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Hydrogen cyanide, which contributes to *Pseudomonas chlororaphis* strain PA23 biocontrol, is upregulated in the presence of glycine

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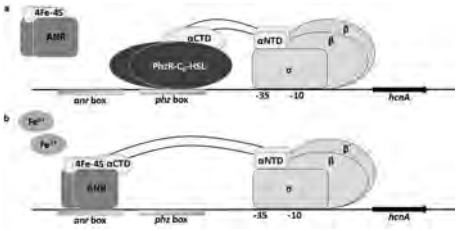
HIGHLIGHTS

- HCN contributes to *Pseudomonas chlororaphis* PA23 biocontrol in vitro and planta.
- Glycine increases production of HCN, phenazine and pyrrolnitrin.
- Glycine upregulates the PhzRI quorum-sensing system.
- In quorum-sensing deficient strains, FeCl3 increases *hcn* gene expression.

GRAPHICAL ABSTRACT



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ABSTRACT

Pseudomonas chlororaphis strain PA23 is a biocontrol agent capable of suppressing the pathogenic fungus *Sclerotinia sclerotiorum*. This bacterium secretes the antibiotics pyrrolnitrin (PRN) and phenazine (PHZ), together with degradative enzymes and siderophores. Strain PA23 also produces hydrogen cyanide (HCN); however, the role of this compound in PA23 antifungal (AF) activity remains unknown. The aim of the current study was to characterize an *hcn* mutant and determine whether HCN contributes to biocontrol. Analysis of an *hcn* mutant revealed decreased AF activity both in vitro and in a newly established model of *S. sclerotiorum* root-rot infection in lettuce. When glycine (20 mM) and ferric chloride (100 μM) were included as media amendments, elevated AF activity was observed. Moreover glycine, but not $FeCl_3$, increased HCN production and *hcnA-lacZ* transcriptional activity in the wild type. As the metabolic precursor for HCN biosynthesis, glycine is expected to enhance HCN production at the post-transcriptional level. We postulated that glycine might be indirectly affecting HCN transcription through the PhzRI quorum-sensing (QS) system. Analysis of *phzR-lacZ* and *phzI-lacZ* activity together with autoinducer levels revealed upregulation by glycine. This compound also increased expression of the QS-controlled *prnA* and *phzA* and their endproducts but, for reasons unknown, $FeCl_3$ exerted a repressive effect. In a QS-deficient background the opposite occurred; $FeCl_3$ increased *hcnA-lacZ* expression whereas glycine had no effect. HCN is under control of QS and the global regulator ANR. We present a model depicting how these regulators, in combination with media amendments, impact PA23 *hcn* expression.

1. Introduction

Fluorescent pseudomonads functioning as biocontrol agents produce a myriad of secondary metabolites; some of these compounds are essential for pathogen suppression while others are expendable (Haas and Keel, 2003). Adding to this complexity is the fact that conditions favouring production of one metabolite

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may be vastly different from those that stimulate expression of another. Accordingly, it is essential to dissect out key compounds involved in pathogen suppression as well as conditions promoting maximal expression. *Pseudomonas chlororaphis* PA23 is an effective biocontrol strain that inhibits the growth of ascomycetes, such as *Sclerotinia sclerotiorum* and *Leptosphaeria maculans* (Savchuk and Fernando, 2004; Poritsanos et al., 2006; Ramarathnam et al., 2011). Among the arsenal of weaponry released by PA23, the antibiotic pyrrolnitrin (PRN) is essential for controlling both of these fungal pathogens (Selin et al., 2010). While phenazines (PHZ) play only a modest role in antifungal (AF) activity, they do contribute to the biofilm-forming ability of this bacterium (Selin et al., 2010).

It has been known for over a decade that PA23 produces hydrogen cyanide (HCN; Poritsanos et al., 2006) and it was recently discovered that production of HCN together with PRN enables PA23 to resist predation by the bacterivorous nematode *Caenorhabditis elegans* (Nandi et al., 2015). HCN in particular was shown to have strong nematicidal effects; thus, we expect this compound to play an important role in facilitating environmental persistence through enabling PA23 to escape predation. At present, the contribution of HCN to PA23 fungal antagonism is unknown.

Previous reports describing the role of HCN in plant-pathogenic fungal suppression have yielded contradictory findings (Flaishman et al., 1996; Nagarajkumar et al., 2004; Pal et al., 2000; Rezzonico et al., 2007; Voisard et al., 1989). Such discrepancies likely result from differences in the experimental parameters employed. Nonetheless, a definitive role for HCN in fungal inhibition has been established for a number of fluorescent pseudomonads (Voisard et al., 1989; Laville et al., 1998; Blumer and Haas, 2000; Michelsen and Stougaard, 2012). HCN exerts its toxic effects through inhibiting cytochrome c oxidase, the final component of the aerobic respiratory chain, as well as other essential metalloenzymes (Knowles, 1976; Solomonson, 1981).

A membrane-bound HCN synthase is responsible for catalysis of glycine into HCN and carbon dioxide (Castric, 1977). A number of biotic and abiotic factors affect expression of the *hcnABC* operon encoding the HCN synthase (Pessi and Haas, 2000; Laville et al., 1998; Castric, 1983, 1994). In *P. fluorescens* and *P. aeruginosa,* for example, HCN expression is under control of ANR (anaerobic regulator of arginine deiminase and nitrate reductase), which is most active under low oxygen conditions (Laville et al., 1998; Zimmermann et al., 1991). ANR binds to a conserved sequence in the promoter of target genes, called the *anr* box, facilitating transcription initiation (Pessi and Haas, 2000). The Gac two-component signal transduction system, which was named in part because of its control over HCN synthesis (Global activator of antibiotic and cyanide synthesis), is another governing element (Lapouge et al., 2008). GacS and GacA work together with a second system, called Rsm, to modulate expression of secondary metabolites and extracellular enzymes during the transition from the logarithmic to the stationary phase of growth (Heeb and Haas, 2001; Bejerano-Sagie and Xavier, 2007). For pseudomonads that employ acyl-homoserine-lactone (AHL) based quorum sensing (QS) as part of their lifestyle, production of secondary metabolites, including HCN, is typically subject to QS control (Fuqua and Greenberg, 1998; Haas and Keel, 2003). At a threshold level, AHLs complex with a cognate LuxR-type protein, which is then able regulate target genes through binding to an upstream *lux* box sequence (Fuqua and Greenberg, 1998). In *P. aeruginosa,* which utilizes two QS systems (Las and Rhl), cooperative binding of LasR, RhlR and ANR is required for maximal *hcnABC* transcription (Pessi and Haas, 2000). For pseudomonads that do not have AHL-based QS systems, ANR and Gac are solely responsible for HCN expression, acting at the transcriptional and translational levels, respectively (Blumer et al., 1999; Blumer and Haas, 2000; Michelsen and Stougaard, 2012).

