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Leaf and powdery mildew colonization by glycolipid-producing *Pseudozyma* species

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ABSTRACT

In this work, we followed the development and compared the biocontrol potential of four phylogenetically related species of *Pseudozyma* (*P. antarctica*, *P. flocculosa*, *P. fusiformata* and *P. rugulosa*) all known to release glycolipids with antifungal activity *in vitro*. To this end, we developed GFP transformants and specific primers for each species and conducted *in situ* observations and quantification of their population sizes over time on both healthy and powdery mildew-infected leaves. Although all species shared many similar features, only *P. flocculosa* antagonized powdery mildew colonies and grew specifically in their presence. Population quantification by qRT-PCR following inundative applications showed a drastic decline in population for all species, including *P. flocculosa* on healthy leaves, but a sharp and steady increase of the latter over 72 h on infected leaves. These results suggest that production of glycolipids, and more specifically flocculosin or ustilagic acid, is not the sole factor dictating biocontrol activity among *Pseudozyma* spp.

Introduction

Powdery mildew fungi are ubiquitous phyllosphere pathogens of numerous field and greenhouse crops. While fungicides remain the method of choice for controlling these pathogens, the development of resistant strains and mounting environmental concerns have prompted the search for biological control methods (Bélanger 2006). Because of their ectotrophic growth, powdery mildews are readily exposed to natural enemies and there are approximately 40 fungal species that have been tested as their potential biocontrol agents (Kiss 2003). Among them, *Pseudozyma flocculosa*, a yeast-like epiphyte belonging to the Ustilaginales, has been extensively documented as an efficient antagonist of powdery mildews (Jarvis *et al.* 2007). On the basis of the reported modes of action for biocontrol agents (BCAs), *P. flocculosa* is described as exerting its antagonistic activity through antibiosis, presumably by releasing flocculosin, an antifungal glycolipid (Cheng *et al.* 2003).

Glycolipids can be categorized as biosurfactants, i.e. amphiphatic compounds of microbial and fungal origins that reduce tension between surfaces (Banat *et al.* 2000). Physiological roles of biosurfactants are rather diverse, ranging from increasing bioavailability of hydrophobic water-insoluble substrates to displaying antimicrobial activity (Ron & Rosenberg 2001). Interestingly, many fungal species within the Ustilaginales have been linked by the production of unusual glycolipids. For instance, *Pseudozyma* spp. produce two classes of glycolipids: mannosylerythritol lipids (MELs) and cellobiose lipids (Fig 1) (Mimee *et al.* 2005; Hewald *et al.* 2005; Hewald *et al.* 2006). Molecules of each class were first isolated from the smut pathogen *Ustilago maydis* (Haskins 1950; Fluharty & Obrien 1969), a basidiomycete phylogenetically related to the *Pseudozyma* genus (Begerow *et al.* 2000). In a recent survey, Morita *et al.* (2007) identified six MEL producers within the genus *Pseudozyma*: *P. antarctica*, *P. fusiformata*, *P. rugulosa*, *P. aphidis*, *P. parantarctica* and *P. tsukubaensis*. For their part,

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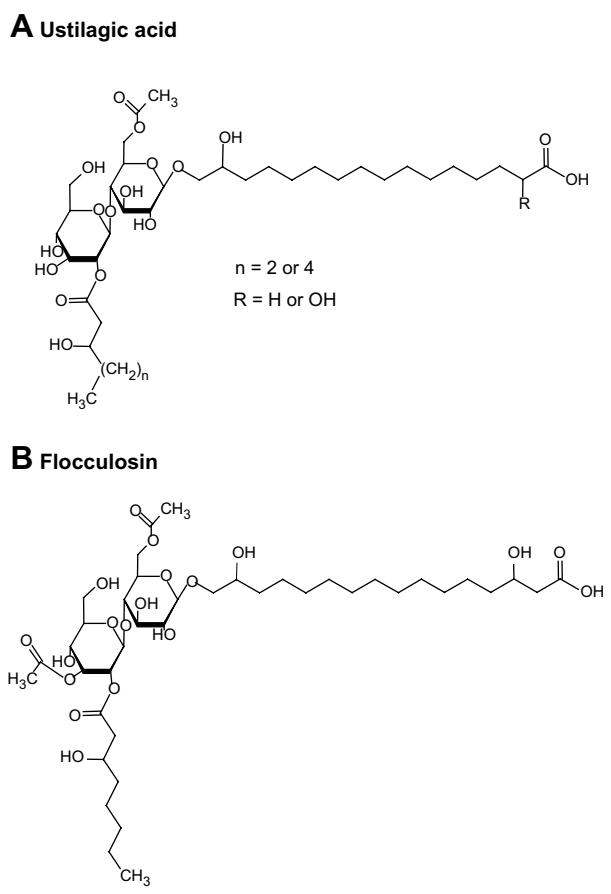

