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Pseudomonas spp. Producing Antimicrobial Compounds Regulate Fungal Communities Inhabiting Wheat Crown in Southern Chile

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Abstract: The 2,4-Diacetylphloroglucinol (2,4-DAPG) and phenazine (PCA)-producing *Pseudomonas* inhibit wheat pathogens' development, but the relationship between communities of pathogens and genotypes of these bacteria has been little studied. Relationships between wheat crown fungi associated with the presence of 2,4-DAPG and PCA-producing pseudomonads were evaluated in four commercial wheat crops located in the La Araucanía and Los Lagos Regions of Chile, during two crops seasons. Portions of the base of the first internode of the culm collected during the grain-filling stage were cultured in an artificial medium for fungal isolation, while roots of the same wheat plants and from plants collected previous harvest, and also used to assess yield and plant height, were used for the detection of 2,4-DAPG and PCA-producing *Pseudomonas* spp. using PCR with specific primers. Genera *Phaeosphaeria*, *Fusarium*, *Rhizoctonia*, and *Microdochium* were repeatedly isolated (52.6%, 22.1%, 7.8%, and 4.9%, respectively) and the genetic composition of 2,4-DAPG and PCA-producing *Pseudomonas* spp. varied between fields and sampling periods. Genetic groups A, B, D, K, L, and P associated with the *phlD* gene were detected. The presence of 2,4-DAPG-producing bacteria benefited crop health, relating their existence with increasing yield and plant height, and the reduction in the incidence and severity of disease caused by pathogenic microorganisms on the first internode of wheat culms.

Keywords: *Pseudomonas*; 2,4-DAPG; phenazine; wheat

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1. Introduction

The development of efficient strategies for managing diseases in plants requires a comprehension of the relationship between pathogens and other organisms and the environment in which they live. Geographic location, climate, cultural practices, relationships among microorganisms, and environmental conditions, are all factors influencing the fungal diversity and the distribution of plant ecosystems [1–4].

Soilborne pathogens are difficult to control and management practices, such as crop rotation, use of resistant varieties, and fungicides, have not been highly effective in controlling different crown and root rot pathogens [5–8]. Therefore, the use of antagonistic microorganisms to control diseases caused by these fungi in wheat might be an efficient alternative for its management [3,8–13]. Among the most important groups of antagonistic microorganisms to reduce crown and root rot diseases in wheat are the bacteria of the genus *Pseudomonas*. These bacteria include widely described species of the *P. fluorescens* group [4,14,15], which suppress soil pathogens, mainly through the production of diverse classes of antibiotics, such as 2,4-diacethylphloroglucinol (2,4-DAPG), phenazine-1-carboxylic acid (PCA), among others [4,9,12,15–17]. These bacteria have a worldwide

distribution [18,19], with fluctuations in population size and diversity depending on the geographical location, type of soil, plant species present in the rotation, and the wheat cultivar used [12,20,21]. The existence of different genetic groups based on the gene *phlD* in the *P. fluorescens* complex has also been determined [14,16,22–24].

Despite the potential of these bacteria in the management of soil phytopathogens, there is no current information on the implication or role played by these microorganisms in the suppression of diseases in wheat crops in South America. In Chile, around 1.5 million tons of wheat is produced on approximately 250,000 hectares. This surface area is mainly concentrated among the Biobío (36°46′ S–73°03′ W), Araucanía (38°54′ S–72°40′ W), Los Ríos (39°48′ S–73°14′ W), and Los Lagos (41°28′ S–72°56′ W) regions. This area is characterized by having soils derived from volcanic ashes (Andisols) that have a high content of organic matter and low pH [25]. These aspects make these soils uncommon around the world. This aspect is relevant when taking into account the recent detection of 2,4-DAPG and PCA-producing bacteria in wheat fields located in this area [26,27]. On the other hand, a recent study evaluated both the distribution and prevalence of the pathogens that cause crown and root rot diseases in commercial wheat crops in southern Chile (Araucanía to Los Lagos region), demonstrating that the severity of these diseases varied between 1.3% and 80% in individual fields. In addition, the most common fungi isolated from individual stems, corresponded to members of the *Gaeumannomyces graminis*, *Fusarium*, and *Phaeosphaeria* (13.9%) complexes [28]. Differences observed in these fields for the populations of fungi present in the first internode of the wheat plants could be influenced by the existence of populations of 2,4-DAPG and PCA-producing bacteria, which might regulate the presence or absence of these phytopathogens in those ecosystems.

Few field studies have evaluated how population dynamics of the 2,4-DAPG and PCA-producing bacteria are environmentally related to both the incidence and severity of different fungi that inhabit the wheat roots, crown, and first internode [29–31]. This information would provide relevant information to elaborate biological control strategies for the management of soilborne pathogens. This research was conducted to establish the relationships between species of soilborne fungal pathogens and populations of 2,4 DAPG and PCA-producing *Pseudomonas* spp. present in four commercial wheat fields in southern Chile. In order to achieve this, species of fungi that colonize the crown and first internode of wheat during the grain-filling stage were identified and quantified. In parallel, the species of 2,4-DAPG-producing *Pseudomonas* spp. and their genetic diversity were identified based on the use of allele-specific primers of *phlD*. Several environmental relationships between bacterial antagonists and fungal phytopathogens isolated from this micro-ecosystem that corresponds to the base of the stem of the wheat plant were determined.

