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Characterising the Influence of First-Year Wheat Cultivar on *Pseudomonas* Selection and Function in a Take-All Infected Field

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Abstract: Wheat, the world's most widely cultivated crop, is highly susceptible to Take-all disease caused by the soil ascomycete fungus *Gaeumannomyces tritici* (*Gt*). Crop rotations using different wheat cultivars can influence Take-all build up (TAB) in the following year's wheat crop. The exact mechanism for this is unknown, but there may be a bacterial biocontrol component associated with TAB control. We cultured and characterised *Pseudomonas* isolates from the rhizosphere and endosphere of second-year field-grown wheat, in plots which, in the previous season, had been used to culture High-TAB cultivar Hereward or Low-TAB cultivar Cadenza. Analysis of two bacterial loci, *wsm* and *fecB*, showed that these genes were significantly and differentially associated with first-year wheat-planting regime: *wsm* was more abundant within the High-TAB Hereward-derived isolates, while *fecB* was more prevalent within the Low-TAB Cadenza-derived isolates. These findings were supported by a *gyrB* phylogenetic analysis and an *in vitro Gt* antagonism assay. These data show that the wheat cultivar grown in the first year plays an especially important role in the selection of the associated *Pseudomonas* spp. found in the second-season wheat-root environment, emphasising the importance of understanding agronomic practice in improving plant health and food security.

Keywords: *Gaeumannomyces*; take-all inoculum build up; L-TAB; H-TAB; *wsm*; *fecB*

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1. Introduction

Bread wheat, *Triticum aestivum*, is one of the four most important crops globally. Wheat is highly susceptible to Take-all disease, caused by the soil-borne ascomycete fungus *Gaeumannomyces tritici* (*Gt*). The disease causes huge economic losses in wheat crop yield and quality [1,2]. Currently, there are a lack of effective fungicide treatments and resistant cultivars to treat this disease. Historically, crop rotation using non-graminaceous species was used to manage Take-all disease [3]. Soil fumigation with methyl bromide has also been used for the control of Take-all disease, but this chemical is now banned due to its negative effects on the environment and human health [4,5]. However, given the great demand for wheat and the lack of economically attractive break crops, wheat is commonly grown in short rotations, resulting in the increased establishment of Take-all disease and associated crop losses. As such, there is a great need to establish alternative practices, which includes biological control approaches for tackling plant diseases.

Take-all decline occurs after a severe outbreak of the disease in very susceptible wheat or barley monoculture fields, which ultimately leads to reduced disease severity

in subsequent wheat [1,4]. The disease suppression is thought to be achieved by a build-up in antagonistic microorganisms [6], such as populations of fluorescent *Pseudomonas* spp. [7]. *Pseudomonas* spp. are bacteria that colonise the root environment of many different plant species and can enhance plant growth via the secretion of molecules such as iron-scavenging siderophores as well as anti-fungal compounds such as phenazine, pyoluteorin and pyrrolnitrin, along with hydrogen cyanide, a volatile metalloenzyme inhibitor [7–9]. *Pseudomonas* spp. such as *P. aureofaciens* and *P. chlororaphis* produce phenazine-1-carboxylic acid (PCA) and derivatives such as 2-hydroxyphenazine-1-carboxylic acid and phenazine-1-carboxamide, respectively, as they have additional genes that modify PCA [10]. *Pseudomonas* chlororaphis YB-10 significantly promoted the growth of wheat seedlings via indole acetic acid and siderophore production and increased activities of the wheat defence-related enzymes [9]. Control of Take-all disease by *P. fluorescens* 2-79 and *P. fluorescens* CHAO was achieved via the production of PCA and 2,4-diacetylphloroglucinol (Phl), respectively [11]. KwakWeller [4] reported that Take-all decline was achieved after a period of wheat monoculture due to an increase in the population of the antibiotic 2,4-Diacetylphloroglucinol (DAPG) producing fluorescent *Pseudomonas* spp. However, in another study, it was found that contrasting wheat cultivars displayed differential ability to tolerate root colonisation by DAPG-producing bacteria due to potential phytotoxicity [12]. Furthermore, McMillan [13] showed the differential ability of wheat cultivars to support Take-all build up (TAB). As such, cultivars were classified as either Low-TAB (L-TAB) or High-TAB (H-TAB). For example, Cadenza and Xi-19 are both L-TAB cultivars, while Hereward is a H-TAB variety. However, the molecular basis of this differential TAB trait is not known [14]. The H-TAB wheat variety Hereward supported a *Pseudomonas* spp. population with traits antagonistic to other bacteria. However, the L-TAB inoculum builder wheat variety Cadenza supported *Pseudomonas* spp. that were better adapted to host communication and nutrient acquisition [15].

In this work, we build on previous work by Mauchline [15] and test the hypothesis that wheat variety grown in the first year impacts the selection of root-associated *Pseudomonas* isolates in the second year of wheat cultivation. We test this by analysing the population diversity, *Gt* suppressive capacity as well as the presence or absence of gene loci associated with rhizosphere colonisation in *Pseudomonas* strains isolated from the roots of second-year wheat. We supplement this approach with *in planta* assays to demonstrate the efficacy of isolates to suppress *Gt*.

2. Materials and Methods

2.1. Sample Collection and Bacterial Isolation

A total of 411 *Pseudomonas* isolates were collected from the root systems of wheat plants from the second year of a two-year field trial run in 2010–2012 at the Rothamsted Research experimental farm, UK. The field design is fully described in Mauchline [15], and consisted of four main blocks each divided into two main plots, with Hereward and Cadenza grown in the first year (Table S1). In the second year, the two main plots were divided into 8 split plots, which were over-sown with a range of wheat cultivars, but only those plots over-sown with Hereward and Xi-19 were sampled (Table S2). As such, four replicates of four planting combinations were sampled: Hereward/Hereward (HH), Hereward/Xi19 (HX), Cadenza/Hereward (CH) and Cadenza/Xi19 (CX). Isolates were purified from the rhizosphere and endosphere of wheat plants as previously described by Mauchline [15], and this isolate collection forms the basis of the current study. Nineteen of these isolates were previously genome sequenced [15]. In total, 103 isolates were collected from CH, 116 from CX, 84 from HH and 108 from HX planting combinations. Furthermore, CH comprised 49 rhizosphere and 54 endosphere isolates; CX 59 rhizosphere and 57 endosphere isolates; HH 43 rhizosphere and 41 endosphere isolates; and HX 60 rhizosphere and 48 endosphere isolates.

