Effect of volatile compounds produced by bacterial biocontrol agent *Pseudomonas chlororaphis* strain PA23 and its transposon mutants against *Sclerotinia sclerotiorum*

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Abstract

Pseudomonas chlororaphis strain PA23 has been found to produce three main volatile compounds, (nonanal, benzothiazole, 2-ethyl-1-hexanol), which have inhibited mycelial, sclerotial and ascospore germination of *Sclerotinia sclerotiorum*. This study was initiated to understand whether phenazine biosynthetic genes or specific regulatory genes would have a negative or positive effect on the production of these compounds. *Tn* mutants of PA23 including PA23-63 (*phzE*-deficient), PA23-754 (*phzC*-deficient), PA23-314 (*gacS*-deficient), PA23-314 (*gacS*) (*gacS* complemented strain), PA23-443 (*ptrA*; transcriptional regulatory mutant) and PA23-443 (*ptrA* complemented strain) were tested for production of volatile compounds and their ability to inhibit the mycelial growth, sclerotial germination and ascospore germination of *S. sclerotiorum*. PA23, PA23-314, PA23-314 (*gacS*), PA23-63, PA23-754 and PA23-443 (*ptrA*) showed a significant reduction (P=0.05) of mycelial growth and sclerotial germination of *S. sclerotiorum* compared to PA23-314, PA23-443 and control. Strain PA23-754 (*phzC* mutant) exhibited the best inhibition of mycelial growth and sclerotial germination, 25.56% and 98.73%, respectively, followed by PA23-63 (*phzE* mutant), 13.33% and 84.84%. Except for PA23-314, all other strains were able to inhibit ascospore germination completely after 48 h. The results highlight the fact that the *gacS* regulatory gene plays a major role in PA23 antibiotic production. Furthermore, the phenazine-deficient mutants PA23-63 and PA23-754, showed enhanced antibiosis, which has been previously observed for production of organic non-volatile compounds. Volatiles produced by each mutant were collected for 120 h in a nutrient rich medium (Tryptic Soy Broth) and in a minimal medium (M9) to investigate whether the production of volatiles is medium dependant. All strains produced the compounds nonanal, benzothiazole, 2-ethyl-1-hexanol regardless of the medium used. We hypothesize that the relative intensity of these compounds may have an effect on the level and rate of inhibition. Our findings do not resolve the exact mechanism underlying regulation of organic volatile production in PA23; consequently, additional studies are underway to elucidate their control.

Key words: *Pseudomonas chlororaphis* (PA23), *Sclerotinia sclerotiorum*, *Tn* mutants, antimicrobial organic volatiles, GC, MS

Introduction

Pseudomonas chlororaphis strain PA23, originally isolated from the root tips of soybean plants, had been found to be successful in biocontrol of *Sclerotinia sclerotiorum* (Lib.) de Bary both in greenhouse and field conditions (Fernando et al, 2007: Savchuk and Fernando 2004; Zhang 2004). The biocontrol activity is mainly due to the production of non-volatile organic compounds like phenazine-1-carboxylic acid (PCA), 2-hydroxyphenazine and pyrrolnitrin (Zhang et al, 2006) and several additional metabolites including protease, lipase, hydrogen cyanide and siderophores (Poritsanos et al, 2007). In addition, this bacterium has been found to produce some volatile organic compounds, like nonanal, benzothiazole and 2-ethyl–1-hexanol, which showed 100 per cent inhibition of mycelial and sclerotial germination of *S. sclerotiorum* (Fernando et al, 2005). Using transposon mutagenesis, six mutants of PA23 have been isolated (Poritsanos et al, 2006). PA23-314 (mutation in *gacS*) and PA23-443 (mutation in LysR-type transcriptional regulatory gene *ptrA*) are no longer capable of inhibiting fungal growth and are deficient in the production of non-volatile antibiotics and other putative antifungal metabolites including HCN (Poritsanos et al, 2006). As expected, addition of the wild-type gene *in trans* restored the biocontrol ability of PA23-314 (*gacS*) (PA23-314 complemented strain) and PA23-443 (*ptrA*) (PA23-443 complemented strain). For mutants PA23-63 (*phzE*-deficient) and PA23-754 (*phzC*-deficient) the ability to inhibit fungal growth of *S. sclerotiorum* remained unchanged. In this study we investigated the impact of the aforementioned mutations on production of volatile compounds, which are inhibitory to mycelial, sclerotial, and ascospore germination of *S. sclerotiorum.* These studies are important for understanding how volatile organic antimicrobial compounds are regulated in PA23.

Materials and Methods

Effect of bacterial volatiles on mycelial growth of S. sclerotiorum (divided plate method): Bacteria were streaked on to one half of the divided plate containing tryptic soy agar (TSA) amended with appropriate antibiotics. The plate was wrapped with parafilm to trap the volatiles produced by bacteria and incubated for 5 days at 28°C. A 5 mm mycelial plug of S.

sclerotiorum was placed on the other half of the divided plate containing PDA and the plates were resealed. Radial mycelial growth was measured every 24 h post-inoculation for 5 days. Ten replicates were analyzed for each treatment and the experiment was repeated twice.