2. Materials and Methods

Sampling protocol: Wheat samples were collected from four fields located between 38°22′41.42″ and 40°41′48.36″ South latitude. Two intensive sample surveys were performed during the season 2012/2013 in fields of the Araucanía and Los Lagos Regions (Potrero 11-Perquenco and Retén-Osorno). Two other survey samplings were conducted during the season 2013/2014 in the Araucanía Region in the fields Potrero 13-Perquenco and Cajón-Temuco (Table 1).

In each field, a first sampling was performed during the grain-filling stage (Feekes stage 10.1–10.5) to determine the species of fungi associated with the crown and the 2,4 DAPG and PCA-producing bacteria. Prior to harvest (Feekes stage 11) the presence of 2,4 DAPG-producing bacteria and yield parameters, such as productivity (Ton ha^{-1}) and plant height, were determined. The method consisted of a modification of that proposed by Moya-Elizondo et al. [32]. Briefly, at each sampling point, whole wheat plants, including tillers and roots up to 25 cm deep, were considered. A total of 24 samples of 1 linear m within an 800 m transect were collected. The transect started 30 m from the edge of the crop, and samples were taken every 30 m approximately, varying its direction depending

on the topographical conditions of each field. All collected samples were stored at 4 °C until being evaluated.

Table 1. Description of the crop location, season, cultivars, crop rotation, and pH range of the soil of the intensively surveyed fields evaluated in southern Chile for this research.

Sampled Farm-Location (Season)	Coordinate	Cultivar	Crop Rotation [1]	Soil pH
Potrero 11-Perquenco (2012/13)	38°22′41,42″ S–72°28′25,59″ W	Ikaro	W-O-W	5.6–5.8
El Reten-Osorno (2012/13)	40°41′48,36″ S–73°15′57,25″ W	Maxi	W-T-W	5.4–5.6
Cajón-Temuco (2013/14)	38°40′04,40″ S–72°29′56,38″ W	Impulso	T-L-T	5.8–6.0
Potrero 13-Perquenco (2013/14)	38°23′05,00″ S–72°28′20,95″ W	Bakan	O-L-T	5.6–5.8

[1] W: Wheat (*Triticum aestivum* L.); O: Oat (*Avena sativa* L.); T: Triticale (X *Triticosecale* Wittmack) and L: Lupine (*Lupinus angustifolius* L.).

Prevalence of diseases and damage severity in the stems of each crop: Previously the 24 collected samples from each field were individually separated and washed. Subsequently, 20 culms from each sample with their respective root systems were randomly selected and leaves cleaned to assess disease incidence and severity (480 stems per field). Incidence was determined by counting the number of symptomatic culms from the total stems selected in each sampling point. Severity was determined by using a crown rot rating scale described by Moya-Elizondo et al. [28], which is based on the extent of the culm-darkness on the first internode of each tiller, where: 1 = 0% or without symptoms; 2 = 1 to 25%, 3 = 25 to 50%, 4 = 50 to 75%, and 5 = 75 to 100% of blackening on the first internode of the culm. Additionally, an internode discoloration severity index (IDSI) was calculated by adding the number of plants in each category class, multiplied by the value for each category and dividing this sum by the total number of plants × 5 (number of categories), and finally multiplying the total number by 100 in order to create an IDSI for each sample [28].

Biodiversity of fungi associated with wheat crown: In order to isolate the fungi associated with wheat plants during the grain-filling stage, a segment of 3 mm was removed from the basal part of the first internode of each one of the selected 20 wheat culms of each sample and they were used to assess the presence of fungal pathogen species through culturing on a general media of 20% Potato Dextrose Agar amended with 2% of lactic acid (aPDA). Prior to plating, culm segments were disinfected in 1% sodium hypochlorite for 1 min and rinsed three times in sterile distilled water. Five disinfected segments were placed on a Petri plate with 20% aPDA, using four plates per sample (96 plates per field = 480 stem segments per field). Plates were incubated at 24 ± 1 °C and monitored daily for fungal growth. The resulting fungal colonies were individually re-isolated on PDA to develop monoxenic cultures by taking hyphal tips. Isolates were identified at the genus level, based on their morphological and cultural characteristics, by using taxonomic keys described by Barnett and Hunter [33]. *Fusarium* isolates were grouped according to their morphological characteristics and identified at the species level by means of the keys described by Nelson et al. [34] and Leslie and Summerell [35]. In parallel, complementing the fungal identification, mycelium from some unidentified fungal isolates was suspended in TE buffer pH 8.0 and stored in 1.5 mL microtubes at 4 °C for subsequent DNA extraction. DNA extraction was performed by means of the method described by Montalva et al. [36]. Fungal DNA was amplified by Polymerase Chain Reaction (PCR) with universal primers 18SF2/pITS4 of the region ITS. This reaction was carried out in a volume of 25 µL per sample. Each sample contained 1 × buffer PCR, $MgCl_2$ 2 mM, 200 µM dNTP, 0.4 µM of each primer, 2 U of Taq polymerase DNA (Invitrogen, Carlsbad, EE.UU.), sterile distilled water, and 25 ng μL^{-1} of fungal isolate DNA. The thermal profile