2.2. Growth Conditions and Media

Pseudomonas bacteria were grown at 27 °C in King's medium B broth (KB, for 16 h with shaking at 2000 rpm) or on agar plates (1.5% agar, King's medium B, KBA) [16] for 48 h. The *Gt* isolate was grown on potato dextrose agar (PDA) (Oxoid) at 24 °C for seven days. For subsequent *Gt* assays, 0.5 cm agar plugs were taken from the hyphal leading edge and transferred to KBA plates.

2.3. In Vitro Gt *Inhibition Studies*

Gaeumannomyces tritici (*Gt*) isolate 16-LH-15d, 16/6/17 used in this work was isolated from Long Hoos 4 field at Rothamsted. The plate inhibition assay was performed on KBA where 5 µL of an overnight bacterial culture was spotted 1 cm from the edge of the plate. Three bacteria were used per plate and a fourth spot used non-inoculated KB as a control. The spots were allowed to soak into the agar until dry before placing a 0.5 cm plug of *Gt* at the centre of the plate. Three replicates were prepared for each combination.

Using the average inhibition zone (cm) of the three treatment replicates, the isolates were classified into three classes of inhibition zone: Class 1 = 0–0.5 cm; class 2 = 0.5–1 cm; and class 3 = >1 cm. The class 3 isolates were then challenged in 1:1 setup with the fungus to validate their *Gt* growth-inhibiting activity. Again, 5 µL of bacterial culture was spotted 1 cm away from the edge of the plate with the fungal plug placed at the centre. A non-inoculated 5 µL of KB was spotted as a control, and each treatment was replicated three times. The plates were incubated at 24 °C and the distance between the edges of bacterial colonies and fungal mycelium after 7 days was computed using ImageJ software [17].

2.4. DNA Extraction

DNA template for PCR was prepared from bacterial colonies using a TE buffer extraction method. Briefly, 1 mL of bacterial broth from an overnight KB culture, prepared from a single colony, was transferred into an Eppendorf tube and centrifuged at 13,000 rpm for 3 min (Microfuge, SCF2, Stuart). The supernatant was discarded, and the pellets were re-suspended in 200 µL of TE buffer (1 M Tris HCl—0.5 M EDTA pH 8). After vortexing, the cell suspension was heated at 100 °C for 10 min on a heating block (Heat block, VWR Scientific). The tubes were chilled on ice for 10 min, followed by a second centrifugation (13,000 rpm for 3 min). The supernatant containing the DNA was then transferred to sterile tubes. Template DNA was either used immediately in a PCR or stored at −20 °C for later use.

2.5. PCR and DNA Sequencing

Primers for amplifying *gyrB*, *phzCD*, *phl*, *wsm* and *fecB* genes are shown in Table 1. The *wsm* and *fecB* primers were designed in this study using NCBI primer design tool; *gyrB* sequences were from Yamamoto [17], *phl* and *phzCD* sequences were from Raaijmakers [11]. PCR was performed in 50 µL reactions containing 25 µL of 2X master mix (PCR Biosystems), 16 µL H_2O, 2 µL of each primer (10 µM) and 5 µL of template DNA. The PCR amplification conditions were as follows: initial denaturation 94 °C for 5 min, 35 cycles of denaturation at 94 °C 1 min, annealing at 63–70 °C for 30 s, extension at 72 °C for 2 min and a final extension step at 72 °C for 7 min. The annealing temperature for isolate 24E/2 was 58 °C.

All PCR products were run on a 1% agarose gel in 0.5X TBE buffer at 90 V for 40 min and were visualised using the G-Box gel documentation system. For sequencing, PCR-amplified products using the primers gyrB SF and gyrB SR [18] were purified using a PCR purification kit (Qiagen) and processed by Eurofins Genomics.

2.6. Sequence Analysis

The resulting sequences were aligned in BioEdit software and were searched using BLASTN with default settings to ensure their identity as *Pseudomonas* spp. [19]. The phylogenetic tree was constructed using the Neighbour Joining method (NJ) after alignment

with MUSCLE using Geneious Prime 2019.0.4 (http://www.geneious.com). The node support was evaluated on 100 bootstrap replications. The *gyrB* sequences of *P. fluorescens* strains SBW25 and F113 were included as references for comparison and the *gyrB* sequence of *P. aeruginosa* PAO1 was used as the out-group.

Table 1. Primers and PCR conditions used in this study.

Locus	Primer Name	Primer Sequence	Tm (°C)	Product Size (bp)
wsm	wsmF	5′ GGCAAYGCCGARHTSATCC 3′	63	603
	wsmR	5′ GCACCARCGSTCYTTRTAYTCRCGGTC 3′		
fecB	fecBF	5′ TGATCGTSGCCGACCTCAAYCG 3′	70	455
	fecBR	5′ CCACARCGGCTGCTTGCTCCAG 3′		
gyrB	gyrBF	5′ CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA 3′	63	888–891
	gyrBR	5′ TGTAAAACGACGGCCAGTGCNGGRTCYTTYTCYTGRCA 3′		
gyrB	gyrBSF	5′ CAGGAAACAGCTATGACC 3′	n/a	n/a
	gyrBSR	5′ TGTAAAACGACGGCCAGT 3′		
phl	Phl2a	5′ GAGGACGTCGAAGACCACCA 3′	67	745
	Phl2b	5′ ACCGCAGCATCGTGTATGAG 3′		
phzCD	PCA2a	5′ TTGCCAAGCCTCGCTCCAAC 3′	67	1150
	PCA3b	5′ CCGCGTTGTTCCTCGTTCAT 3′		

2.7. Seed Sterilisation

Wheat (*Triticum aestivum*) seeds of cultivars Cadenza and Hereward were surface sterilised via immersion in a 2.5% (*v*/*v* with sterile deionised water (SDW)) solution of bleach for 3 min followed by 3 rinses in SDW. The seeds were allowed to air dry for up to 3 h in a class 2 safety cabinet (Labogene) [20].

