Multiple mechanisms of biocontrol by *Pseudomonas chlororaphis* PA23 affect stem rot of canola caused by *Sclerotinia sclerotiorum*

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Abstract

Pseudomonas chlororaphis strain PA23 has consistently reduced stem rot of canola in greenhouse and field experiments. PCR analysis and Southern blotting detected the presence of phenazine and pyrrolnitrin biosynthetic genes in this bacterium. High performance liquid chromatography confirmed the production of phenazine, 2-hydroxy phenazine and pyrrolnitrin antibiotics. PA23 produces three organic volatiles (nonanal, benzothiozole, 2-ethyl, 1-hexanol) that are capable of completely inhibiting mycelial growth, and significantly reducing ascospore and sclerotia germination. Induction of higher activity of chitinase and β-1,3 glucanase was observed, when the bacterium was challenge inoculated with the pathogen. Western blot analysis confirmed the induction of a 34 kDa chitinase protein. Transposon mutant PA23-314, which has a Tn5 insertion within the *gacS* gene, lacked antifungal activity in lab and greenhouse assays. Complementation with the wild-type *gacS* allele *in trans* restored antifungal activity, phenazine production, exoprotease, and *N*-acylhomoserine lactone to levels of the wild type. Another mutant PA23-63, a phenazine non-producer, mutated at the *phzE* gene of the phenazine biosynthetic operon, still produced pyrrolnitrin and exhibited antifungal activity suggesting that pyrrolnitrin could play a vital role in the antifungal activity of the bacterium. PA23 strain is capable of forming a biofilm that imparts resistance to lethal antibiotics. Strain PA23, a bacterium with several weapons in its armoury against *Sclerotinia sclerotiorum,* with the ability to grow as a biofilm is an excellent biological control agent for management of stem rot of canola.

Key Words: Biocontrol, *Sclerotinia*, *Pseudomonas chlororaphis* strain PA23, phenazine, pyrollnitrin, volatiles, chitinase, β-1,3 glucanase, Gac S, Tn-mutants, biofilms, phyllosphere, PCR, biosynthetic genes

Introduction

Modern day crop protection relies heavily on the use of chemical pesticides. Increasing public concern over the risk these chemicals pose to the environment and human health has resulted in the need for safer alternatives. Biological control using bacteria that naturally produce antibiotics to inhibit disease-causing pests represents a safer, more environmentally friendly substitute to chemical pesticides. *Pseudomonas chlororaphis* strain PA23, a bacterium that is able to inhibit the fungal pathogen *Sclerotinia sclerotiorum* has been the focus of our studies for the last few years (Savchuk 2002; Zhang 2004). *S. sclerotiorum* is an ever-present, soil-borne fungus capable of infecting more than 400 plant hosts and causing significant loss of economically important crops. We are specifically interested in the ability of PA23 to control *S. sclerotinia*-incited stem rot of canola (Savchuk and Fernando, 2004). Because there is no known resistance against *S. sclerotiorum* in commercially grown canola cultivars, management of this pathogen is essential. Biological approaches to disease management have previously shown inconsistent results in the field. It is therefore essential to elucidate the molecular mechanisms mediating biocontrol activity in strain PA23 so that expression and activity of the pathogen-suppressing factors can be optimized.

Materials and Methods:

Tn*5*-OT182 transposon mutagenesis and biocontrol assaysin greenhouse and field: PA23 mutants were derived as described by Poritsanos (2005). Sequence analysis enabled us to elucidate the genetic region where the transposon had randomly inserted. *In vitro* biocontrol assays were performed with PA23 and its mutants on potato dextrose agar (PDA) plates to investigate the nature of biocontrol of *S. sclerotiorum* radial mycelial growth. This was followed by greenhouse studies with the same organisms and ascospores of the pathogen (10^4 spores ml^{-1}). All field tests were carried out as described in Fernando et. al (2007).

Testing for compounds related to biocontrol: HCN, homoserine lactone production, protease production, lipase activity and phenazine production were tested using published protocol (Poritsanos et al., 2006).

Identification of antibiotic biosynthetic genes: PA23 DNA was isolated using standard procedures and then subjected to PCR amplification to identify biosynthetic genes for phenazines, pyrrolnitrin, 2,4 diacetyl phloroglucinol and pyoluteorin using relevant PCR primers (Zhang 2004). The PCR products obtained weresubjected to sequence analysis using blastn and blastx databases. Southern analysis was performed for further confirmation (Zhang et al, 2006).

Analysis for chitinase and β-1, 3-glucanase: The effect of PA23 on the induction of hydrolytic enzymes chitinase and

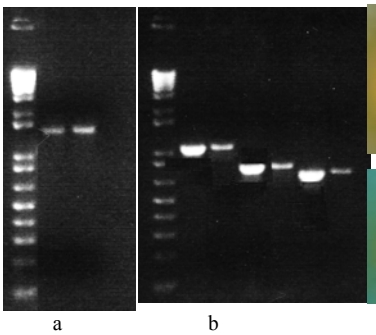
β-1, 3-glucanase was investigated under greenhouse conditions and confirmed by western blot analysis in the lab (Fernando et al, 2007).

