Iran. Small pieces of infected mushroom caps or stipes (about 0.5 g) were immersed in 0.25% sodium hypochlorite solution for 30 s and rinsed in sterile distilled water. Each piece was macerated in 2–3 mL sterile water and the resulting suspension was streaked onto King,s B (KB) and nutrient agar (NA) media. The plates were incubated at 26–28 °C for 72 h and the presumptive colonies were further purified by streaking on the same medium. Phenotypic characterization of the isolates was carried out by using standard procedures (Lelliott and Stead, 1987). Testing of isolates for white line production was carried out according to the procedure previously described (Wong and Preece, 1979).

2.2. Pathogenicity test

Pathogenicity tests on *A. bisporus* cubes of cap tissue or stipe were performed according to the methods previously described (Wong and Preece, 1979; Inglis et al., 1996). In brief, 20 µL of the bacterial suspension (density of about 1×10^8 CFU/mL) was inoculated onto caps or stipe bases prewashed with sterile water, then incubated in a humid chamber at room temperature. Disease symptoms were recorded up to 72 h. Mushroom tissues inoculated with sterile water was used as a control. All experiments were conducted three times with three replications.

2.3. Isolation, characterization and identification of endofungal bacteria

Healthy wild growing mushrooms were collected from different locations of Kurdistan Province during July to September 2015. Samples were used immediately or kept in a refrigerator until used. Different parts of mushrooms including cap, stipe and hyphae were washed with tap water, surface sterilized by immersing in 1% sodium hypochlorite solution for 1 min, washed three times with sterile-distilled water, macerated in 5 mL sterile water and 100 µL of suspension was inoculated onto NA, KB and LB agar media. The plates were incubated at 26–28 °C up to 14 days and observed daily. Colonies growing on the media were subcultured to get pure cultures. To check the efficiency of sterilization, inoculation of 100 µL of the water from the final wash onto nutrient agar media was used as a negative control. Preliminary phenotypic properties of all isolates such as gram reaction, oxidase activity, catalase production and fluorescent pigment production were characterized according to the standard method (Schaad et al., 2001) and identification of the selected isolates was further confirmed by partial nucleotide sequencing of the *16s rRNA* gene using PCR using two universal primers, fD2 (5′-AGA GTT TGA TCA TGG CTC AG-3′, position 8–27) and rP1 [5′-ACG GTT ACC TTG TTA CGA CTT-3′, position 1512–1492 (*Escherichia coli*)] following the method described by Weisburg et al. (1991). The PCR products were sequenced using an ABI3730XL DNA sequencer (Applied Biosystems). The *16s rRNA* sequences obtained were aligned and manually adjusted where necessary by using BioEdit Sequence Alignment Editor 7.0.9.0 software (Hall, 2011). *16s rRNA* sequences obtained were further subjected to BLAST analysis with other sequences deposited in the NCBI database using BlastN program. The neighbor-joining phylogenetic analysis was performed using PAUP version 4.0b10 (Swofford, 2003) and a phylogenetic tree was constructed (bootstrap analysis with 1000 replicates was conducted).

2.4. In vitro antagonistic properties of the isolated endofungal bacteria

The ability of isolates for siderophore production on CAS medium was screened using methods previously described by Schwyn and Neilands (1987). Protease, lipase and HCN production was assessed according to the procedure of Sgroy et al. (2009), Sierra (1957) and Alstrom and Burns (1989), respectively. To check the ability of bacterial isolates for antibiotic production, overnight growth of bacteria was spectrophotometrically adjusted to the density of approximately 1×10^8 CFU/mL ($OD_{600nm} \simeq 0.1$), and a 20 µL was spotted directly onto nutrient agar (NA) medium. Plates were incubated at 28 °C for 48 h, after which bacterial colonies were swept from the plates using sterile cotton before treated with chloroform vapor for 1 h. Thereafter, 300 µL of *P. tolaasii* and *E. americana* strains ($OD_{600nm} \simeq 0.3$ for bacterial concentration of about 2×10^8 CFU/mL) were spread onto the medium. The plates were kept at 26–28 °C for 48 h and the diameter of the inhibition zones was calculated. Sterile water spotted in the plates inoculated with the bacterial pathogen was used as a control. The bioassay was conducted with four replications (Vanneste et al. 1992).

2.5. In vivo biocontrol activity of endofungal bacteria

The antagonistic activity of the bacterial isolates against *P. tolaasii* and *E. americana* was evaluated according to the method described by Fermor and Lynch (1988) with some modifications. Suspensions of the bacterial pathogens and endofungal bacteria (approximately 10^6 and 10^8 CFU/mL, respectively) were mixed in equal volume and spot-inoculated (100 µL) on the mushroom blocks or injected into stipe using sterile syringe. Tissues inoculated with the pathogen only or sterile water were used as positive and negative controls, Respectively, All inoculated tissues were maintained in a humid chamber at 25–26 °C for 48 h, and the diameter or length of the lesions on the caps or stipes of the mushroom was scored. The experiment was repeated twice and carried out as completely randomized design with three replications.

2.6. Statistical analysis

Data analysis was performed using the SAS 8.2 program and the comparison of means was carried out using the Duncan test at 5% probability level for the biocontrol assays. Graphs were plotted using Excel software.

3. Results

3.1. Identification of bacterial pathogens

In total, eighteen isolates were obtained from infected button