2.8. In Planta *Assays*

In total, 1 mL of the overnight culture of selected bacterial isolates was pelleted via centrifugation at 5000 rpm for 8 min. The pelleted cells were washed twice in 1 mL phosphate-buffered saline (PBS) and the Optical Density (OD600) was adjusted to 0.1 in PBS. Twelve seeds were soaked in bacterial suspension in sterilised 50 mL tubes and placed in an orbital shaker set at 200 rpm, 27 °C for 1 h. After this time, the seeds were left to air dry for 2 h. Sterile 50 mL Falcon tubes were prepared for seed transplantation with the addition of 5 g sterile fine vermiculite. For each test isolate, 3 replicates were prepared with one seed placed 1 cm below the vermiculite surface. Each tube was watered with 25 mL SDW and covered with parafilm. The tubes were incubated in a temperature-controlled room set at 21 °C with 16 h light–dark cycle and 70% humidity. Sterile seeds soaked in PBS were used as a control. The parafilm was removed from all treatments after 5 days. The seedlings were watered with 5–10 mL SDW on day 6 and day 13 and harvested at day 21. Following harvest, seedling biomass, shoot height, root length and fresh weight was measured.

2.9. In Planta Gt *Antagonistic Assay*

The six most antagonistic isolates to control *Gt* in an *in vitro* setup were tested for ability to suppress *Gt* with an *in planta* assay. The growth chamber assay was slightly modified from the methods described by [6]. Briefly, sterile seeds of Cadenza and Hereward were soaked in bacterial OD600 0.1. Five *Gt* plugs (0.5 cm) were placed 2 cm below the sown seed in sterile vermiculite. Initial analysis showed that 5 plugs was sufficient to cause disease symptoms. One seed was sown per 50 mL Falcon tube and watered and incubated as above. The percentage of infected roots was determined by comparing the total number of roots and the infected roots.

2.10. Statistical Analysis

GENSTAT (17th edition, VSN International Ltd., Hemel Hempstead, UK) was used to analyse the data. The category class 1–3 inhibition zone data were analysed by fitting a generalised linear model (GLM) to account for the blocks in the experiment and to test the main effects and interactions between the factors year 1 (H or C cultivar), year 2 (H or Xi-19 cultivar) and Niche (Rhizosphere (R) or Endosphere (E)). ANOVA was also performed on the inhibition zone data of isolates, which was averaged per plot. A Poisson distribution was assumed, and a log link function was incorporated. The fitted model was Block+ (Year 1*Year 2*Niche). There was no evidence of over-dispersion for this model (F pr. < 0.001 with dispersion parameter estimated to be 0.231 from the residual deviance). Predicted means on the log scale were used or obtained to enable comparisons using the standard error of the difference (SED) between means on the residual degrees of freedom from the model, along with the least-significant difference (LSD) at the 5% level of significance. GLM was fitted to the PCR data for both genes. The fitted model was Block+ (Year 1*Year 2*Niche). There was no evidence of over-dispersion for the data using this model for either gene and the outcomes of each locus were analysed separately. A GLM was used to analyse the infected root data. The model fitted was cultivar*Isolate with the total number of roots as the binomial total. For the *in planta* assay mixed model, Restricted Maximum Likelihood variance components were used to analyse the plant biomass data.

3. Results

3.1. Screening of Isolates for Gt *Antagonism and Classification of Potency*

Pseudomonas isolates were phenotypically and genotypically screened to examine whether wheat-planting regime influenced their selection in the root microbiome. The isolates were profiled for *Gt* antagonism using a two-stage *in vitro* antagonism screen. In the first stage, *Gt* was inoculated in the centre of a Petri dish and three randomly selected isolates were separately inoculated to challenge the fungus in a spatially separated manner. The resultant inhibition zone measurements were grouped into three classes. There were 164 class 1 isolates (0–0.5 cm), 200 class 2 isolates (0.5–1 cm) and 47 class 3 isolates (>1 cm) (Table S3). Out of the 47 class 3 isolates, 40 (85%) were from the year 1 Cadenza background and 7 (15%) were from the year 1 Hereward background. This equates to 18.2% of first-year Cadenza isolates and 3.6% of first-year Hereward isolates being class 3 inhibitors. In addition, a higher proportion of first-year Hereward isolates (49%) were assigned as class 1 inhibitors compared with first-year Cadenza isolates (32%) (Figure 1). The 47 class 3 isolates were further challenged in a 1:1 setup with *Gt* to confirm their antagonistic ability. The six isolates (24E-2, 24E-4, 25R-7, 28R-9, 30R-11, 44E-7) displaying the strongest antagonism of *Gt* came from a diverse range of field plots (Table 2). Four of the six isolates were from the Cadenza/Xi-19 (C, Xi-19) planting combination, with one isolate from Hereward/Hereward (H, H) and one from Hereward/Xi-19 (H, Xi-19) planting combinations (Tables S3 and S4).

The regression analysis of the category class 1–3 data showed some evidence of a two-way interaction between year 1 and year 2 factors (F = 4.86 on 1 and 410, d.f.; $p = 0.028$) (Tables S5 and S6 and Figures S1 and S2), but no effect of niche. Secondly, ANOVA analysis on the average inhibition zone data per plot showed the main effect was driven by year 1 cultivar (F = 0.031, d.f. = 1) regardless of which variety was grown in year 2 or niche (Table S7). The most antagonistic isolates (with the largest inhibition zone values) were largely derived from a Cadenza background in year 1 as opposed to Hereward (Table S8).