Effect of bacterial volatiles on sclerotial germination of S. sclerotiorum: Instead of mycelia, sclerotia were placed on the other half of the PDA plate. Ten replicates were analyzed for each treatment and the experiment was repeated twice.

Effect of bacterial volatiles on ascospore germination of S. sclerotiorum: A 20 µL-aliquot of ascospore suspension (5×10^4 spores/mL in 0.1 M phosphate buffer pH 7.0) was placed on a cavity slide. The slide was then placed inside the bottom dish of a sterile petri plate**.** Another bottom dish containing 24 h old bacterial culture on TSA was inverted on the dish containing the cavity slide and the two dishes were sealed together using parafilm. After 24 and 48 h of incubation at room temperature, the slides were observed for spore germination under a microscope. Four replicates of each bacterial strain were analyzed and the experiment was repeated once.

Collection and identification of volatile organic compounds: Headspace volatiles produced by each bacterium were collected using a setup described by Fernando et al, 2005. Volatiles in the volatile trap were extracted into glass vials with 500 µL of methylene chloride and analysed through gas chromatography and mass spectrometry.

Results and Discussion

Mutants PA23-314 (*gacS*), PA23-63, PA23-754, PA23-443 (*ptrA*) showed a significant (P=0.05) inhibition in both mycelial growth and sclerotial germination of *S. sclerotiorum* compared to control. PA23-754 had the best inhibition of 25.56% and 98.73% in mycelial growth and sclerotial germination respectively (Fig. 1). Mutants PA23-314 and PA23-443 did not show any inhibitory effect. These results are comparable with studies done with radial diffusion assays using these mutants (Poritsanos, 2005).

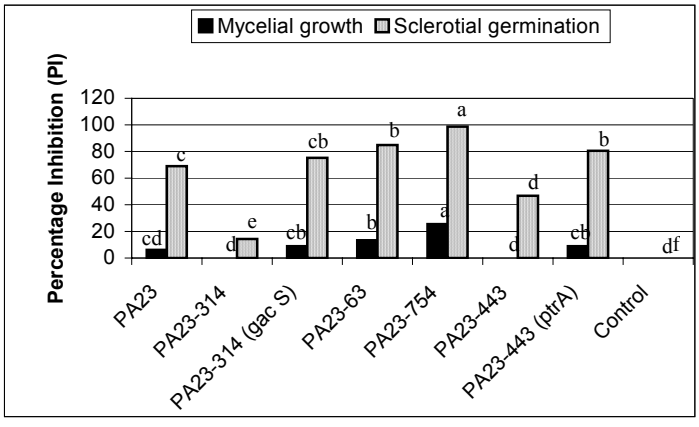

Fig. 1. Inhibition of mycelial growth and sclerotial germination on TSA/PDA.

The chromatograms obtained for PA23 and PA23-314 revealed that both strains produce the same three organic volatile compounds, nonanal, benzothiazole, 2-ethyl-1-hexanol regardless of the medium used (Fig. 2). Similar findings were obtained for PA23-314 (*gacS*), PA23-63, PA23-754, PA23-443 and PA23-443 *(ptrA)* (data not shown). Therefore this study suggests that the variation in inhibition observed is not dependent upon media or production of different volatile compounds. Differences in the quantity of one or more volatile, which was not detectable by the method used, may have been responsible for the variations in antifungal activity. According to Poritsanos (2005), PA23-314 and PA23-443 do not produce HCN, while the other strains do. Therefore another reason for this discrepancy may be the lack of production of HCN, which is an inorganic volatile. Our findings do not resolve the exact mechanism underlying regulation of organic volatile production in PA23; consequently, additional studies are underway to elucidate their control.