In terms of abiotic factors that impact HCN production, oxygen and iron exert their effects primarily through ANR (Laville et al., 1998; Blumer and Haas, 2000). Blumer and Haas (2000) proposed that the active form of ANR is a dimer containing two [4Fe-4S] clusters. In the presence of oxygen, the [Fe-S] cluster is believed to convert to [2Fe-2S] rendering ANR non-functional, similar to oxygen-mediated inactivation of FNR (Beinert and Kiley, 1999; Blumer and Haas, 2000). Despite the initial proposal that oxygen impedes ANR function, recent reports suggest a regulatory role for ANR under atmospheric oxygen conditions (Ibrahim et al., 2015; Nandi et al., 2017). Not surprisingly, iron sufficiency favors [4Fe-4S] assembly and ultimately HCN production (Castric, 1975, 1983). Studies have shown that media composition profoundly affects HCN expression; for example glycine, the metabolic precursor for HCN biosynthesis, stimulates production of this compound by *P. aeruginosa* (Castric, 1977). Moreover, strains propagated on nitrogen-rich media (King's B; Lysogeny broth) exhibit elevated HCN production compared to growth on the more carbohydrate-rich potato dextrose agar (PDA) (Paulin et al., 2009; Michelsen and Stougaard, 2012).

The focus of the current study was to reveal whether HCN contributes to PA23 biocontrol both in vitro and in a newly established model of Sclerotinia root-rot infection in lettuce. Moreover, we were interested in determining the impact of glycine and $FeCl_3$ addition on HCN as well as other metabolites produced by PA23.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains, plasmids and primers used in this study are listed in Table 1. *Escherichia coli* was cultured at 37 °C on lysogeny broth (LB, Lennox formulation) (Difco Laboratories, Detroit, MI, USA). *Pseudomonas* strains were cultured on LB or M9 minimal salts medium supplemented with 0.2% glucose (M9-Glc) at 28 °C. Antibiotics were used at the following concentrations: ampicillin (Amp; 100 μg/mL), gentamicin (Gm; 15 μg/mL), tetracycline (Tc; 15 μg/mL) for *E. coli,* and piperacillin (Pip; 40 μg/mL), Gm (20 μg/mL), Tc (15 μg/mL) and rifampicin (Rif; 50 μg/mL) for *P. chlororaphis* PA23. All antibiotics were obtained from Research Products International Corp. (Mt. Prospect, IL, USA).

2.2. Growth rate analysis

The PA23 wild type was inoculated at a starting OD_{600} of 0.01 in M9-Glc, M9-Glc supplemented with 100 μM ferric chloride ($FeCl_3$), 20 mM glycine, or both. OD_{600} readings were taken at 10 h, 12 h and 15 h, followed by readings every 2 h thereafter for a total of 33 h. Samples were analyzed in triplicate and the experiment was repeated twice.

2.3. Generation of an hcnA-lacZ transcriptional fusion

To monitor PA23 *hcn* gene expression, an *hcnA-lacZ* transcriptional fusion was created using primers *hcnA*-FOR and *hcnA*-REV (Table 1). The *hcnA* promoter region was PCR amplified and a 963-bp amplicon was cloned into pCR2.1, generating pCR2.1-*hcnA*. The pCR2.1-*hcnA* construct was digested with *Hin*dIII and *Xba*I and the insert was subcloned into the same sites of pLP170, creating *hcnA-lacZ*. Standard techniques for cloning, purification, electrophoresis and other manipulations of nucleic acid were performed according to Sambrook et al. (1989).

Table 1
Bacterial strains, plasmids and primers used in this study.

Strain, plasmid, or primer	Relevant genotype, phenotype or sequence	Reference or source
Strains		
P. chlororaphis		
PA23	PRN$^+$ PHZ$^+$ Rif^R; wild-type (soybean root tip isolate)	Savchuk and Fernando (2004)
PA23*hcn*	PA23 with the pKNOCK-Tc vector inserted into the *hcn* gene	Nandi et al. (2015)
PA23-6863	PA23 carrying pME6863; AHL deficient	Selin et al. (2012)
PA23*phzR*	PA23 with Gm^R marker inserted into *phzR* gene	Selin et al. (2012)
P. aeruginosa		
QSC105	Strain carrying pEAL01 (*lasB-lacZ* transcriptional fusion), Carb^R	Ling et al. (2009)
E. coli		
DH5α	*supE44* Δ*U169* (*Φ80lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco
Chromobacterium violaceum CVO26	Autoinducer synthase (*cviI*) mutant from *C. violaceum* ATCC 31,532 autoinducer biosensor	Latifi et al. (1996)
Plasmids		
pME6863	pME6000 carrying the *aiiA* gene from *Bacillus* sp.A24 under the constitutive P_{lac} promoter	Reimmann et al. (2002)
pCR2.1	TA cloning vector, Amp^R	Invitrogen
pCR2.1-*hcnA*	963 bp fragment containing the *hcnA* promoter in pCR2.1	This study
pLP170	*lacZ* transcriptional fusion vector	Preston et al. (1997)
hcnA-lacZ	963 bp fragment containing the *hcnA* promoter in pLP170	This study
prnA-lacZ	*prnA* promoter in pLP170	Selin et al. (2010)
phzA-lacZ	*phzA* promoter in pLP170	Selin et al. (2010)
phzI-lacZ	674 bp fragment containing the *phzI* promoter in pLP170	Selin et al. (2012)
phzR-lacZ	1.1 kb fragment containing the *phzR* promoter in pLP170	Selin et al. (2012)
Primers		
hcnA-FOR	5′-atgctgttgcaggcggtg-3′	This study
hcnA-REV	5′-atgcggttgacctcggtctg-3′	This study

2.4. Analysis of PA23 transcriptional fusions

The activity of an *hcnA-lacZ* transcriptional fusion was determined in PA23, the *phzR* mutant and the AHL-deficient PA23 (pME6863). Strains carrying the *lacZ*-fusion plasmids were grown at 28 °C for 24 h in M9-Glc alone or supplemented with $FeCl_3$ (100 μM), glycine (20 mM), or both prior to β-galactosidase analysis (Miller, 1972). The activity of *phzI*-, *phzR*-, *phzA*- and *prnA-lacZ* transcriptional fusions was also measured in PA23 under the same conditions. Samples were analyzed in triplicate and the experiment was repeated three times.