3.2. Biomarker Screening to Differentiate Antagonistic Bacteria

PCR screening of the six strongest antagonistic isolates along with the least antagonistic isolate 37R/15, for genes relating to the production of 2,4-diacetylphloroglunicol (DAPG) and phenazine-1-carboxylic acid using primers corresponding to genes involved in their biosynthesis, namely, *phl* for DAPG and *phzCD* for phenazine-1-carboxylic acid, revealed that none of these isolates produced a PCR amplicon for either gene. *Pseudomonas protegens*

strain Pf-5 (*Phl*$^+$) was included as a positive control for the 2,4-DAPG gene. We then extracted the *wsm* (coding for lipopolysaccharide synthesis) and *fecB* (coding for a ferric citrate ABC transporter solute binding protein, involved in Fe uptake) gene sequences from the 19 *P. fluorescens*-sequenced genomes [15]. PCR screening of the 411 isolates using the *wsm* and *fecB* degenerate primers was performed and isolates were scored for the presence or absence of these genes. For both genes, the wheat grown in the first year had a strong selection effect ($p < 0.001$, F-test, Table S9). It was found that the *wsm* locus was more associated with first-year Hereward, whereas *fecB*-encoding strains were more prevalent in first-year Cadenza ($p < 0.001$, F-test) (Table S10). Furthermore, when considering the interaction between niche and first-year cultivar, it was found that for first-year Cadenza-derived plants *fecB* and *wsm* genes were more prevalent in rhizosphere as opposed to endosphere isolates (Figure S3). The opposite was found to be the case for first-year Hereward-derived plants.

3.3. fecB *and* wsm *Incidence Correlate with Fungal Inhibition*

When comparing the presence of *fecB* and *wsm* and pathogen suppression, we found that a positive correlation existed with the presence of *fecB* and the ability of isolates to supress *Gt*. The opposite was found to be the case with the presence of *wsm* (Figure 2, Pearson correlation: inhibition category versus *fecB*: 0.231681, inhibition zone diameter versus *fecB*: 0.284845; inhibition category versus *wsm*: −0.19152, inhibition zone diameter versus *wsm*: −0.16932).

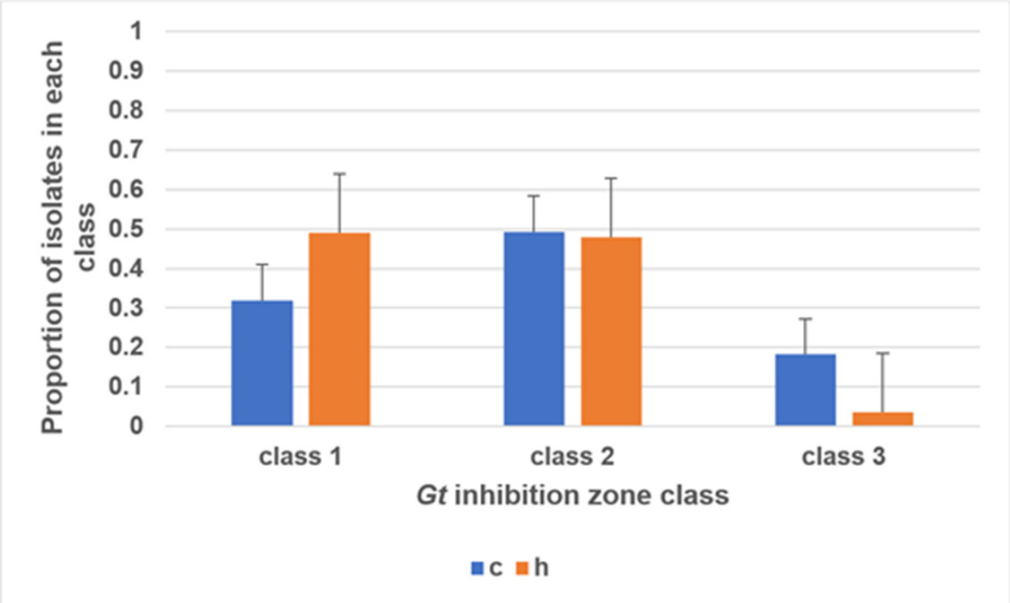
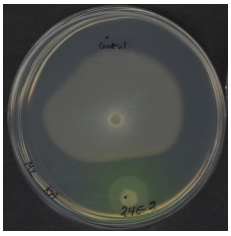
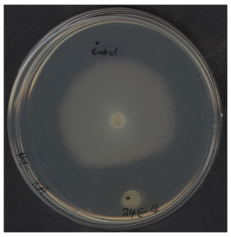
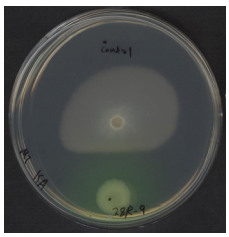
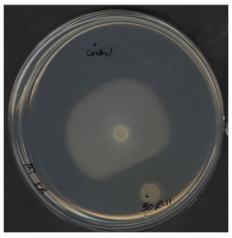
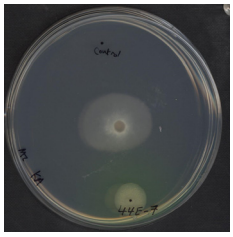


Figure 1. Proportion of *Gaeumannomyces tritici* (*Gt*) inhibited by *Pseudomonas* isolates associated with first-year wheat cultivar Cadenza (c) or Hereward (h). The inhibition zone measurements were grouped into three classes: class 1 (0–0.5 cm), class 2 (0.5–1 cm) and class 3 (>1 cm). Values are the mean of 3 replicates and error bars are the standard error of the mean.

Table 2. List of six *Pseudomonas* isolates with most antagonistic activity against *Gaeumannomyces tritici* after *in vitro* growth inhibition assay.