Detection of volatile antibiotics: A divided plate assay was used to identify the volatile inhibition of *S. sclerotiorum* as described in Fernando and Linderman (1994). The volatiles were isolated and identified through GC/MS methods and confirmed using pure products of the compounds (Fernando et al., 2005).

Biofilm Formation: The ability of PA23 to form a biofilm was studied using the methods described by O'Toole and Kolter (1998). Biofilm susceptibility to clinical antibiotics was tested using methods of Parkins et al (2001).

Results and Discussion:

This is the first bacterial biocontrol agent to be used against ascospore infection of *Sclerotinia sclerotiorum* in canola. To our knowledge, this is also the first time a successful phyllosphere biological control system has been developed for a field crop in Canada. Thus, it becomes important to further elucidate the nature of biocontrol by determining the mechanisms underlying antifungal activity. PCR analysis (Fig 1) and Southern blotting (data not shown) detected the presence of phenazine and pyrrolnitrin biosynthetic genes in this bacterium. HPLC confirmed the production of phenazine, 2-hydroxy phenazine and pyrrolnitrin antibiotics (data not shown). PA23 produced three organic volatiles, nonanal, benzothiozole, 2-ethyl, and 1-hexanol responsible for inhibiting mycelial growth, reducing ascospore and sclerotia germination (Fernando et al 2005). Higher activity of chitinase and β-1,3 glucanase was observed, when the bacterium was challenge inoculated with the pathogen on canola (Fig 3 & 4). Western blot analysis confirmed the induction of a 34 kDa chitinase protein (Fernando et al 2007). Transposon mutant PA23-314, which has a Tn5 insertion within the *gacS* gene, lacked antifungal activity in lab and greenhouse assays (Fig 6). Complementation with the wild-type *gacS* allele *in trans* restored antifungal activity, phenazine production, exoprotease, and *N*-acylhomoserine lactone to wild-type levels (Fig 2, Tables 1). Another mutant PA23-63, a phenazine non-producer, mutated at the *phzE* gene of the phenazine biosynthetic operon, still produced pyrrolnitrin and exhibited antifungal activity suggesting that pyrrolnitrin could play a vital role in the antifungal activity of the bacterium (Fig 5,6). Strain PA23 is capable of forming a biofilm that is resistant to lethal antibiotic challenge (Table 2), a property which should promote bacterial sustainability in the environment. In conclusion, strain PA23,a bacterium with several antifungal weapons in its armoury, and the ability to form antibiotic-resistant biofilms is an excellent biological control agent for management of stem rot of canola caused by *S, sclerotiorum.*



a b

Figure 1: PCR analysis for presence of antibiotic biosynthetic genes for phenazine biosynthetic genes. (1a) Lane 1; ladder, lane 2; PA23, lane 3; strain 2-79 (positive control), lane 4; negative con. and pyrrolnitrin (1b) Lane 1; DNA ladder; Lane 2; PA23, Lane 3; Pf-5 (positive control) – note done with 3 PCR primers.

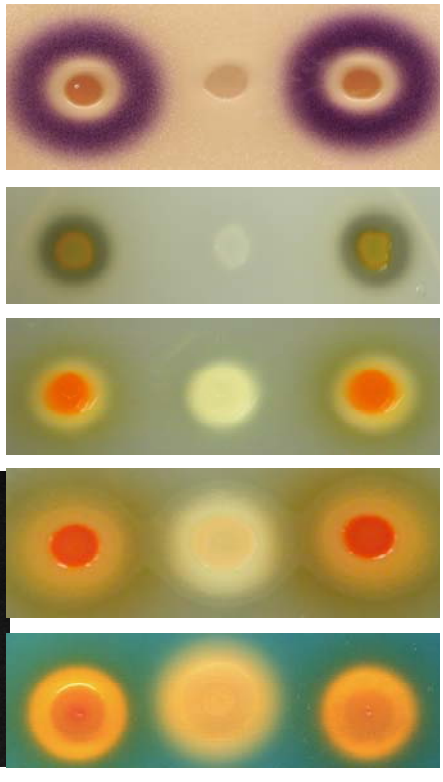


Figure 2: Extracellular metabolites believed responsible for biocontrol in *P. chlororaphis* PA23. Lane 1. PA23 WT; Lane 2. PA23-314 *gacS* mutant; Lane 3. PA23-314 (*gacS* complemented). From top to bottom. 1. Homoserine lactone (HSL) production; 2. Extracellular protease; 3. Lipase hydrolysis 24 h; 4. 72 h and 5. siderophore production. Except for siderophores, there is reduced activity in PA23-314.