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ORIGINAL ARTICLE

In vitro study of biological activity of four strains of *Burkholderia gladioli* pv. *agaricicola* and identification of their bioactive metabolites using GC–MS

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Abstract This research was carried out to study *in vitro* antibacterial activity of 4 strains of *Burkholderia gladioli* pv. *agaricicola* (*Bga*) against G + ve *Bacillus megaterium* and G− ve *Escherichia coli*, haemolytic activity against the cell membrane of erythrocytes, the production of extracellular hydrolytic enzymes and finally, the pathogenicity against *Agaricus bisporus* flesh blocks. Chemical structure of bioactive substances of the most bioactive strain (ICMP 11096) was established using gas chromatography–mass spectrometry (GC–MS). All the studied *Bga* strains inhibited the growth of the two tested bacteria although some growing substrates negatively influenced the antimicrobial substance production. The same *Bga* strains showed highly haemolytic activity and were able to produce 3 hydrolytic enzymes, i.e. chitinase, glucanase and protease. In pathogenicity assays, the considered *Bga* strains resulted virulent for *A. bisporus*. The GC–MS for compounds from *Bga* ICMP 11096 were compatible with the structure of two bioactive fatty acids identified as methyl stearate and ethanol 2-butoxy phosphate with mass spectrum m/e 298 and 398, respectively.

1. Introduction

The public concern to search novel microbial natural biocides has recently been increasing in order to avoid the negative impact of synthetic pesticides either on the environment and/or animal and human health. Many *Burkholderia* spp. produce *in vitro* secondary metabolites with relevant biological activities and potential practical applications. The genus *Burkholderia* contains several species which have a wide host range, including many clinically important microorganisms as well as

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phytopathogens (Sawana et al., 2014), and inhabit a wide range of ecological niches, ranging from soil to human respiratory tract (Yabuuchi et al., 1992; Jiao et al., 2003; Coenye and Vandamme, 2003).

Burkholderia gladioli Yabuuchi is a species that causes disease in human, plants and fungi (Coenye and Vandamme, 2003). In particular, *B. gladioli* pv. *agaricicola* (*Bga*) is considered a dangerous pathogen in the mushroom industry (Gill, 1995). In fact, it causes soft rot disease on a number of commercially important mushrooms such as *Lentinula edodes* (Berkeley) Pegler, *Pleurotus ostreatus* (Jacq.) P. Kumm, *Flammulina velutipes* (Curtis) Singer, *Pholiota nameko* (T. Itô) S. Ito & S. Imai, *Hypsizygus marmoreus* (Peck) H.E. Bigelow and *Grifola frondosa* (Dicks.) Gray in Japan and different cultivated *Agaricus* species in New Zealand and Europe (Chowdhury and Heinemann, 2006).

The biocontrol effect exhibited by diverse microorganisms depends on their antagonistic action against phytopathogenic microbes which operates through synthesis of antibiotics with fungicidal effect and cell-wall degradation enzymes as well as production of siderophores (Glick, 1995; Ciccillo et al., 2002; Lucy et al., 2004; Elshafie et al., 2013).

Most of *Burkholderia* species can potentially be used as biocontrol agents against phytopathogenic fungi, bacteria, protozoa and nematodes in several different crops such as corn, sweet corn, cotton, grapevine, pea, tomato, pepper and some citrus and apple fruit trees (Cain et al., 2000; Perin et al., 2006; Scuderi et al., 2009) due to the production of antimicrobial substances (El-Banna and Winkelmann, 1998; Elshafie et al., 2010, 2012; Lamorte et al., 2010).

B. gladioli has been indicated for *in vitro* and *in vivo* diseases biocontrol because it can completely inhibit conidial germination of *Penicillium digitatum* Sacc., *Rhizoctonia solani* (Cooke) Wint., and *Botrytis cinerea* Pers. (Walker et al., 2001; Elshafie et al., 2013). Metabolites produced by *B. gladioli* also induced a significant inhibition of *P. expansum* Link growth (Altindag et al., 2006). The mode of action of *B. gladioli* as biocontrol agent is apparently the consequence of the synergic combination between its competition for nutrients and/or space and production of antimicrobial metabolites (Altindag et al., 2006). Previous studies on the biological and chemical characterization of toxic metabolites produced by *Bga* ICMP 11096 suggested the lipodepsipeptide nature of the above bioactive molecules (Andolfi et al., 2008).