No.	Isolate *	Planting Combination	Mean Inhibition Zone (cm)	*Gt* 1:1 Antagonistic Assay
1	24E-2	(C, Xi-19)	1.24	
2	24E-4	(C, Xi-19)	1.05	
3	25R-7	(C, Xi-19)	1.10	
4	28R-9	(H, Xi-19)	1.08	
5	30R-11	(C, Xi-19)	1.23	
6	44E-7	(H, H)	1.44	

* Number = field plot, E = Endosphere, R = Rhizosphere, wheat cultivar C: Cadenza, H: Hereward.

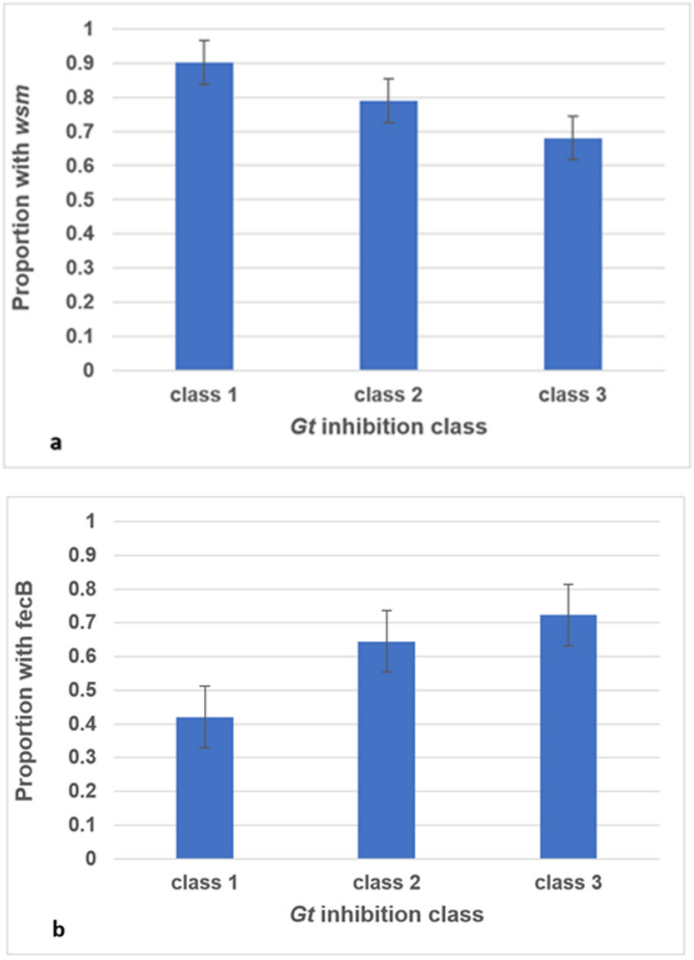


Figure 2. Proportion of *Gaeumannomyces tritici* (*Gt*) inhibiting *Pseudomonas* isolates possessing *wsm* (**a**) and *fecB* (**b**) genes. Values are the mean of 3 replicates and error bars are the standard error of the mean.

3.4. gyrB *Phylogeny*

A subset of 25 *Pseudomonas* isolates were genotypically characterised using the gyrase B subunit (*gyrB*) gene (Table 1) to further investigate the relationship between the planting combinations, *Pseudomonas* phylogeny and the *Gt* antagonism pattern of the isolate. This included the six strongest antagonists, the two weakest antagonists and 17 strains randomly chosen to cover the different planting combinations and niches. *Pseudomonas aeruginosa* was included as an outgroup and SBW25 and F113 were also included for comparison. The phylogenetic tree of the *gyrB* sequences shows that the isolates grouped into two clusters: group A, with the least antagonistic isolates, as well as group B, which was primarily made up of *Gt*-antagonistic isolates (Figure 3). This observation implies that the antagonistic status of isolates is deep-rooted and associated with the core genome of *Pseudomonas*, as it can be discriminated by phylogeny when based on a housekeeping gene whose function is unrelated to *Gt* suppression. In group A, there were 12 isolates, 9 derived from year 1 Hereward and 3 from year 1 Cadenza, while group B comprised 13 isolates, 9 from year 1 Cadenza and 4 from year 1 Hereward (Table 3). We also correlated the relationship between *Gt* inhibition, presence or absence of *wsm*/*fecB* as well as *gyrB* genotype for these 25 isolates (12 derived from first-year Hereward and 13 from first-year Cadenza). It was found that 85% of the first-year Cadenza-derived isolates possessed the *fecB* gene, whereas for first-year Hereward-derived isolates this was only 42%. However, for the *wsm* gene, 92% and 75% of first-year Cadenza and Hereward isolates, respectively, possessed this gene.