2.5. In vitro antifungal assay

To determine the role of HCN in PA23 suppression of *S. sclerotiorum*, radial diffusion assays were performed. A 5-μL aliquot of overnight cultures of wild-type PA23 and the *hcn* mutant were spotted onto one-fifth strength potato dextrose agar (1/5th PDA) alone or supplemented with 100 μM $FeCl_3$ and 20 mM glycine separately as well as in combination. An agar plug containing fungal mycelia was then placed in the centre of the plate, which was incubated for 3 days at 22 °C to allow for growth of *S. sclerotiorum*. Plates were parafilmed to avoid the escape of volatile HCN. Fungal inhibition was assessed by measuring the zone of clearing between the edge of the bacterial colony and the fungal growth front as described by Poritsanos et al. (2006). Five replicates were analyzed and the experiments were repeated three times.

2.6. Qualitative HCN analysis

Qualitative analysis of HCN production was performed using Cyantesmo paper (Machery-Nagel GmbH & Co., Germany), which turns blue in the presence of HCN. Three replicates were analyzed and the experiments were repeated twice.

2.7. Qualitative and quantitative autoinducer assays

Production of AHL molecules was assessed qualitatively by spotting 5 μL of an overnight culture onto LB agar plates seeded with the biosensor strain *Chromobacterium violaceum* CV026. This AHL-deficient bacterium is able to detect exogenous AHLs with carbon chain length ranging from C_4 to C_8, resulting in a purple halo (violacein pigment) surrounding the colonies (Latifi et al., 1996). The diameter of the purple zones was measured at 24 h. Five replicates were analyzed and experiments were repeated three times.

Total autoinducer production was monitored according to Ling et al. (2009) with the following modifications. PA23 was grown for 18 h at 28 °C in 30 mL M9-Glc, M9-Glc supplemented with 100 μM ferric chloride ($FeCl_3$), 20 mM glycine, or both. Cells were pelleted and cell-free supernatants were extracted twice with an equal volume (30 mL) of acidified ethyl acetate. The ethyl acetate fractions were pooled and concentrated to a final volume of 1 mL. For AHL quantification, 100 μL aliquots of each extract were tested using the biosensor strain *P. aeruginosa* QSC105 following the method of Selin et al. (2012). Samples were analyzed in triplicate and the experiments were repeated three times.

2.8. Quantitative analysis of PHZ

Production of PCA and 2-OH-PHZ was quantified according to the methods outlined by Chancey et al. (1999). Cultures (5 mL) were grown overnight at 28 °C in M9-Glc, M9-Glc supplemented with 100 μM ferric chloride ($FeCl_3$), 20 mM glycine, or both. Cell supernatants were collected and subjected to PHZ extraction. Spectrophotometric quantification was performed at 367 nm and 490 nm for PCA and 2-OH-PHZ, respectively (Maddula et al., 2008). Samples were tested in triplicate and the experiments were repeated twice.

2.9. Quantitative analysis of PRN

Production of PRN was quantified by HPLC according to the methods outlined by Selin et al. (2010). Briefly, 20 mL cultures of PA23 were grown for 5 days at 28 °C in M9-Glc, M9-Glc supplemented with 100 μM ferric chloride ($FeCl_3$), 20 mM glycine, or both. Cells were combined with an equal volume of ethyl acetate (20 mL) and 5 mL toluene (internal control). Following 30 min of

shaking at RT, the organic layer was removed and dried under air. Peaks corresponding to toluene and PRN were analyzed by UV absorption at 225 nm using a Varian 335 diode array detector. PRN peaks were detected at 4.8 min. Samples were analyzed in triplicate.

2.10. Greenhouse assay

The PA23 wild type and an *hcn* mutant were assessed for their ability to control lettuce drop disease under greenhouse conditions. Lettuce drop is typically caused by two species of *Sclerotinia*, namely *S. sclerotiorum* and *S. minor* (Koike and Davis, 2007). *S. sclerotiorum* was used as the fungal pathogen in the following experiment. Lettuce seeds (*Lactuca sativa* L. cv. Grand Rapids) were germinated in a seedling flat (21″ × 11″) filled with sterile potting soil kept in a growth chamber (24/16 °C, 16-h photoperiod). Approximately three weeks after the seeds were sown, plants were treated with bacteria as follows. Three hundred ml cultures of PA23 were grown in LB, LB plus 100 μM $FeCl_3$, LB plus 20 mM glycine, or LB supplemented with both $FeCl_3$ and glycine. Cultures of the *hcn* mutant were propagated in LB. Bacteria were allowed to grow for 16 h at 28 °C with shaking, after which cells were pelleted, washed once with phosphate-buffered saline and resuspended in sterile LB to a final concentration of 2.0×10^8 CFU mL^{-1}. Lettuce roots were soaked in the bacterial suspension for 45 min, before plants were transferred to pots (5″ × 5″) containing sterile soil (70–80% Canadian sphagnum peat moss; 20–30% dolomite limestone and perlite). After transfer, the soil was irrigated with 100 mL of bacterial culture diluted 1:4 with LB (final concentration 0.5×10^7 CFU mL^{-1}). Control plants were treated with sterile LB broth. Plants were incubated in the growth chamber at 73% relative humidity for 2 weeks before challenge with *S. sclerotiorum* mycelia. *S. sclerotiorum* slurries were prepared by slicing agar plugs harboring fungal mycelia into small sections that were then transferred to a petri dish containing 25 mL of potato dextrose broth. After 5 days growth at 22 °C, broth was decanted from the hyphae before drying for 4 h in a biosafety cabinet. Two g aliquots of mycelia were ground using a mortar and pestle and then added to 30 mL of distilled water. Homogenization of the mycelial slurry was accomplished by passing multiple times through a 10 ml syringe. A 10-ml suspension of mycelial slurry was applied to the soil surrounding the base of the stem of each plant. Plants were incubated in the growth chamber for an additional two weeks after which symptom development on the crown, stem and leaves was scored (Subbarao, 1998; Rabeendran et al., 2006; Koike and Davis, 2007; Chen et al., 2016). Six plants were used for each treatment and the plant study was repeated two times.

2.11. Statistical analysis

All statistical analysis was performed using an unpaired Student's *t* test in Microsoft Excel.

