Functional characteristics of New Zealand wheat rhizosphere *Pseudomonas fluorescens* isolates and their potential to inhibit *in-vitro* growth of *Gaeumannomyces graminis* var. *tritici*

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Abstract *Pseudomonas fluorescens* are soil-inhabiting plant growth-promoting rhizobacteria (PGPR) linked with suppression of take-all of wheat, a soilborne disease caused by *Gaeumannomyces graminis* var. *tritici* (Ggt). PGPR increase plant growth by direct stimulation, producing metabolites (such as 2,4-diacetylphloroglucinol, 2,4-DAPG) to inhibit plant pathogens, or by inducing host defence mechanisms. Forty-three New Zealand *P. fluorescens* isolates collected from wheat rhizospheres of different cropping histories, were characterised for secondary metabolite production using biochemical assays and PCR analysis. Their ability to inhibit the growth of Ggt was determined in dual plate assays. All of the bacterial isolates produced siderophores, and 10 isolates produced hydrogen cyanide (HCN). However, none of the isolates produced indole acetic acid, and the *phlD* gene responsible for the production of 2,4-DAPG was not detected. Isolates that showed at least 60% inhibition of Ggt growth were found to produce either HCN or high levels of siderophore. The results suggest HCN and siderophores could play a role in suppressing Ggt and managing take-all in New Zealand.

Keywords take-all decline, secondary metabolites, 2,4-DAPG, *PhlD*, disease suppression, hydrogen cyanide, indole acetic acid, PGPR

INTRODUCTION

Take-all, caused by the soil-borne fungus *Gaeumannomyces graminis* var. *tritici* (Ggt), is one of the most important root diseases of wheat (*Triticum aestivum* L.) (Hornby et al. 1998; Cook 2003). The disease is difficult to control, with no host plant resistance or cost-effective fungicide treatments available. Management is limited to crop rotation, or the continuous

growing of wheat in the same field which can induce spontaneous suppression of the disease, known as take-all decline (TAD) (Cool 2007).

Overseas, increases in wheat rhizosphere *Pseudomonas fluorescens* populations have been linked to the onset of TAD (Cook 2007). The bacterium is commonly isolated from New Zealand soils. The existence of TAD has been confirmed in some New Zealand soils, with *Pseudomonads*

reported as one of the major microbial groups associated with it (Chng et al. 2015).

Populations of *P. fluorescens* are members of the plant growth-promoting rhizobacteria (PGPR) communities found in association with host plant roots. PGPR can enhance plant growth, either by directly stimulating plant growth or by suppressing soilborne pathogens. Isolates producing the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) are thought to play a key role in suppressing *Ggt* (Thomashow et al. 1996; Raaijmakers et al. 1997). The gene *phlD* is involved in the biosynthesis of 2,4-DAPG (Mazzola et al. 2004). Other characteristics including the production of secondary metabolites such as hydrogen cyanide (HCN), siderophores, or indole acetic acid (IAA), are important traits of *P. fluorescens* contributing to plant growth and disease control as well (Weller et al. 2002).

Although *P. fluorescens* has been identified as one of the major groups of microorganisms associated with TAD soils globally and in New Zealand, there is currently no information available on the functional characteristics of *P. fluorescens* populations associated with New Zealand wheat rhizospheres. The aim of the present study was to investigate some of the major PGPR characteristics of *P. fluorescens* isolates collected from wheat rhizospheres of different cropping histories across Canterbury, which is New Zealand's major wheat growing area. Isolates were screened for their ability to inhibit the growth of *Ggt in-vitro*, and their production of 2,4-DAPG, HCN, IAA and siderophores were assessed using biochemical assays and polymerase chain reaction (PCR) analyses.