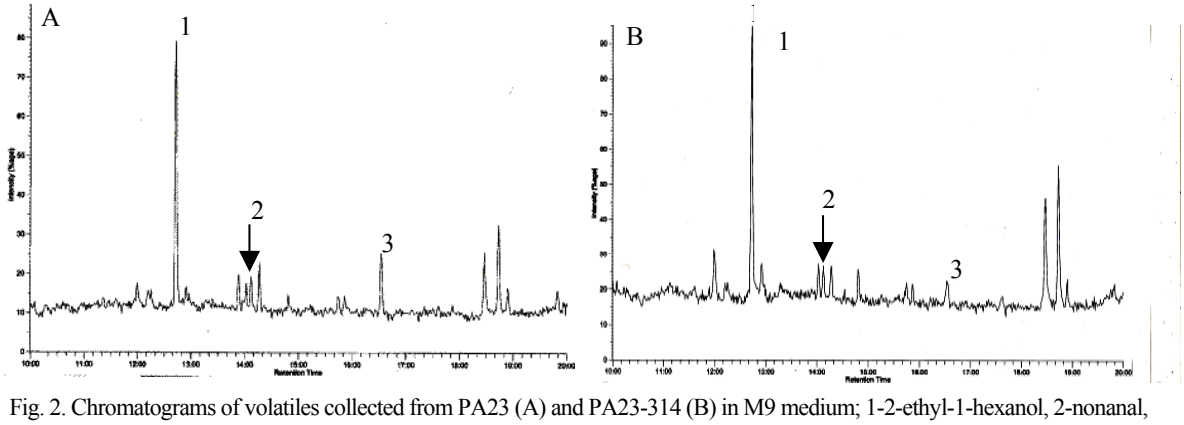

Fig. 2. Chromatograms of volatiles collected from PA23 (A) and PA23-314 (B) in M9 medium; 1-2-ethyl-1-hexanol, 2-nonanal, 3-benzothiazole.

Except for strain PA23-314, all other mutants completely inhibited (100%) ascospore germination of *S. sclerotiorum*. Strain PA23-314 showed 28.25% ascospore inhibition, which were significantly different (P=0.05) from other treatments and the control.

Effect of bacterial organic volatiles is now being intensively studied in terms of growth promotion and induced systemic resistance (ISR) in *Arabidopsis thaliana* (Ryu et al, 2003, 2004). In addition the antimicrobial nature of bacterial organic volatiles of *Pseudomonas* spp. and *Bacillus* spp. has been demonstrated in several studies. (Fernando et al, 2005; Fernando & Linderman, 1994; Fiddaman & Rossall, 1993). Few similar studies as of current study are reported where the volatiles produced by some growth and ISR promoting *Bacillus* spp. have been identified through gas chromatography (Ryu et al, 2004) and 3-hydroxy-2-butanone and 2,3-butanediol have been the main volatiles with other compounds such as decane, undecane, decanal, dodecane and pyrazine derivatives, which are also reported to be produced by PA23 (Fernando et al, 2005). Knockout mutants of 2,3-butanediol synthesis has blocked the production and growth promotion in *A. thaliana* (Ryu et al, 2003). Fiddaman and Rossall, (1994) found that volatile activity is enhanced with the addition of D-glucose. Also, Fernando et al, (2005) reported increased volatile production in TSA. Our study shows that the types of volatiles produced are not dependant on nutrient availability of the medium. However the M9 minimal medium used in the current study consisted of 2% D-glucose, which might have contributed to the increased volatile production similar to TSA.

References

Fernando W.G., Ramarathnam R., Krishnamoorthy A.S. and Savchuk S.C. (2005). Identification and use of potential bacterial organic antifungal volatiles in biocontol. Soil Biology & Biochemistry 37: 955-964.

Fernando, W.G.D. and Linderman, R. (1994). Inhibition of *Phytophthora vignae* and root rot of cowpea by soil bacteria. Biological Agriculture and Horticulture 12:1-14.

Fernando, W.G.D., Zhang, Y**.,** Nakkeeran, S., and Savchuk, S. (2007). Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on canola petals. Crop Protection (accepted and *in press*) – available on-line June 5, 2006.

Fiddaman, P.J. and Rossall, S. (1993). The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology 74:119-126.

Fiddaman, P.J. and Rossall, S. (1994). Effect of substrate on the production of antifungal volatiles from bacillus subtilis. Journal of applied Bacteriology 76(4):395-405.

Poritsanos, N. (2005). Molecular mechanisms involved in secondary metabolite production and biocontrol of *Pseudomonas chlororaphis* PA23. M.Sc. Thesis. Dept. Microbiology. University of Manitoba. Winnipeg, Manitoba.

Poritsanos, N., Selin, C., Fernando, W.G.D, Nakkeeran, S., and de Kievit, T.R. (2007). A GacS deficiency does not affect *Pseudomonas chlororaphis* PA23 fitness when growing on canola, in aged batch culture or as a biofilm. Canadian Journal of Microbiology 52:1177-1188

Rye, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Kloepper J.W. and Pare P.W. (2004). Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiology 134(3): 1017-1026.

Rye, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Wei, H.X., Pare P.W. and Kloepper J.W. (2003). Bacterial volatiles promote growth in *Arabidopsis*. Proceedings of the National Academy of Sciences 100:4927-4932.

Savchuk S.C. and Fernando W.G.D. (2004). Effect of timing of application and population dynamics on the degree of biological control of *Sclerotinia sclerotiorum* by bacterial antagonists. FEMS Microbiology Ecology 49: 379-388.

Zhang, Y. (2004). Biocontrol of *sclerotinia* stem rot of canola by bacterial antagonists and study of biocontrol mechanisms involved. M.Sc.Thesis. Dept. Plant Science