Fig 1 – Chemical structures of (A) ustilagic acid, (B) flocculosin and (C) mannosylerythritol lipid, three glycolipids produced by some Ustilaginales species.

cellobiose lipids are known to be produced by only two *Pseudozyma* spp., *P. fusiformata* (Kulakovskaya *et al.* 2005) and *P. flocculosa* (Cheng *et al.* 2003), which respectively produce ustilagic acid and flocculosin.

The *in vitro* antibiotic activity of glycolipids secreted by the Ustilaginales has been reported in several separate studies. Kitamoto *et al.* (1993) showed that MELs from *P. antarctica* were particularly active against Gram-positive bacteria. Deml *et al.* (1980) reported that MELs accumulating in *Schizonella melanogramma* were active against some fungal strains. Ustilagic acid from *P. fusiformata* also showed a broad antifungal spectrum (Golubev *et al.* 2001), similar to that of ustilagic acid from *U. maydis* (Haskins & Thorn 1951). Moreover, several pathogenic yeasts were found to be sensitive to flocculosin, whose chemical structure is almost identical to that of ustilagic acid (Mimee *et al.* 2005). In addition, *P. antarctica*, *P. fusiformata* and *P. flocculosa* all displayed *in vitro* antagonistic activity against a few fungal species (Buzzini & Martini 2000; Golubev *et al.* 2001; Cheng *et al.* 2003). Thus, glycolipid production and *in vitro* antagonistic activity by *Pseudozyma* spp. appear to be closely linked. If it is indeed the case, this would suggest that glycolipid production might confer biocontrol activity to other *Pseudozyma* spp. Incidentally, some earlier studies (Jarvis *et al.* 1989; Hajlaoui & Bélanger 1991) did report that *P. rugulosa* had antagonistic activity against powdery mildews of rose and cucumber, although Avis *et al.* (2001) could not confirm these results. Therefore, the role played by glycolipids in the development and/or biocontrol activity of *Pseudozyma* spp. remains poorly understood.

Considering their production of glycolipids with intriguing biological properties and our limited knowledge of the ecology of *Pseudozyma* spp., the main objective of this study was to compare the development of *P. antarctica*, *P. rugulosa* and *P. fusiformata* to that of *P. flocculosa* following inundative applications on both healthy and powdery mildew-infected leaves. To this end, we took advantage of the Green Fluorescent Protein (GFP) (Neveu *et al.* 2007a) and quantitative real-time PCR (qRT-PCR) technologies to conduct *in situ* observation, and quantification of their population over time, and to assess their respective biocontrol activity.

Materials and methods

Fungal material

The fungal isolates used in this study all came from the Centraalbureau voor Schimmelcultures (CBS; Utrecht, NL) and were *P. flocculosa* (CBS 167.88), *P. antarctica* (CBS 516.83), *P. fusiformata* (CBS 423.96) and *P. rugulosa* (CBS 170.88). All four species together with *Phomopsis* sp. were maintained on potato-dextrose-agar (PDA) at 4 °C and subcultured every two weeks. *Pseudozyma* transformants were grown on PDA amended with 300 μg ml^{-1} hygromycin for all species but *P. flocculosa* (100 μg ml^{-1}). Powdery mildews were maintained on their respective host plants: *Podosphaera xanthii* (Px) on *Cucumis sativus* cv Corona and *Blumeria graminis* f. sp. *tritici* (Bgt) on *Triticum aestivum*. All plants were grown in growth chambers at Université Laval and were subjected to a 16 h photoperiod.

Fungal transformation and plasmid material

PEG/$CaCl_2$-mediated transformation for each *Pseudozyma* spp. was performed using the gene transfer system established by Cheng *et al.* (2001) to obtain GFP-expressing transformants. Briefly, protoplast preparation was achieved using a 15 % Glucanex solution as described by Cheng & Bélanger (2000). Transformation was then completed using either both pSceI-Hyg and pCAct.GFP (10 μg per plasmid) or only pSPF.GFP (10 μg)