MATERIALS AND METHODS

Bacterial isolates

Forty-three *P. fluorescens* isolates (Table 1) were collected from the rhizospheres of wheat plants from four fields across Canterbury. Wheat plants from field A were first year wheat, B and C were second year wheat, and field D wheat plants were part of a 12-year continuous wheat system. Isolations were carried out by shaking

excess soil from the wheat roots and suspending the unwashed root systems in 0.1 M magnesium sulphate before plating following the protocol of King et al. (1954). King's medium B (KB) agar was modified with 20 mg/L of trimethoprim. All isolates were screened for fluorescence under ultraviolet light and characterised with LOPAT profile determinative tests, as described by Lelliott et al. 1966. Five additional cultures isolated from kiwifruit and cherries were included as references.

DNA extraction

Genomic DNA (gDNA) was isolated from overnight nutrient broth *P. fluorescens* cultures, using a Geneaid gSYNC DNA mini kit (Dnature, New Zealand), following the manufacturer's tissue extraction protocol.

PCR analysis

A PCR to differentiate *Pseudomonas fluorescens* from other *Pseudomonas* species was carried out using primer set 16SPSEfluF (5'-TGCATTCAAAACTGACTG-3') and 16SPER (5'-AATCACACCGTGGTAACCG-3') (Scarpellini et al. 2004). All PCRs were performed in 25 µl reaction mixtures containing 50 ng of gDNA, 2.5 µl of 10×PCR buffer, 200 µM of each dNTP, 2 mM of MgCl₂, 0.5 µM of each primer and 0.5 U of HOT FIREPol® DNA Polymerase (Dnature, New Zealand). Amplifications were performed with an Eppendorf Mastercycler Gradient using thermal cycling conditions of: 3 min at 95°C; 35 cycles of 95°C for 30 s, 60°C for 40 s, 72°C for 1 min; final extension of 72°C for 7 min. All PCR products were analysed by electrophoresis on a 1% agarose gel. A single fragment of 850 bp was amplified for *P. fluorescens* only.

Identification of isolates carrying the *phlD* gene, and hence the ability to produce 2,4-DAPG, was determined by PCR following the methods of Raaijmakers et al. (1997). The primer set Phl2a (5'-GAGGACGTCTGAAGACCACCA-3') and Phl2b (5'-ACCGCAGCATCGTGTATGAG-3') were used, with the PCR conditions described above.

Figure 1 Siderophore production test using chrome azurol S (CAS) agar. Four *Pseudomonas fluorescens* isolates and a sterile water control assayed per plate. Zones of iron depletion resulting in orange halos around colonies indicate siderophore production.

Amplified DNA products were run on 1.2% agarose. Positive reactions amplify a 745 bp fragment.

In addition to LOPAT determinative tests, the 16s rRNA region of several isolates was amplified following standard PCR protocols, and sequenced using primers 27F and 1492R (Lane 1991). The resulting nucleotide sequences were aligned to generate a consensus sequence, which was then subjected to a Genbank BLASTn search (NCBI, <http://www.ncbi.nlm.nih.gov>) using Geneious® 6.1.8 (Biomatters Ltd, New Zealand) to identify the species.

Siderophore production

Siderophore production was tested using a chrome azurol S (CAS) agar assay. CAS indicator solution and basal agar medium were prepared following the methods of Velloré (2001) and Schwyn & Neilands (1987). Four isolates and a water control were dispensed (20 µl each) onto each plate (Figure 1). There were three replicates, with isolates allocated to plates according to a 4-Latinized resolvable block design. Plates were incubated at ambient temperature for 48 h. A zone of iron depletion resulting in an orange halo around a colony indicated siderophore

production. Diameter (mm) of the colony and the total diameter of colony plus the surrounding halo were measured at 24 h and at 48 h. Siderophore production (halo diameter) was calculated using the formula: (colony + halo diameter) – colony diameter. The assay was repeated twice.

IAA production

IAA production was determined colorimetrically (Clarke & Cowan 1952). Isolates were grown at 35°C for 48 h in 5 ml tubes of Fisher BioReagents LB Miller broth (Thermo Fisher Scientific, New Zealand). Five drops of Remel™ Kovacs Indole reagent (Thermo Fisher Scientific, New Zealand) were added directly to each tube, and tubes lightly shaken. A pink colour development indicated the presence of IAA, while negative reactions remained colourless or light yellow. Detection of IAA production was repeated twice, with three replicates per isolate.