3. Results and discussion

3.1. HCN contributes to PA23 AF activity in vitro

To assess the role of HCN in PA23 fungal suppression, an *hcn* mutant was monitored for its AF activity in vitro. Radial diffusion assays on 1/5th PDA revealed a significant ($P < 0.0001$) decrease in the zone of inhibition surrounding the *hcn* mutant (1.2 ± 0.4 mm) compared to that of the wild type (3.8 ± 0.3 mm). No difference in AF activity was observed when strains were plated on full-strength PDA (PA23: 1.8 ± 0.3 mm versus PA23*hcn*: 1.5 ± 0.4 mm). Thus, HCN contributes to fungal antagonism under some but not all conditions. As media composition has a profound effect on expression of biocontrol compounds, including HCN, variable degrees of pathogen inhibition are not surprising. Michelsen and Stougaard (2012) reported similar findings for *P. fluorescens* In5 wherein HCN was found to contribute to suppression of *Rhizoctonia solani* and *Pythium aphanidermatum* when propagated on nitrogen-rich LB and KB. Conversely, cultivation on carbohydrate-rich PDA resulted in a lack of hyphal inhibition by either strain. We have found that on LB and KB, *S. sclerotiorum* grows poorly with mycelia migrating 3–4 mm from the fungal plug at which point growth ceases and sclerotial bodies are formed. Consequently, these media were not employed in the current study. Contradictory conclusions regarding the contribution of HCN to the suppression of plant-pathogenic fungi are expected to result, at least in part, from differences in the experimental conditions employed

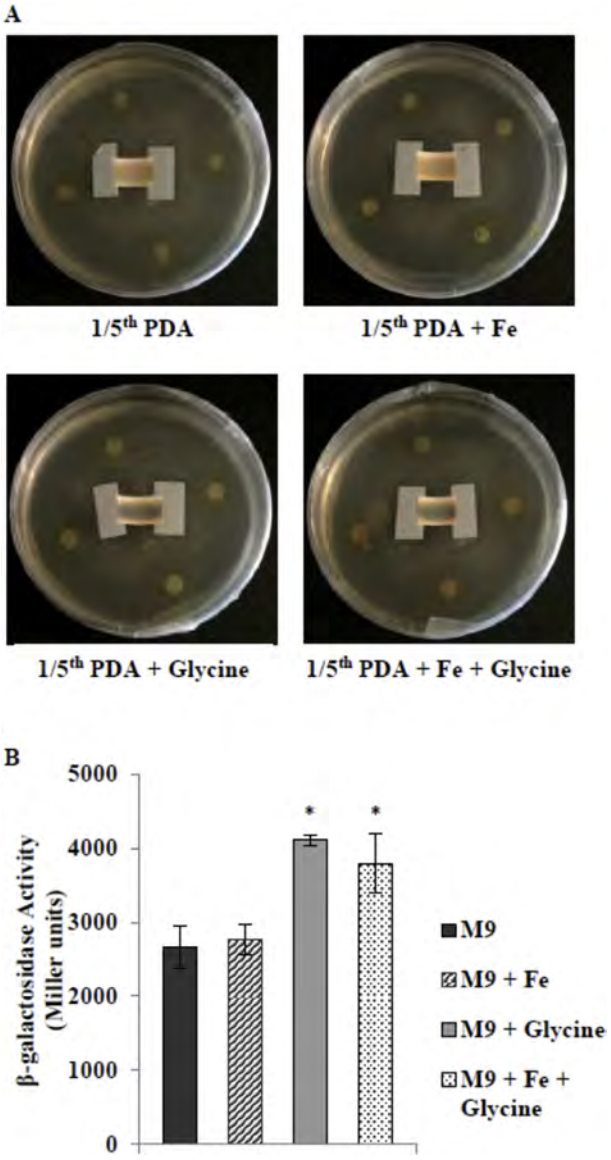

Fig. 1. HCN production and *hcnA* gene activity in *Pseudomonas chlororaphis* strain PA23. (A) Qualitative assessment of HCN production using cyantesmo paper. Cultures were spotted onto 1/5th PDA alone, 1/5th PDA supplemented with $FeCl_3$ (100 μM), glycine (20 mM), or both. Pictures were taken at 48 h. (B) PA23 harboring an *hcnA-lacZ* fusion were grown in the presence and absence of media additions. Cells were assayed for β-galactosidase activity at 24 h. Each value represents the mean from three biological replicates ± standard error. Data points marked with an asterisk (*) are statistically significant ($P < 0.05$).

(Flaishman et al., 1996; Pal et al., 2000; Nagarajkumar et al., 2004; Rezzonico et al., 2007).

3.2. Fungal antagonism is elevated in the presence of $FeCl_3$ and glycine

$FeCl_3$ is able to elevate HCN production through its effect on ANR (Michelsen and Stougaard, 2012) and as the metabolic precursor for HCN synthesis, glycine amendment leads to enhanced production of this metabolite (Castric, 1977). We were interested, therefore, to determine the impact of these two compounds on PA23 AF activity. We observed significantly increased ($P < 0.001$) zones of fungal inhibition surrounding PA23 colonies on 1/5th PDA amended with $FeCl_3$ (5.1 ± 0.6 mm), glycine (5.3 ± 0.4 mm), or both (5.7 ± 0.7 mm) compared to PDA alone (3.8 ± 0.4 mm). Thus, the presence of either one or both of these compound increases the ability of PA23 to antagonize *S. sclerotiorum*.

3.3. Glycine increases HCN production in PA23

To determine whether glycine and $FeCl_3$ amendment impacts HCN, cyantesmo paper was used in qualitative assays. As indicated in Fig. 1A, elevated HCN production occurred when PA23 was cultured on glycine-supplemented media. These findings are consistent with earlier reports that in *P. aeruginosa,* glycine (20 mM) elevated HCN production fivefold (Castric, 1977). $FeCl_3$, on the other hand, had no effect (Fig. 1A). Analysis of an *hcnA-lacZ* transcriptional fusion revealed similar findings; gene expression was elevated 1.5-fold in the presence of glycine but not $FeCl_3$ (Fig. 1B). As glycine is the metabolic precursor for the HCN synthase, it is expected to enhance HCN production at the post-translational level rather than through increasing *hcnABC* transcription. These findings lead us to postulate that the effects of glycine are being mediated indirectly through transcriptional regulators of the *hcn* operon, the most obvious being the PhzRI QS system. Accordingly, we monitored *phzI* and *phzR* expression in PA23 grown in the presence and absence of glycine as well as $FeCl_3$. Similar to what was observed for *hcnA-lacZ* activity, both genes were significantly upregulated when glycine was added to the media, whereas $FeCl_3$ had no effect on gene expression (Fig. 2A). Qualitative analysis using CVO26-seeded agar revealed significantly higher AHL production by PA23 grown in the presence of glycine (Table 2). Similarly glycine amendment produced 1.6-fold higher AHL levels in quantitative assays (Fig. 2B); thus, our endproduct analysis supports glycine-mediated upregulation of *phzI* expression. Collectively, these findings suggest that the impact of glycine on PA23 HCN expression occurs indirectly through upregulation of the PhzRI QS system. In a study examining environmental conditions influencing phenazine-1-carboxamide (PCN) synthesis, glycine supplementation resulted in a 7-fold increase in PCN production by *P. chlororaphis* PCL1391 (van Rij et al., 2004). Conditions that increased PCN levels also increased AHL production, leading to the conclusion that the effects were being channelled through QS (van Rij et al., 2004), in keeping with the findings presented herein.