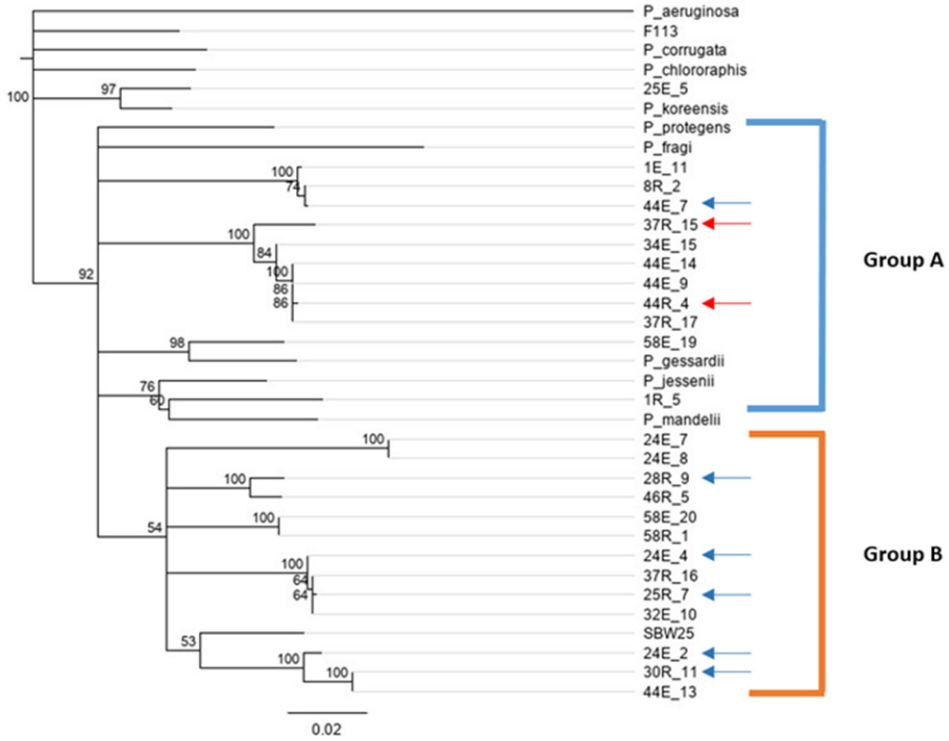


Figure 3. *gyrB* phylogeny of the 25 *Pseudomonas* isolates from field GH2 in comparison with *Pseudomonas fluorescens* SBW25 and F113. *Pseudomonas aeruginosa* PAO1 was used as an outgroup. The tree was constructed using the Neighbour Joining Method after alignment with MUSCLE using Geneious Prime. The numbers on the nodes indicate bootstrap probabilities. The bar is the nucleotide substitution per site. The six most antagonistic isolates are indicated with blue arrows and the two least antagonistic isolates are indicated with red arrow. Group A represents the least antagonistic isolates, group B represents the most antagonistic isolates. Supplementary Table S3 describes the isolate naming pattern.

3.5. In Planta *Take-All Disease Control Assays*

Six *Pseudomonas* isolates displaying strong *Gt* antagonism as well as two control isolates with poor *Gt*-suppression capacity were selected for *in planta* assays with cultivars Hereward and Cadenza in the presence or absence of *Gt*. In addition, a mixture of all six antagonistic isolates as well as a no-bacterial-inoculant treatment (sterile) was set up. When wheat was grown without *Gt* inoculation, the roots were healthy, and no blackening was observed. The mixture of six isolates reduced the number of infected roots in comparison to the no-*Pseudomonas* (sterile) control (Figure 4). There was a significant difference in the number of infected roots between the two cultivars in the presence of the isolates ($F = 0.031$) (Table S11). In the *Gt*-only infected treatments, the mean disease levels were high regardless of cultivar: those for Cadenza were 11.754 (s.e. = 93.6) and those for Hereward 11.762 (s.e. = 75.57). When assessing the prediction from the regression model, inoculation of either wheat cultivar with isolates 24E/4 and 30R/11 led to a reduced root infection for Cadenza and Hereward (Table S12). Although both isolates were derived from year 1 Cadenza selection, this finding demonstrates that isolates selected by Cadenza can provide protection to another wheat cultivar. With regard to the control inoculants, it was found that 37R/15 was unable to prevent *Gt* disease; however, control isolate 44R/4 resulted in reduced disease for both cultivars.

Table 3. The identification was based on BLAST outcomes in NCBI. Class: inhibition zone class 1 (0–0.5 cm), class 2 (0.5–1 cm) and class 3 (>1 cm); Y1: year 1; Y2: year 2.

No	Isolate	Class	Y1	Y2	*wsm*	*fecB*	Identified as
	25E/5	1	C	Xi	0	1	*Pseudomonas* R-42020
	1E/11	2	H	H	1	1	*Pseudomonas fluorescens*
	8R/2	1	C	H	1	1	*Pseudomonas orientalis*
	44E/7	3	H	H	1	1	*Pseudomonas orientalis*
	37R/15	1	H	H	1	0	*Pseudomonas* sp. GH1-PS70
	34E/15	2	H	Xi	1	1	*Pseudomonas* sp. RZ109
	44E/14	1	H	H	1	0	*Pseudomonas marginalis*
	44R/4	1	H	H	1	0	*Pseudomonas* sp. Ra3
	44E/9	3	H	H	1	0	*Pseudomonas marginalis*
	37R/17	1	H	H	1	0	*Pseudomonas* sp. RZ109
	58E/19	1	C	H	0	0	*Pseudomonas* sp. GH1-PS43
	1R/5	1	H	H	0	0	*Pseudomonas* sp. GH1-PS83
	24E/7	3	C	Xi	1	1	*Pseudomonas poae*
	24E/8	1	C	Xi	1	1	*Pseudomonas poae*
	28R/9	3	H	Xi	1	1	*Pseudomonas orientalis*
	46R/5	3	C	H	1	1	*Pseudomonas orientalis*
	58E/20	2	C	H	1	1	*Pseudomonas* sp. GH1-PS43
	58R/1	1	C	H	1	1	*Pseudomonas* sp. GH1-PS43
	24E/4	3	C	Xi	1	1	*Pseudomonas salmonii*
	37R/16	2	H	H	1	0	*Pseudomonas fluorescens*
	25R/7	3	C	Xi	1	1	*Pseudomonas* sp.
	32E/10	3	H	H	1	1	*Pseudomonas poae* 36C8
	24E/2	3	C	Xi	1	1	*Pseudomonas* sp. R-41739
	30R/11	3	C	Xi	0	0	*Pseudomonas orientalis*
	44E/13	2	H	H	1	1	*Pseudomonas fluorescens*