HCN production

The ability of isolates to produce HCN was assessed qualitatively using CYANTESMO™ test paper (Macherey-Nagel, Germany). This test paper is commonly used for detecting HCN gas released from plant material (Dalefield 2000). Isolates grown overnight in FMM were streaked onto KB agar, which was dispensed in one side of a divided

Figure 2 *Gaeumannomyces graminis* var. *tritici* (Ggt) interaction plate set-up. Area inoculated with *Pseudomonas fluorescens* shown by cross hatching. Arrows (A and B) show position and direction of measurement of Ggt growth.

Table 1 *Pseudomonas fluorescens* isolates used in this study and their PGPR characteristics¹.

<i>Pseudomonas</i> Isolate number	Numbering for study	Source	Field ²	<i>PhlD</i> status	IAA	HCN ³	Siderophore
<i>Pseudomonas fluorescens</i>		Wheat					
1202/33-1	1		A	-	-	-	+
1205/37-3	2		A	-	-	-	+
1201/30-1	3		A	-	-	-	+
1204/37-2	4		A	-	-	-	+
1203/37-1	5		A	-	-	-	+
1206-1	6		B	-	-	-	+
1207-2	7		B	-	-	-	+
1208-1	8		B	-	-	-	+
1208-4	9		B	-	-	-	+
1208-6	10		B	-	-	-	+
1208-7	11		B	-	-	-	+
1208-9	12		B	-	-	-	+
1208-10	13		B	-	-	-	+
1208-12	14		B	-	-	-	+
1208-13	15		B	-	-	-	+
1212	16		C	-	-	-	+
1215	17		C	-	-	-	+
1216	18		C	-	-	-	+
1218	19		C	-	-	-	+
1219	20		C	-	-	-	+
1220	21		C	-	-	-	+
1221	22		C	-	-	-	+
PF13/12-11	23		D	-	-	-	+
PF13/12-14	24		D	-	-	-	+
PF13/12-20	25		D	-	-	-	+
PF13/12-29	26		D	-	-	-	+
PF13/12-34	27		D	-	-	-	+
PF13/12-54	28		D	-	-	-	+
PF13/12-61	29		D	-	-	-	+
PF13/12-64	30		D	-	-	+ ^Δ	+
PF13/12-69	31		D	-	-	-	+
PF13/12-70	32		D	-	-	-	+
PF13/12-71	33		D	-	-	+	+
PF13/12-72	34		D	-	-	+ ^Δ	+
PF13/12-76	35		D	-	-	-	+
PF13/12-77	36		D	-	-	+ ^Δ	+
PF13/12-79	37		D	-	-	+ ^Δ	+
PF13/12-87	38		D	-	-	+ ^Δ	+

Table 1 (continued) *Pseudomonas fluorescens* isolates used in this study and their PGPR characteristics¹.

<i>Pseudomonas</i> Isolate number	Numbering for study	Source	Field ²	<i>PhlD</i> status	IAA	HCN ³	Siderophore
PF13/12-89	39		D	-	-	+	+
PF13/12-92	40		D	-	-	+ ^Δ	+
PF13/12-93	41		D	-	-	+ ^Δ	+
PF13/12-94	42		D	-	-	-	+
PF13/12-95	43		D	-	-	-	+
ICMP 3636	44	Kiwifruit		-	-	-	+
ICMP11288	45	Kiwifruit		-	-	-	+
ICMP2431	46	Cherry		-	-	+	+
ABAC62	47	Kiwifruit		-	-	-	+
<i>Pantoea</i> sp.							
M&R regular	48	Cherry		-	+	-	+

¹ Detection (+) presence or (-) absence of the *phlD* gene, indole acetic acid (IAA) hydrogen cyanide (HCN) production, or siderophore

² Field A = first year wheat, B and C = second year wheat, D = 12 year continuous wheat

³ “+^Δ” indicates strong HCN production.