3.4. QS control of HCN production and relationship to media amendments

Analysis of an *hcnA-lacZ* transcriptional fusion in the PA23-6863 (AI deficient) and Δ*phzR* strains revealed decreased expression under all media conditions (Fig. 3), consistent with our earlier conclusion that HCN is under QS control (Nandi et al., 2015). In other studies, $FeCl_3$ has been reported to elevate HCN expression through its effects on ANR (Blumer and Haas, 2000; Michelsen and Stougaard, 2012). Fe is believed to promote cofactor assembly, which in turn enables ANR to bind to the *anr* box upstream of *hcnA*. While $FeCl_3$ modestly increased *hcn-lacZ* activity in a QS-deficient background, no change in expression was observed in the parental strain (Fig. 3). Glycine, on the other hand, did not elevate expression of this fusion in the QS-deficient background (Fig. 3). Putative *anr* and *phz* boxes have been previously identified within the *hcnA* promoter region (Fig. 4; Nandi et al., 2017). These regulatory sequences are in close proximity, with only 33 bp separating the two (Nandi et al., 2017). We hypothesize that when PhzR-C_6-HSL

Table 2
Quantification of phenazines (PHZ), pyrrolnitrin (PRN) and autoinducer molecules present in the cultures of PA23 grown in M9-glc (M9) alone, or supplemented with 100 μM $FeCl_3$ and 20 mM glycine, separately or in combination.

Media	PRN (μg/ml)[a]	PHZ (μg/ml)[a]	Autoinducer (mm)[b]
M9	2.72 (0.02)	43.57 (5.38)	0.67 (0.29)
M9 + $FeCl_3$	0.56 (0.08)[c]	19.33 (4.82)[d]	1.10 (0.28)[e]
M9 + glycine	3.94 (0.06)[d]	60.61 (2.04)[d]	2.0 (0.5)[f]
M9 + $FeCl_3$ + glycine	2.47 (0.36)[e]	36.47 (4.31)[e]	2.0 (0.5)[f]

[a] Mean (SD) from three triplicates.
[b] Mean (SD) of the zones of activity (mm) from six replicates.
[c] Significantly different from M9 ($P < 0.001$).
[d] Significantly different from M9 ($P < 0.01$).
[e] Not significantly different from M9.
[f] Significantly different from M9 ($P < 0.05$).

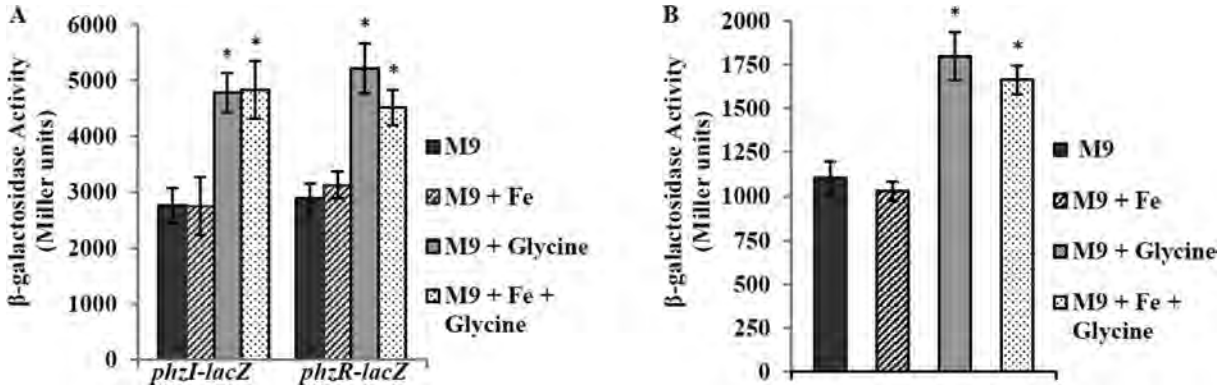

Fig. 2. The effect of $FeCl_3$ and glycine on *phzI* and *phzR* expression and AHL production. (A) PA23 harboring *phzI*- and *phzR-lacZ* fusions was grown in M9-glc alone, M9-glc supplemented with $FeCl_3$ (100 μM), glycine (20 mM), or both. Cells were assayed for β-galactosidase activity at 24 h. (B) PA23 cultures grown in the presence and absence of media additions were subjected to AHL extraction. AHLs were added to cultures of *P. aeruginosa* QSC105 (pEAL01), followed by β-galactosidase analysis at 18 h. Media additions were the same for both panels A and B. Each value represents the mean from three biological replicates ± standard error. Data points marked with an asterisk (*) are statistically significant within each group ($P < 0.05$).

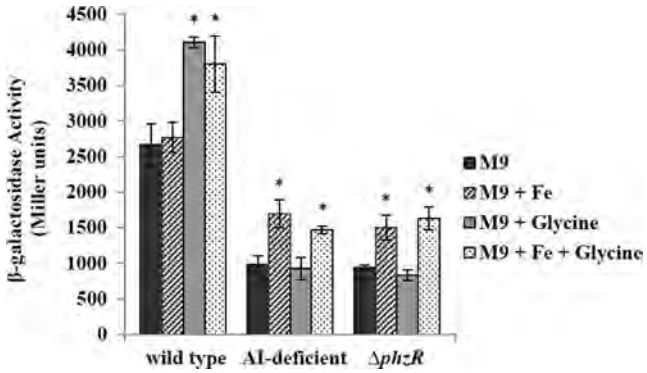

Fig. 3. The effect of quorum sensing, $FeCl_3$ and glycine addition on *hcnA-lacZ* activity. Cultures of PA23, the AI-deficient PA23 (pME6863) and Δ*phzR* strains harboring an *hcnA-lacZ* fusion were grown in M9-glc alone, M9-glc supplemented with $FeCl_3$ (100 μM), glycine (20 mM), or both. Cells were assayed for β-galactosidase activity at 24 h. Each value represents the mean from three biological replicates ± standard error. Data points marked with an asterisk (*) are statistically significant within each group ($P < 0.05$).

binds to the *hcn* promoter, it blocks access to the *anr* box; consequently, any effect of $FeCl_3$ on ANR is masked in a wild-type background. In the absence of QS, the *anr* box is available resulting in increased *hcn* promoter activity due to $FeCl_3$-mediated ANR assembly. In Fig. 4, a model depicting control of *hcn* expression by PhzR-C_6-HSL and ANR is presented.

3.5. Effect of media amendments on the regulation of PRN and PHZ

In PA23, both PHZ and PRN are under QS control (Selin et al., 2012); as such, we were interested in analyzing whether media additions affect expression of these antibiotics. Glycine was found to upregulate the *phzA*- and *prnA-lacZ* transcriptional fusions, whereas $FeCl_3$ downregulated expression of both genes (Fig. 5). Moreover our endproduct analysis closely mirrored transcriptional findings. Production of both antibiotics was elevated 1.4-fold in the presence of glycine, while $FeCl_3$ was found to decrease PHZ and PRN production by more than 2- and 4-fold, respectively (Table 2). In the presence of both amendments, antibiotic levels were similar to or below that of M9-Glc alone (Table 2), indicating that the repressive effect of $FeCl_3$ overrides any increases in antibiotic production associated with glycine. As described above, we observed an increase in vitro AF activity upon $FeCl_3$ supplementation, which is surprising considering that this compound decreases expression of the *hcn, phz* and *prn* genes and their encoded products. We speculate that $FeCl_3$ is affecting other yet-to-be identified traits associated with PA23 biocontrol.

