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Biocontrol activity of surfactin A purified from *Bacillus* NH-100 and NH-217 against rice bakanae disease

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ABSTRACT

The potential of the *Bacillus* genus to antagonize phytopathogens is associated with the production of cyclic lipopeptides. Depending upon the type of lipopeptide, they may serve as biocontrol agents that are eco-friendly alternatives to chemical fertilizers. This study evaluates the biocontrol activity of surfactin-producing *Bacillus* (SPB) strains NH-100 and NH-217 and purified surfactin A from these strains against rice bakanae disease. Biologically active surfactin fractions were purified by HPLC, and surfactin A variants with chain lengths from C12 to C16 were confirmed by LCMS-ESI. In hemolytic assays, a positive correlation between surfactin A production and halo zone formation was observed. The purified surfactin A had strong antifungal activity against *Fusarium oxysporum, F. moniliforme, F. solani*, *Trichoderma atroviride* and *T. reesei.* Maximum fungal growth suppression (84%) was recorded at 2000 ppm against *F. moniliforme*. Surfactin A retained antifungal activity at different pH levels (5–9) and temperatures (20, 50 and 121 °C). Hydroponic and pot experiments were conducted to determine the biocontrol activity of SPB strains and the purified surfactin A from these strains on Super Basmati rice. Surfactin production in the rice rhizosphere was detected by LCMS-ESI at early growth stages in hydroponics experiments inoculated with SPB strains. However, the maximum yield was observed with a consortium of SPB strains (T4) and purified surfactin A (T5) treatments in the pot experiment. The outcomes of the present study revealed that surfactin A significantly reduced rice bakanae disease by up to 80%. These findings suggest that purified surfactin A could be an effective biocontrol agent against bakanae disease in rice and should be incorporated into strategies for disease management.

1. Introduction

The excessive usage of chemical fungicides over the years is not only responsible for the development of resistance in the target fungal strains but has also adversely affected non-target populations of microbes, resulting in disruption of the rhizobial microbial community, which leads to decreased soil health (Yang et al., 2011). Therefore, the development of eco-friendly substitutes for pathogen control is a prerequisite to achieve sustainable agricultural production (Kaur et al., 2016). Biological control approaches based on the usage of normal microbial populations and their secondary metabolites is a promising and safe alternate to chemical fungicides used for pathogen control and might be successfully used in conjunction with chemicals (Dubey et al., 2015). The use of plant growth-promoting rhizobacteria (PGPR) and their products as biocontrol agents has received considerable attention; however, to develop incorporated biocontrol strategies, the production of antifungal secondary metabolites produced by PGPR such as *Bacillus* spp., *Pseudomonas* spp. and *Serratia* spp. must be explored (Killani et al., 2011).

The use of native microorganisms to control pathogenic fungal species has received emphasis in the past years, as they can provide effective defense against pathogens due to their ability to colonize roots and their valuable plant interactions that can stimulate host defense responses against pathogen attack (Whipps, 2007). Different Gram-negative and Gram-positive bacterial strains have been reported to produce different antifungal and antimicrobial compounds (Borris, 2011; Haas and Defago, 2005; Toure et al., 2004). The members of *Bacillus* genus are well recognized for their production of cyclic lipopeptides (CLPs), including a wide range of antifungal products (Falardeau et al., 2013; Borris, 2011).

The CLPs have a cyclic structure comprising a β-hydroxy or β-amino fatty acid chain incorporated through peptide bonds and synthesized

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via non-ribosomal peptide synthetases (NRPSs). The genes of these NRPSs can move via horizontal gene transfer, which results in the production of different isomers of CLPs having significant effects on the bacterial strains that produce them (Stein, 2005). The different classes of lipopeptides may produce CLPs with differences in fatty acid chain lengths, amino acids sequences, and the cyclization type of the peptide sequence (Hamley, 2015). Different members of *Bacillus* species, such as *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus coagulans*, *Bacillus pumilus* and *Bacillus licheniformis* produce CLPs that are classified into the iturin, surfactin and fengycin families based upon their structures (Koumoutsi et al., 2004; Mukherjee and Das, 2005; Ongena and Jacques, 2008; Roongsawang et al., 2010).

Members of the surfactin A family are heptapeptides that form a lactone ring structure due to linkage with a β-hydroxy fatty acid. The size of the fatty acid chain may vary from C12 to C15, and different isomeric compounds are frequently coproduced (Peypoux et al., 1999). Surfactins are potent biosurfactants and play significant roles in the development of persistent biofilms (Bais et al., 2004). In this study, Leu7-surfactin, reported as surfactin A, one of the major surfactins, was purified (Snook et al., 2009; Figs. 4 and 5). Surfactin B and C differ in the amino acids at carbon-7 and contain valine and isoleucine, respectively. Lichenysin and esperin are also surfactins that contain Gln instead of Glu at carbon 1 or other linkages, respectively. These strong biosurfactants are toxic to viruses, fungi, mites and bacteria (Desai and Banat, 1997; Peypoux et al., 1999; Seydlova and Svobodova, 2008). The iturins mainly consist of heptapeptides containing a C14–C17 β-amino fatty acid chain and show limited antibacterial activity and strong antifungal activity (Moyne et al., 2004). Fengycins are decapeptides with a C14–C19 β-hydroxy fatty acid chain that exhibit strong antifungal activity, especially toward filamentous fungi (Wu et al., 2007; Li et al., 2013).