Petri dish (90 mm). Two strips of CYANTESMO™ paper 2–3 cm long were placed in the agar-free side of the Petri plate. Plates were sealed with Parafilm™ and incubated at 28°C for 4 days. CYANTESMO™ strips showed a colour change from pale green to deep blue in the presence of HCN. Detection of HCN production was repeated twice, with three replicates per isolate.

Inhibition of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* isolates

Screening of inhibition against a pathogenic *Ggt* isolate was assessed in dual plate assays. Agar plugs (5 mm each) were cut from actively-growing edges of a 10-day-old *Ggt* culture (A3SL4, previously tested for pathogenicity on wheat, Chng et al. (2005)), and placed in the centre of half-strength Potato Dextrose agar (PDA) plates. Plates were incubated with a diurnal cycle of 23/20°C, 14 h light/10 h dark, for 48 h. An inoculation loop of each *P. fluorescens* grown overnight in FMM was then streaked on the opposite side of each plate, at a distance of 2.25 cm from the middle of the plate (Figure 2). Negative control plates were streaked with sterile water only. There were

three replicate plates prepared for each bacterial isolate. Plates were returned to the incubator and arranged in a 2-Latinized resolvable block experimental design. Measurements of *Ggt* growth (mm) across the centre of the inoculation plug in two perpendicular directions (A & B, Figure 2) were recorded for each plate after 4 days. Inhibition assays were repeated twice. The average percent inhibition was calculated for each isolate using the formula: % inhibition = (growth control-growth treatment)/growth control × 100.

RESULTS

Biochemical assays

All of the *P. fluorescens* isolates were categorised as group IVb or group Vb pseudomonads, using the LOPAT determinative test (data not shown). All the *P. fluorescens* isolates showed siderophore production on CAS agar. Halo diameter varied substantially between isolates ($P < 0.001$). At 48 h, isolate 15 had the largest halo (16.1 mm diameter), followed by isolate 8 (14.0 mm) (Figure 3). Halo diameter at 48 h was fairly well correlated with that at 24 h ($r = 0.76$). Ten isolates (23% of those

Figure 3 Average siderophore halo width (mm) of *Pseudomonas fluorescens* isolates at 48 h against the halo width at 24 h. Each number is an isolate. Averages taken from three replicates.

tested) produced HCN. Seven of them (isolates 30, 34, 36, 37, 38, 40, and 41) showed strong HCN production, with a colour change observed at 24 h, while three isolates (33, 39, and 46) showed moderate production, with HCN detected on the fourth day (Table 1). All wheat rhizosphere

P. fluorescens isolates that produced HCN produced low levels of siderophore (Figure 5). None of the isolates, except the positive control, produced IAA.

PCR Analysis

Primers 16SPSEfluF & 16SPER, specific for the detection of *P. fluorescens* from other Group 1 pseudomonads, amplified an 850 bp product from all isolates. A fragment of the same size was amplified from DNA of all the included *P. fluorescens* reference isolates, with no product amplified in the *Pantoea* negative control.

All 16S region sequencing results had either a 100% or 99.9% sequence match to *P. fluorescens* (Accession numbers CP003041, EU169191 or EU364534).

No PCR product specific for the *phlD* gene was amplified in any samples, other than the positive control. The positive control was gDNA from a *phlD* carrying *P. fluorescens* isolate (FD6), supplied by the College of Plant Protection and Horticulture, Yangzhou University, Yangzhou 225009, PR China (Zhang et al. 2015).

Inhibition of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* isolates

All isolates showed at least some inhibition of *Ggt* growth compared to control plates (Figure 4). Isolates 33 and 16 had the highest level of inhibition ($\approx 80\%$), while isolates 42, 39, and 3 had the lowest

Figure 4 Average *Gaeumannomyces graminis* var. *tritici* (*Ggt*) growth inhibition (%) by *Pseudomonas fluorescens* in two interaction plate assays. Dotted line indicates 60% inhibition.