At present, the molecular mechanisms underlying the repressive effect of $FeCl_3$ on *phz* and *prn* expression remain unclear. Analysis of PA23 growth under these different media conditions revealed that in the presence of $FeCl_3$, PA23 achieved a 1.5-fold higher OD_{600} compared to cells grown in M9-Glc with or without glycine supplementation (Supplemental Fig. 1). It is possible that in the presence of $FeCl_3$, cells undergo primary metabolism for an

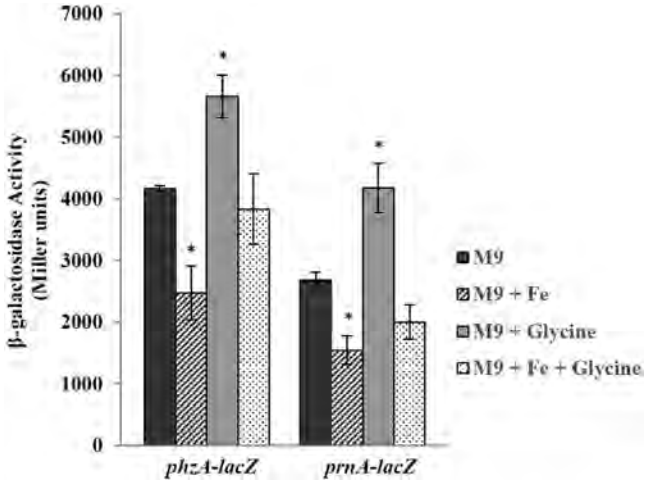

Fig. 5. The effect of $FeCl_3$ and glycine on *phzA* and *prnA* gene expression. PA23 cultures harboring *phzA*- and *prnA-lacZ* fusions were grown in M9-glc alone, M9-glc supplemented with $FeCl_3$ (100 μM), glycine (20 mM), or both. Cells were assayed for β-galactosidase activity at 24 h. Each value represents the mean from three biological replicates ± standard error. Data points marked with an asterisk (*) are statistically significant within each group ($P < 0.05$).

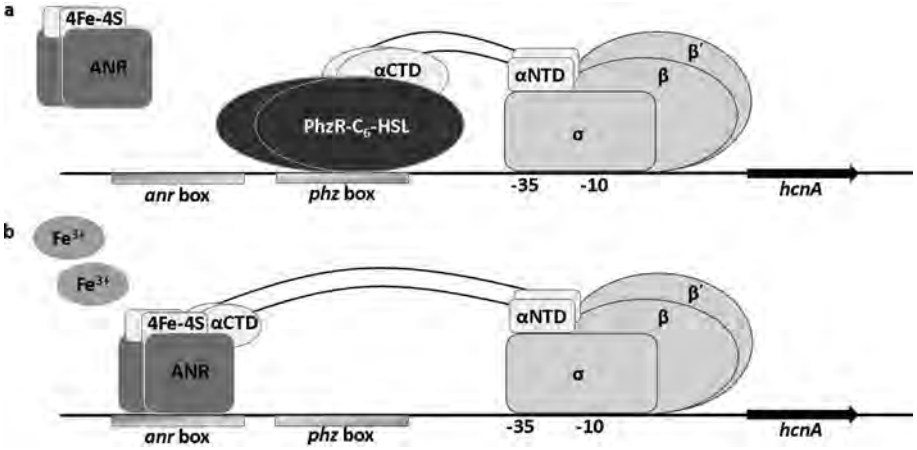

Fig. 4. Schematic model of promoter recognition by the transcriptional regulators PhzR-C_6-HSL and ANR through their interaction with the αCTD (C-terminal domain of the alpha subunit) and αNTD (N-terminal domain of the alpha subunit) of RNA polymerase. The αCTD and αNTD are joined by a linker. The *anr* and *phz* boxes found within the *hcnA* promoter region are in close proximity with a 33-bp separation. Panel (A) depicts that the PhzR-C_6-HSL complex is sufficient for activation of *hcnA* transcription and, when present, occludes binding of ANR to the *anr* box. Panel (B) shows the binding of ANR to the *anr* box in the QS-minus background leading to *hcnA* promoter activation. The presence of iron (Fe^{3+}) favors [4Fe-4 S] cofactor assembly and ANR binding to the *anr* box, leading to increased transcription. This model has been modified from that proposed by Pessi and Haas (2000).

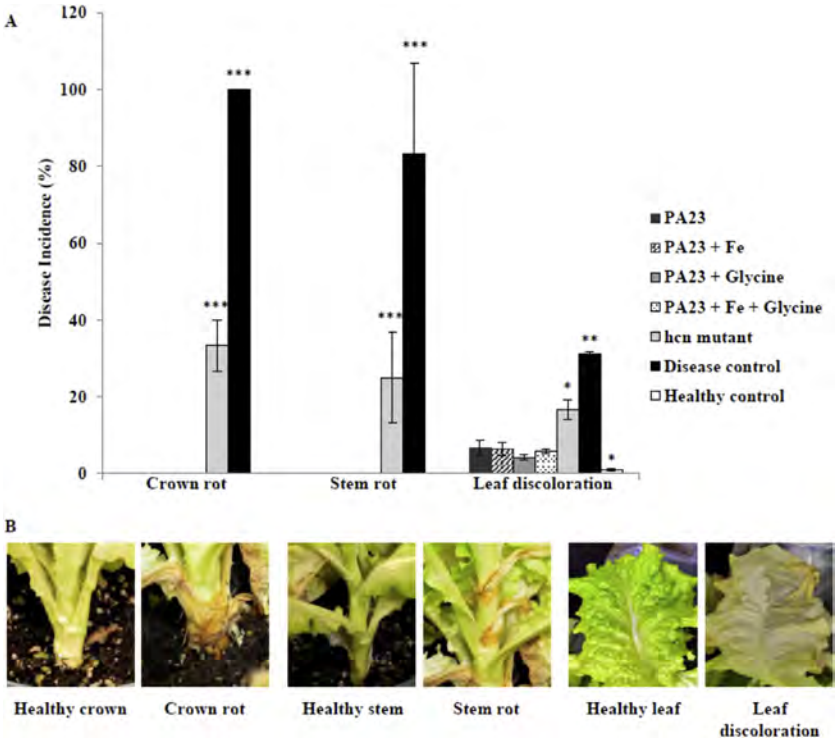

Fig. 6. Effect of ferric chloride, glycine and an *hcn* mutation on *P. chlororaphis* PA23 in managing *Sclerotinia sclerotiorum* infection on lettuce plants. (A) The disease incidence reflecting crown rot, stem rot and leaf discolouration is shown. For all treatments except the healthy control, plants were infected with a *S. sclerotiorum* mycelial slurry. Six plants were used per treatment. Treatment groups that differ significantly from wild type are indicated with an asterisk (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (B) Symptoms of lettuce infection caused by *Sclerotinia sclerotiorum*.

extended period before the onset of secondary metabolism, when production of antifungal metabolites is maximal.