The cyclic lipopeptides control pathogenic fungal species either directly by targeting the membranes of fungal pathogens or indirect systemic resistance induction in the host plant (Patel et al., 2011; Jourdan et al., 2009). The selective antifungal activity of different CLPS from *Bacillus* spp. has been acknowledged due to their interactions with fungal cell membranes, resulting in osmotic differences of the cellular components. Still, the mechanism of interaction varies and depends upon the structure of CLPs and fungal cell membrane, thus resulting to differential activities (Falardeau et al., 2013). The synergistic interactions among different lipopeptide families and their isomers by different *Bacillus* strains may lead to the effective fungal pathogen control (Cawoy et al., 2015; Romero et al., 2007; Maget-Dana and Peypoux, 1994).

Rice is a staple food crop that is grown worldwide (Rana et al., 2015). Rice yield suffers a regular loss of 10–50% due to *Fusarium moniliforme*, which causes bakanae or foot rot disease (Anon., 2015). Moreover, this fungus produces a diversity of mycotoxins comprised mainly of fumonisin B1 (FB1) during the progression of growth and incursion of rice grains (Lizárraga-Sánchez et al., 2015). FB1 is not only responsible for significant economic losses but has also been correlated with high occurrences of liver and esophageal cancer in numerous areas of the world (Chen et al., 2015). Naturally, *F. moniliforme* responsible for FB1 production has a similar environment as other microorganisms (Lizárraga-Sánchez et al., 2015), such as *Bacillus subtilis*, *B. velezensis* and *B. cereus*, which are capable of inhibiting the production of fumonisin B1. This inhibition may result from several factors such as competition for nutrients, space and the secondary metabolites present in nature (De Melo et al., 2015).

Several strains of *Bacillus* have been proven as effective biocontrol agents due to the production of several classes of antibiotic lipopeptides, mainly surfactin, fengycin, and iturin. These lipopeptides are effective in the suppression of numerous phytopathogens, such as species of *Rhizoctonia*, *Fusarium*, *Pythium*, *Phytophthora*, *Sclerotinia* and *Verticillium* (Ongena and Jacques, 2008; Nagorska et al., 2007). They exhibit good antagonistic activity by direct suppression of plant pathogens and are capable of facilitating root colonization or supporting the resistance of the host plant through stimulating induced systemic resistance (ISR). *Bacillus subtilis* NH-100 and *Bacillus* sp. NH-217 suppressed *Colletotrichum falcatum*, which is responsible for sugar cane red rot (Hassan et al., 2010, 2014). In the present study we evaluated the potentials of surfactin-producing *B. subtilis* NH-100 and *Bacillus* sp. NH-217, along with their lipopeptide surfactin A, to control the rice bakanae disease caused by *F. moniliforme*. The objective was to differentiate and assess the efficacy of lipopeptides that are responsible for the antifungal activity exhibited by *Bacillus* species.

Table 1
Microorganisms used in this study.

Bacterial strains	Source/reference
Bacillus subtilis NH-100 EU627167	Applied Microbiology and Biotechnology (AMB) Lab, CIIT, Islamabad
Bacillus sp. NH-217 EU627170	AMB Lab, CIIT
Bacillus amyloliquefaciens FZB42 NC009725 (reference strain)	AIT (Tulln, Vienna), HBD, Koumoutsi et al., 2004; BGSC
Bacillus subtilis 168	AIT, *Bacillus stock center*
Escherichia coli M5	Microbiology and Public Health (MPH) Lab, CIIT
Staphylococcus aureus ATCC 25923	MPH Lab, CIIT
Pseudomonas aeruginosa ATCC 9027	MPH Lab, CIIT
Enterobacter sp. 18T	MPH Lab, CIIT
Lactococcus lactis JX119011	MPH Lab, CIIT
Fungal strains	Source/reference
Fusarium moniliforme KJ719445	AMB Lab, CIIT; rice bakanae disease
Fusarium oxysporum 166	Punjab University
Fusarium solani ofio601As5	AIT, Strain collection, Tulln Vienna
Fusarium solani P302	AIT, Strain collection, Tulln Vienna
Fusarium solani SAN1077	AIT, Strain collection, Tulln Vienna
Trichoderma atroviride P150907	AIT, Strain collection, Tulln Vienna
Trichoderma reesei Qm6a	AIT, Strain collection, Tulln Vienna
Trichoderma atroviride IMI206040	AIT, CBS-KNAW collection, Tulln Vienna

2. Materials and methods

2.1. Microorganisms and culture conditions

The bacterial strains capable of producing surfactin and fungal strains used in this study are listed in Table 1. Pure bacterial cultures stored at −80 °C in 20% glycerol were revived on Luria Bertani (LB) agar plates (Somasegaran and Hoben, 1994). Pure bacterial cultures were collected from the surface of LB agar plates using sterile disposable loops after 24 h incubation at 28 °C and used as liquid starter cultures of the *Bacillus* strains in 10 mL of LB broth.

Characterized strains of pathogenic *Fusarium* and *Trichoderma* species were obtained from different culture collections (Table 1). The emerging cultures of each fungus were selected with a disposable loop, placed onto Potato Dextrose Agar (PDA) plates and incubated for 5–7 days at 25 °C. A single fungal colony was picked up and sub cultured onto a new PDA plate for purification.

2.2. Inhibition of fungal growth by Bacillus strains

Bacillus strains were assessed for antagonism against *F. moniliforme, F. solani, F. oxysporum, T. atroviride* and *T. reesei* by a dual culture test (Hassan et al., 2010). A freshly grown 5-day old, 5 mm fungus disk was positioned in the PDA plate center. *Bacillus* cells were streaked on both sides of the fungal plug. PDA plates with fungal disks were used as controls. The plates were incubated for seven days at 28 °C, the inhibition of mycelial growth of the respective fungal strains was measured in centimeters, and the percentage inhibition of growth was calculated. This experiment was performed in triplicate for each