In the current research, the biological characterization of four *Bga* strains (11096, 11097, 12220 and 12322) from the International Collection of Microorganisms from Plants (ICMP) has been carried out. The antibacterial activity of the above studied strains was evaluated against *Escherichia coli* (Migula) Castellani & Chalmers (*E. coli*) and *Bacillus megaterium* de Bary (*B. megaterium*), together with their haemolytic, hydrolytic activities and eventually their pathogenicity against *A. bisporus* flesh blocks. The current research studied also the main bioactive substances produced by *Bga* ICMP 11096 by Gas Chromatographic and Mass Spectrometry (GC–MS) analysis.

2. Materials and methods

2.1. Bacterial strains used in this study

The following *Bga* strains, obtained from ICMP, were used in this study: 11096, 11097, 12220 and 12322. The target microorganisms *B. megaterium* ITM100 and *E. coli* ITM103 (ITM: Institute of Tropical Medicine in Antwerp) have been obtained from stock cultures of the same prokaryotes kept freeze-dried in collection at the Laboratory of Mycology of School of Agricultural, Food-Forestry and Environmental Sciences of University of Basilicata (Potenza, Italy), recultured on King B (KB) media (King et al., 1954) and stored at 4 °C.

2.2. In vitro antibacterial assay

The studied *Bga* strains were evaluated for their ability to inhibit the growth of target organisms in dual agar plate assay following the method of Lavermicocca et al. (1997) with some minor modifications. More specifically, single small masses from fresh *Bga* cultures were transferred in the center of 9 cm diameter Petri dish series, each containing 14 ml of three different nutrient media, i.e. KB, potato dextrose agar (PDA) and minimal mineral agar (MMA). The different plate series were then sprayed with a target bacterial suspension containing 10^8 - CFU ml^{-1} of *B. megaterium* or *E. coli* and incubated for 48 h at 24 ± 2 °C. The antagonistic activity was registered measuring the diameter of bacterial inhibition zone after a 72 h incubation period at room temperature and expressed using the following equation: $BIP\ (\%) = 100 - [(GC - GT)/GC \times 100]$, where BIP represents the bacterial inhibition percentage, GC the average diameter of bacterial grown in control plate in cm and GT the average diameter of inhibition zone in cm. The test was repeated twice with three replicates.

2.3. Biological characterization of Bga strains

2.3.1. Haemolytic assay

The haemolytic activity of studied *Bga* strains was evaluated against cell membrane of erythrocytes (RBCs) using blood agar base (BAB) media supplemented with fresh bovine blood following the method reported by Munsch and Alatossava (2002) and Lo Cantore et al. (2006) with some minor modifications. Blood sample was treated with heparin 25 µl 1000 U/5 ml blood, washed three times in buffer (0.72 g Tris–HCl, 1.16 g NaCl, 0.07 g EDTA at pH 7) and then centrifuged at 20.000 g for 3 min at room temperature. RBCs were successively added at 0.25% to BAB. Ten ml of the suspension was later poured in each Petri dish. A loopful of bacterial mass of studied *Bga* strains was added in the Petri dish and incubated at 24 ± 2 °C. Diameter of haemolysis zone was scored after 48 h. The test was repeated twice with three replicates.

2.3.2. Extracellular hydrolytic enzymes assay

Chitinase and protease activities of studied *Bga* strains were determined according to Tahtamouni et al. (2006) on plates of KB containing chitin 1% and skim milk 1%, respectively. Cellulase activity was detected according to the method of Essghaier et al. (2009) using carboxymethyl cellulose 0.4%. Glucanase activity was detected according to the method of Teather and Wood (1982) using lichenan 0.2%. Amylase, pectinase and polygalacturonase activities were detected using soluble starch 1%, pectin 0.5% and polygalacturonic acid 1%, respectively (Sung et al., 1993; Bhardwaj and Garg, 2010). After a five day incubation at 28 ± 2 °C, plates were flooded with specific staining solutions: congo red 0.03% for chitinase, cellulase and glucanase; lugol solution for amylase; CTAB 2%