Figure 5 Relationship between siderophore production and average percentage inhibition of *Gaeumannomyces graminis* var. *tritici* (*Ggt*). Each number refers to a *Pseudomonas fluorescens* isolate. Isolates which produced HCN are circled.

level of inhibition at 30–40%. There was no inhibition of *Ggt* in the sterile water control plates. The average percentage inhibition in the two assays was similar. Twenty nine isolates showing at least a 60% inhibition of *Ggt* growth in both assays either produced HCN (five isolates) or high amounts of siderophore in regard to halo width (Figure 5).

DISCUSSION

Although previous studies have found an association between *Pseudomonads* and TAD, no characterisation of the metabolites or antibiotics produced by *P. fluorescens* associated with New Zealand wheat rhizospheres has been undertaken. It is widely known that *Pseudomonads* differ considerably in the type and number of metabolites they produce. In this study, most New Zealand *P. fluorescens* isolates were found to have similar functional profiles, irrespective of their field of origin, with the exception of HCN production. Ten isolates

were found to produce HCN, with all of these isolates collected from a 12-year continuous wheat field. This suggests that TAD could be developing in this field. The development of TAD soils are characterised by initially low take-all levels during the early cropping years, an outbreak of the disease between 2 and 7 years, and subsequently a decline of disease with increased yields (Chng et al. 2015). Eight of these isolates showed 50% to 80% growth inhibition of *Ggt* (Figure 5). This indicated that the suppression of *Ggt* by *P. fluorescens* originating from this field may be attributed to HCN.

HCN has been known to effectively block the cytochrome oxidase pathway, so is highly toxic to all aerobic microorganisms at picomolar concentrations (Pal & McSpadden-Gardener 2006). For instance, the production of HCN by certain fluorescent *Pseudomonads* is believed to be involved in the suppression of root pathogens. *Pseudomonas fluorescens* strain CHA0 produces antibiotics, siderophores and HCN, but suppression of black rot of tobacco caused by *Thielaviopsis basicola* appeared to be due primarily to HCN production (Voisard et al. 1989). Hence, the production of HCN may provide isolates with the potential for plant protection, including protection against other soilborne organisms not included in this study. Future research should include screening the HCN-producing isolates against soilborne pathogens other than *Ggt*.

All isolates in this study were found to produce siderophores. Siderophores are small, high-affinity iron-chelating compounds produced by various microorganisms. Siderophore producing bacteria are thought to facilitate biocontrol by sequestering iron from pathogens, limiting their growth, and increasing the availability of stable iron for uptake by plant roots (Alexander & Zuberer 1991). In the present study, a group of siderophore producing isolates gave at least 60% inhibition of *Ggt* growth (Figure 5). These isolates could be used as PGPR in the control of *Ggt*. Further work to determine the different siderophore types produced by isolates in this study, such as phosphatase soluble or pyoverdine, would be beneficial in identifying specific siderophores

that may have an effect on *Ggt* suppression.

In this study, the *phlD* gene was not detected from any of the *P. fluorescens* isolates, suggesting that these isolates may produce other functional products, such as phenazines, which contribute to pathogen suppression. Phenazine-1-carboxylic acid (PCA), produced by *P. fluorescens* isolates 2-79 and 30-84, has been shown to suppress take-all (Thomashow et al. 1990). In future research, the production of PCA by New Zealand isolates should be determined. This could be achieved using specific primers targeting genes responsible for phenazine production (Raaijmakers et al. 1997). In addition, screening for other functional characteristics, such as phosphate solubilisation and nitrogenase activity, and the use of high performance gas/liquid chromatography, may be useful for identifying other beneficial compounds produced.

This study provides an initial assessment into some of the functional characteristics contributing to the suppression of *Ggt* by New Zealand wheat rhizosphere *P. fluorescens* populations. Two groups of isolates were identified as potential PGPR candidates for managing take-all and other soilborne diseases.

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