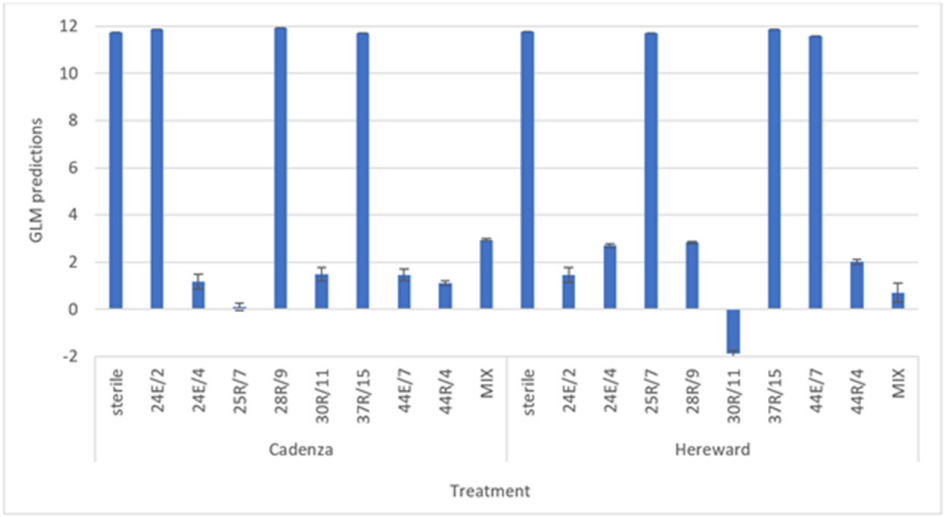


Figure 4. Effect of *Pseudomonas* isolates on 21-day-old wheat root seedlings infected with *Gaeumannomyces tritici* (*Gt*), cultivar Cadenza and Hereward. Treatments included *Gt* with sterile seeds and in combination with the isolates 24E/2, 24E/4, 25R/7, 30R/11, 28R/9, 44E/7, mixture of the six along with two least effective antagonists 37R/15 and 44R/4. Values are the mean of 3 replicates and error bars are the standard error of the mean. GLM: generalised linear model.

4. Discussion

Here, we show that first-year Cadenza cropping selects for pseudomonads with the ability to suppress *Gt* to a greater extent than isolates from first-year Hereward in the subsequent wheat crop. There are a wide range of mechanisms deployed by pseudomonads to antagonise *Gt*. In many fluorescent *Pseudomonas* spp., PCA and Phl antibiotics have been identified as the main determinants of biocontrol against soil-borne plant pathogens such as Take-all disease [21]. None of the isolates tested in this study had any of these antibiotic genes, suggesting these are not the only modes of action for *Gt* suppression by pseudomonads. Indeed, Yang [10] reported that 13 fluorescent *Pseudomonas* isolates controlled *Gt in vitro* and under greenhouse conditions, and none of the isolates had *phlD*, *prnC* and *pltB*, the genes involved in the biosynthesis of DAPG, pyrrolnitrin and pyoluteorin antibiotics, respectively. Furthermore, Yang [5] showed that a viscosin-like cyclic lipopeptide of *P. fluorescens* strain HC1-07 was linked to growth inhibition of *Gt*. However, wheat-seed treatment with *P. synxantha* (formerly *P. fluorescens*) 2-79, which naturally produces the antibiotic phenazine-1-carboxylic acid and encodes the biosynthesis of the antibiotic pyrronitrin, suppressed both Take-all and *Rhizoctonia* root rot of wheat [22]. The correlation analysis of phenotypic loci by Mauchline [15] placed *Pseudomonas* populations into two groups: those that were primarily derived from first-year Hereward and effective for actinomycete suppression, with genes for viscosin, type IV pili, Wsm lipopolysaccharide production and LapA/BapA adhesin operons, whereas those primarily from first-year Cadenza had genes for hemophore, acetoin catabolism, as well as siderophore production and secretion, but with limited predicted antibacterial capability. Mazzola and Gu [23] investigated a range of wheat cultivars for the establishment of *Rhizoctonia*-suppressive apple orchard soils. They highlighted that host genotype is an important factor in establishing disease-suppressive soils through supporting the colonisation of a specific population of pseudomonads. The finding that more isolates that we identified as class 3 inhibitors were found in pseudomonads isolated from first-year Cadenza plants, and that more isolates of class 1 inhibitors were found in pseudomonads isolated from first-year Hereward plants suggests that the observed bacterial selection is implicated in the *in planta* antagonism of the Take-all fungus, and that host genotype is an important factor in our study. In addition, the *wsm* locus was more associated with isolates derived from first-year Hereward, while *fecB* was more associated with isolates derived from first-year Cadenza. As such, the first-year wheat seems to be implicated in the selection of the associated *Pseudomonas* spp. in our experimental system, which agrees with the results of the correlation analysis by Mauchline [15]. In addition, we found that more *fecB*-encoding isolates were present in the rhizosphere than the endosphere of first-year Cadenza-derived plants and that this trend was reversed for first-year Hereward-derived plants. One possible explanation for this is that in a L-TAB situation the fungal inoculum is controlled in the rhizosphere environment and does not enter the root system to cause disease. However, in a H-TAB situation, where there is a higher fungal load in the root system, it is possible that the host plant selects for a higher proportion of *fecB*-encoding microbes in this niche as a last line of defence. The observation that 72% of class 3 *Gt* antagonists possess the *fecB* gene as opposed to 42% of class 1 antagonists provides evidence that the *fecB* gene is implicated in the control of *Gt* and that the microbially mediated suppression of the fungus in the L-TAB environment is not necessarily dependent on the production of secondary metabolites such as DAPG or phenazine. This observation could prove to be important if bacterial antibiotic molecules are also phytotoxic. As such, the competition for nutrients offers an alternative strategy for pathogen biological control. The phylogenetic analysis of the *gyrB* gene showed that the isolates with the largest inhibition zones grouped separately from those with smaller inhibition zones. It was found that 11/13 of group B isolates were antagonistic to *Gt* and that 9 out of the 11 antagonistic isolates in group B possessed the *fecB* gene. In comparison, in group A 4/11 isolates were antagonistic to *Gt*, with all 4 of these isolates possessing the *fecB* gene. These observations suggest that the presence of *fecB* is associated with *Gt* suppression and that an interaction with first-year wheat cropping

regime influences this phenomenon. The presence or absence of *fecB* could be used to develop a possible molecular indicator of *Gt* antagonism potential in a given isolate, or in a microbial community. Furthermore, this grouping agreed with the inhibition zone data, which showed that isolates from first-year Cadenza had larger inhibition zones than those from first-year Hereward. In addition, isolates derived from the (H, H) planting scheme were associated with the smallest *Gt* inhibition zone. This suggests that Cadenza selects for antagonistic *Pseudomonas* isolates in the development of the L-TAB status. Given the selective association of highly *Gt*-antagonistic species of *Pseudomonas* with Cadenza, which are also positive for the *fecB* genotype, it follows that iron deprivation could be implicated as a mechanism for Take-all disease control within the rhizosphere. Comparative genomics, transcriptomics and mutagenesis of candidate genes for isolates designated as inhibitory to *Gt* will enhance understanding of factors leading to differential fungal inhibition.