3.6. Contribution of HCN to PA23 biocontrol of S. sclerotiorum in the greenhouse

Until now our investigation of PA23-mediated disease suppression has involved phyllosphere application of this BCA to the aerial surfaces of canola, where *S. sclerotiorum* infection begins. However HCN is a volatile at >25 °C, and if temperatures in the greenhouse reach or exceed this threshold, HCN will be converted to the gaseous form. As such, the phyllosphere represents a sub-optimal arena for evaluating the contribution of HCN to PA23 disease control. In the past, research focused on HCN has typically involved BCA application to the soil or seeds, followed by assessment of disease inflicted by root-rot pathogens (Flaishman et al., 1996; Laville et al., 1998; Voisard et al., 1989). While Sclerotinia does not attack the roots of canola, lettuce roots are susceptible to infection by this fungus, resulting in lettuce drop disease (Koike and Davis, 2007). Consequently, a lettuce infection model was used to explore whether HCN plays a role in PA23 biocontrol of *S. sclerotiorum*. Employing this pathosystem, disease severity was assessed by monitoring the following symptoms: (i) crown rot (soft, brown decay on the plant crown tissue); (ii) stem rot (brown lesion on stem); and (iii) leaf discoloration (Fig. 6). Crown rot and stem rot were found to develop on plants treated with the *hcn* mutant, whereas PA23-treated plants exhibited neither disease symptom (Fig. 6). In terms of leaf discoloration, plants treated with PA23*hcn* showed significantly higher symptoms in comparison to the wild type (Fig. 6). Moreover, glycine, but not $FeCl_3$ was found to reduce the incidence of leaf discoloration compared to the wild type (Fig. 6). Taken together, these findings indicate that HCN plays a role in PA23-mediated biocontrol of *S. sclerotiorum* infection of lettuce, and glycine increases the biocontrol potential of PA23 presumably through enhanced production of HCN, PRN and PHZ.

4. Conclusions

We have established that HCN contributes to PA23 AF activity both in vitro and in a newly established model of Sclerotinia root infection of lettuce. While media amendments have the potential to increase biocontrol capacity, they can also increase formulation costs. A thorough understanding of the molecular mechanisms underlying any beneficial effect is important to fully appreciate the value of such additions. Glycine was found to enhance PA23 AF activity by indirectly increasing HCN, PRN and PHZ through upregulation of the Phz QS system. We have previously established that PRN is the primary antibiotic responsible PA23-mediated biocontrol (Ramarathnam et al., 2011; Selin et al., 2010). PHZs, on the other hand, contribute little to fungal antagonism but they do facilitate biofilm formation (Selin et al., 2010). More recently, we have discovered that production of HCN and PRN enables PA23 to escape predation by the bacterivorous nematode *C. elegans* (Nandi et al., 2015). Accordingly, amendments that increase production of these metabolites may have the added value of facilitating environmental persistence through promoting biofilm formation and reducing predation pressure.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2017.02.008.