The *in planta* study showed that regardless of wheat cultivar, inoculation with the synthetic community of six isolates resulted in a consistent reduction in *Gt* disease for both wheat cultivars. This might be due to a combined or synergistic effect achieved by the mixture. We also found that S4E/4, 30R/11 and 44R/4 were consistently able to suppress disease build up. It was interesting that the control isolate 44R/4 (*fecB*-negative and did not suppress *Gt in vitro*) was associated with reduced *Gt* disease *in planta* regardless of crop genotype. This isolate could be associated with alternative mechanisms of *Gt* suppression, such as activation of innate plant defence pathways, and highlights the importance of a holistic approach to biocontrol of plant pathogens. However, Mehrabi [24] demonstrated that increased richness of *Pseudomonas* mixtures resulted in a reduction in Take-all inhibition under in vitro conditions. This was thought to be due to increased competition between isolates. In our study, isolates 24E/2, 24E/4, 25R/7, 28R/9 and 44E/7, which were among the most antagonistic isolates, gave positive PCR diagnostic bands for *wsm* and *fecB* loci, indicating the importance of these loci in host colonisation and pathogen suppression, respectively. In addition, the *gyrB* phylogeny indicated a clustering of five of these isolates in group B, indicating high genetic similarity between these isolates (i.e., low species richness), and the high Take-all suppression with the most complex inoculum suggests that they are not antagonistic to each other. However, more research is required to determine whether or not this is the case. On the other hand, Jousset [25] stated that genetically dissimilar microbial communities are better equipped to combat pathogen invasion by providing greater resource competition through more efficient utilisation of resources and that increasing the genotypic richness could also result in a greater range of antifungal metabolite production. As such, it is not clear what the best strategy is for the development of synthetic microbial communities for *Gt* suppression.

Interestingly, Yu [26] demonstrated that deletion of a 90 bp sequence upstream of the divergently orientated *phzX* and *phzR* promoters resulted in the up-regulation of phenazine production and associated improvement in Take-all disease inhibition, biofilm formation, extracellular DNA release and suppression. As such, an alternative approach for biocontrol could be the amendment of pseudomonads to be more effective nutrient scavengers. For example, transformation of microbes with genes for high-affinity metal-uptake ABC transporters is an alternative and more subtle way of enhancing inoculants as opposed to diversifying the secondary metabolite production of inoculants. This could be a good strategy, as microbes better equipped for nutrient scavenging would likely be highly competitive rhizosphere colonisers and potentially suppress pathogen development through niche exclusion. However, it is likely that many biotic and abiotic factors need to be considered and in future work it will be interesting to explore relationships between *Pseudomonas* isolate phylogenetic distance and compatibility for the optimisation of synthetic microbial communities for *Gt* suppression.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/crops3030019/s1, Table S1. Field layout of Great Harpenden 2 (GH2) at Rothamsted in the first year.; Table S2. Field layout of Great Harpenden 2 (GH2) at Rothamsted in the second year; Table S3. Inhibition zone class data for the 411 bacterial isolates on *Gaeumannomyces tritici*; Table S4. Great Harpenden 2 (GH2) treatment structure; Table S5. Accumulated analysis of deviance from generalised linear model of *Pseudomonas* isolates' inhibition zone class on *Gaeumannomyces tritici*; Table S6. Table of predicted means from generalised linear model analysis for *Pseudomonas* isolates' inhibition zone class on *Gaeumannomyces tritici*; Table S7. ANOVA analysis of averaged *Pseudomonas* isolates' inhibition zone on *Gaeumannomyces tritici* per plot; Table S8. Table of means from ANOVA analysis of averaged *Pseudomonas* isolates' inhibition zone data on *Gaeumannomyces tritici* per plot; Table S9. Generalised linear model table of accumulated deviance for *Pseudomonas* isolates' *wsm* gene PCR outcome; Table S10. Generalised linear model table of accumulated deviance for *Pseudomonas* isolates' *fecB* gene PCR outcome; Table S11. Accumulated analysis of deviance from generalised linear model on *Gaeumannomyces tritici*-infected wheat root data; Table S12. Prediction from the regression model on the reduction of *Gaeumannomyces tritici* wheat root infection by *Pseudomonas* isolates; Figure S1. Proportion of *Gaeumannomyces tritici* inhibiting *Pseudomonas* isolates associated with the four planting combinations, (c, h), (c, x), (h, h) and (h, x); Figure S2. Proportion of *Gaeumannomyces tritici* inhibiting *Pseudomonas* isolates associated with first-year wheat cultivars Cadenza (c) or Hereward (h) and niche; Figure S3. Proportion of *Gaeumannomyces tritici* inhibiting *Pseudomonas* isolates possessing *wsm* (a) and *fecB* (b) genes associated with first-year wheat cultivar Cadenza (C) or Hereward (H) and niche.

Author Contributions: M.A.Z., V.M., T.H.M. and R.W.J. conceived the study. M.A.Z. performed the experiments. M.A.Z., M.R., T.H.M. and V.M. performed data analysis. M.A.Z., M.R., V.M., L.J.S., K.H.-K., J.G.M., T.H.M. and R.W.J. contributed to the experimental design, interpretation of results and preparation of the article. M.R., M.A.Z. and T.H.M. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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