References

Beinert, H., Kiley, P.J., 1999. Fe-S proteins in sensing and regulatory functions. Curr. Opin. Chem. Biol. 3 (2), 152–157.
Bejerano-Sagie, M., Xavier, K.B., 2007. The role of small RNAs in quorum sensing. Curr. Opin. Microbiol. 10 (2), 189–198.
Blumer, C., Heeb, S., Pessi, G., Haas, D., 1999. Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. Proc. Natl. Acad. Sci. U.S.A. 96, 14073–14078.
Blumer, C., Haas, D., 2000. Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Arch. Microbiol. 173 (3), 170–177.
Castric, P.A., 1975. Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. Can. J. Microbiol. 21 (5), 613–618.
Castric, P.A., 1977. Glycine metabolism by *Pseudomonas aeruginosa*: hydrogen cyanide biosynthesis. J. Bacteriol. 130, 826–831.
Castric, P.A., 1983. Hydrogen cyanide production by *Pseudomonas aeruginosa* at reduced oxygen levels. Can. J. Microbiol. 29, 1344–1349.
Castric, P.A., 1994. Influence of oxygen on the *Pseudomonas aeruginosa* hydrogen cyanide synthase. Curr. Microbiol. 29, 19–21.
Chancey, S.T., Wood, D.W., Pierson 3rd, L.S., 1999. Two-component transcriptional regulation of N-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. Appl. Environ. Microbiol. 65 (6), 2294–2299.
Chen, X., Pizzatti, C., Bonaldi, M., Saracchi, M., Erlacher, A., Kunova, A., Berg, G., Cortesi, P., 2016. Biological control of lettuce drop and host plant colonization by rhizospheric and endophytic streptomycetes. Front. Microbiol. 7 (714), 1–12.
Flaishman, M.A., Eyal, Z., Zilberstein, A., Voisard, C., Haas, D., 1996. Suppression of *Septoria tritici* blotch and leaf rust of wheat by recombinant cyanide-producing strains of *Pseudomonas putida*. Mol. Plant-Microbe Interact. 9 (7), 642–645.
Fuqua, C., Greenberg, E.P., 1998. Self perception in bacteria: quorum sensing with acylated homoserine lactones. Curr. Opin. Microbiol. 1 (2), 183–189.
Haas, D., Keel, C., 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. Annu. Rev. Phytopathol. 41, 117–153.
Heeb, D., Haas, D., 2001. Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. Mol. Plant-Microbe Interact. 14, 1351–1363.
Ibrahim, S.A., Crack, J.C., Rolfe, M.D., Borrero-de Acuña, J.M., Thomson, A.J., Le Brun, N.E., Schobert, M., Stapleton, M.R., Green, J., 2015. Three *Pseudomonas* putida FNR family proteins with different sensitivities to O2. J. Biol. Chem. 290 (27), 16812–16823.
Knowles, C.J., 1976. Microorganisms and cyanide. Bacteriol. Rev. 40 (3), 652–680.
Koike, S., Davis, R., 2007. UC IPM Pest Management Guidelines: Lettuce – Disease Section. University of California Agriculture and Natural Resources. Publication 3450.
Lapouge, K., Schubert, M., Allain, F.H., Haas, D., 2008. Gac/Rsm signal transduction pathway of γ-proteobacteria: from RNA recognition to regulation of social behaviour. Mol. Microbiol. 67 (2), 241–253.
Latifi, A., Foglino, M., Tanaka, M., Williams, P., Ladzdunski, A., 1996. A hierarchial quorum-sensing cascade in *Peudomonas aeruginosa* links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary phase sigma factor RpoS. Mol. Microbiol. 21, 1137–1146.
Laville, J., Blumer, C., Von Schroetter, C., Gaia, V., Défago, G., Keel, C., Haas, D., 1998. Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. J. Bacteriol. 180 (12), 3187–3196.
Ling, E.A., Ellison, M.L., Pesci, E.C., 2009. A novel plasmid for detection of *N*-acyl homoserine lactones. Plasmid 62, 16–21.
Maddula, V.S.R.K., Pierson, E.A., Pierson III, L.S., 2008. Altering the ratio of phenazines in Pseudomonas chlororaphis (aureofaciens) strain 30–84, effects on biofilm formation and pathogen inhibition. J. Bacteriol. 190, 2759–2766.
Michelsen, C.F., Stougaard, P., 2012. Hydrogen cyanide synthesis and antifungal activity of the biocontrol strain *Pseudomonas fluorescens* In5 from Greenland is highly dependent on growth medium. Can. J. Microbiol. 58, 381–390.
Miller, J.H., 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 351–355.
Nagarajkumar, M., Bhaskaran, R., Velazhahan, R., 2004. Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani*, the rice sheath blight pathogen. Microbiol. Res. 159 (1), 73–81.
Nandi, M., Selin, C., Brassinga, A.K.C., Belmonte, M.F., Fernando, W.G.D., Loewen, P. C., de Kievit, T.R., 2015. Pyrrolnitrin and hydrogen cyanide production by *Pseudomonas chlororaphis* strain PA23 exhibits nematicidal and repellent activity against *Caenorhabditis elegans*. PloS One. http://dx.doi.org/10.1371/Journal.poine.0123184.
Nandi, M., Selin, C., Brawerman, G., Fernando, W.G.D., de Kievit, T.R., 2017. The global regulator ANR is essential for *Pseudomon as chlororaphis* strain PA23 biocontrol. Microbiology 162, 2159–2169.
Pal, K.K., Tilak, K.V.B.R., Saxena, A.K., Dey, R., Singh, C.S., 2000. Antifungal characteristics of a fluorescent *Pseudomonas* strain involved in the biological control of *Rhizoctonia solani*. Microbiol. Res. 155 (3), 233–242.
Paulin, M.M., Novinscak, A., St-Arnaud, M., Goyer, C., DeCoste, N.J., Privé, J.-P., et al., 2009. Transcriptional activity of antifungal metabolite-encoding genes *phlD* and *hcnBC* in *Pseudomonas* spp. using qRT-PCR. FEMS Microbiol. Ecol. 68 (2), 212–222.
Pessi, G., Haas, D., 2000. Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. J. Bacteriol. 182, 6940–6949.
Poritsanos, N., Selin, C., Fernando, W.G.D., Nakkeeran, S., de Kievit, T.R., 2006. A GacS deficiency does not affect *Pseudomonas chlororaphis* PA23 fitness when growing on canola, in aged batch culture or as a biofilm. Can. J. Microbiol. 52 (12), 1177–1188.
Preston, M.J., Seed, P.C., Toder, D.S., Iglewski, B.H., Ohman, D.E., Gustin, J.K., Goldberg, J.B., Pier, G.B., 1997. Contribution of proteases and LasR to the virulence of *Pseudomonas aeruginosa* during corneal infections. Infect. Immun. 65, 3086–3090.
Rabeendran, N., Jones, E.E., Moot, D.J., Stewart, A., 2006. Biocontrol of *Sclerotinia* lettuce drop by *Coniothyrium minitans* and *Trichoderma hamatum*. Biol. Cont. 39 (3), 352–362.
Ramarathnam, R., de Kievit, T.R., Fernando, W.G.D., 2011. The role of antibiosis and induced systemic resistance, mediated by strains of *Pseudomonas chlororaphis*, *Bacillus cereus* and *B. amyloliquefaciens*, in controlling blackleg disease of canola. Biol. Cont. 56, 225–235.
Reimmann, C., Ginet, N., Michel, L., Keel, C., Michaux, P., Krishnapillai, V., Zala, M., Heurlier, K., Triandafillu, K., Harms, H., Défago, G., Haas, D., 2002. Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. Microbiology 148, 923–932.
Rezzonico, F., Zala, M., Keel, C., Duffy, B., Moënne-Loccoz, Y., Défago, G., 2007. Is the ability of biocontrol fluorescent pseudomonads to produce the antifungal metabolite 2,4-diacetylphloroglucinol really synonymous with higher plant protection? New Phytol. 173 (4), 861–872.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Press, New York.
Savchuk, S., 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 Microbiol. Ecol. 49 (3), 379–388.
Selin, C., Habibian, R., Poritsanos, N., Athukorala, S.N., Fernando, W.G.D., de Kievit, T. R., 2010. Phenazines are not essential for *Pseudomonas chlororaphis* PA23 biocontrol of *Sclerotinia sclerotiorum*, but do play a role in biofilm formation. FEMS Microbiol. Ecol. 71 (1), 73–83.
Selin, C., Fernando, W.G.D., de Kievit, T.R., 2012. The PhzI/PhzR quorum-sensing system is required for pyrrolnitrin and phenazine production, and exhibits cross-regulation with RpoS in *Pseudomonas chlororaphis* PA23. Microbiology 158, 896–907.
Solomonson, L.P., 1981. Cyanide as a metabolic inhibitor. In: Vennesland, B., Conn, E.E., Knowles, C.J., Westley, J., Wissing, F. (Eds.), Cyanide in Biology. Academic Press Ltd., London, England, pp. 11–28.
Subbarao, K.V., 1998. Progress towards integrated management of lettuce drop. Plant Dis. 82, 1068–1078.
van Rij, E.T., Wesselink, M., Chin-A-Woeng, T.F., Bloemberg, G.V., Lugtenberg, B.J.J., 2004. Influence of environmental conditions on the production of phenazine-1-carboxamide by *Pseudomonas chlororaphis* PCL1391. Mol. Plant-Microbe Interact. 5, 557–566.
Voisard, C., Keel, C., Haas, D., Defago, G., 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root-rot of tobacco under gnotobiotic conditions. EMBO J. 8 (2), 351–358.
Zimmermann, A., Reimmann, C., Galimand, M., Haas, D., 1991. Anaerobic growth and cyanide synthesis of *Pseudomonas aeruginosa* depend on *anr*, a regulatory gene homologous with *fnr* of *Escherichia coli*. Mol. Microbiol. 5, 1483–1490.