of the 35 PCR cycles (MultiGeneTM Gradient Thermal Cycler, Labnet International Inc., Woodbridge, New Jersey, USA) consisted of an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 57 °C for 45 s, 72 °C for 60 s, and the final elongation at 72 °C for 5 min. PCR products were run in 1% agarose gels 0.1 µL ml^{-1} of ethidium bromide in 0.5 × TBE buffer at 100 V for 20 min. Subsequently, amplicons were observed and photographed in a UV light transilluminator. The identification of *Fusarium* isolates was corroborated through the utilization of specific primers and methods described by Scott et al. [37]. Isolates from the genus *Gaeumannomyces* were confirmed by means of the specific primers NS5 and GGT.RP and protocols described by Fouly and Wilkinson [38].

PCR products resulting from DNA amplification of the fungi were submitted for sequencing in both directions to MACROGEN Laboratories (Seoul, Korea). Sequences obtained for each genomic region were compared in their homologies with those existing in the database of the European Bioinformatics Institute (www.ebi.ac.uk) and in the BLAST public database present on the website of the National Center of Biotechnology Information (NCBI) of the United States (http://www.ncbi.nlm.nih.gov/BLAST/ (accessed on 26 January 2022).

Detection and quantification of bacterial populations of 2,4 DAPG and PCA-producing *Pseudomonas* spp.: Root samples of the previously selected wheat plants were used to identify and to detect 2,4-DAPG and PCA producing *Pseudomonas* spp., by means of a PCR-based dilution end-point assay proposed by McSpadden Gardener et al. [39]. The detection of 2,4-DAPG-producing *Pseudomonas* spp. was performed by direct PCR based on the specific primers B2BF/BPR4, designed for identifying *phlD*, which is key to the production of this antibiotic [40]. In order to identify the populations of PCA-producing bacteria, the specific primers PCA2a/PCA3b designed to identify the gene *phzCD* involved in PCA synthesis were used, based on protocols described by Raaijmakers et al. [40]. As positive or negative controls for the presence of *phlD*, strains of *P. fluorescens* Q8r1 and Woody1R (W1R) were used. On the other hand, for *phzCD*, strain 2–79 was used. *phlD* and *phzCD* control strains were grown in 1/3KB broth at 30 °C for 24 h and stored in 1.5 mL micro-centrifuge tubes containing 300 µL of sterile glycerol at −80 °C.

Genotypes of the populations of *Pseudomonas* with the gene *phlD+* were determined by use of the method proposed by De La Fuente et al. [18], based on amplification of the allele-specific PCR of *phlD*. This technique allows the detection of genotypes A, B, D, K, L, and P of 2,4-DAPG-producing *Pseudomonas*, by means of the utilization of the set of primers A-align/A-gen-exten, B-genlong/B2BF, D-gen-2/B2BF, K-gen/BPR4, L1-extent/B2BF, and P-gen/BPR4, respectively.

All primer sequences used in this research are available in Supplementary Table S1.

Statistical analysis: Ecological relationships associated with fungal communities isolated from the first internode of the wheat plants were determined through the calculation of the Shannon–Wiener Index and the ecological indices of abundance and evenness. Differences in the incidence and severity of damage on the culms were analyzed by using the Kruskal–Wallis non-parametric sum rank test. The frequency of pathogens and bacterial populations determined from the 24 sampling points was compared between fields with the Chi-square test (χ^2). Spearman correlation tests were used to determine relationships between the incidence and severity of the disease with yield parameters and plant height, as a function of the populations of 2,4 DAPG and PCA-producing bacteria. These non-parametric correlations were determined during grain fill and harvest and considered the total of samples, years of separation, and individual fields. Moreover, both the incidence and severity of the disease in the wheat culms were correlated with the diversity indices of Shannon–Wiener obtained for each sampling point as a function of positive and negative sampling points for 2,4 DAPG and PCA bacteria. These evaluations were performed through the package "Rcmdr" of the R-Software (R Foundation for Statistical Computing, Vienna, Austria). In order to explore relationships between groups of fungi present in the fields under study, a principal component analysis was performed, based on the presence or absence of *phlD*. Thus, the function "princomp" of the 'stats' package was

used. All statistical analyses were performed with the statistical packages of R-Software (http://www.r-project.org/ (accessed on 1 October 2017), R Foundation for Statistical Computing, Vienna, Austria).

3. Results

The percentage of samples with the presence of fungi in the first internode of wheat in four commercial fields in southern Chile during the grain-filling period was 67.9% (n = 1920), where fungi of the genus *Phaeosphaeria* were the most common (54.8% of total isolates), followed by *Fusarium* spp. (21.4% of total isolates) (Figure 1).

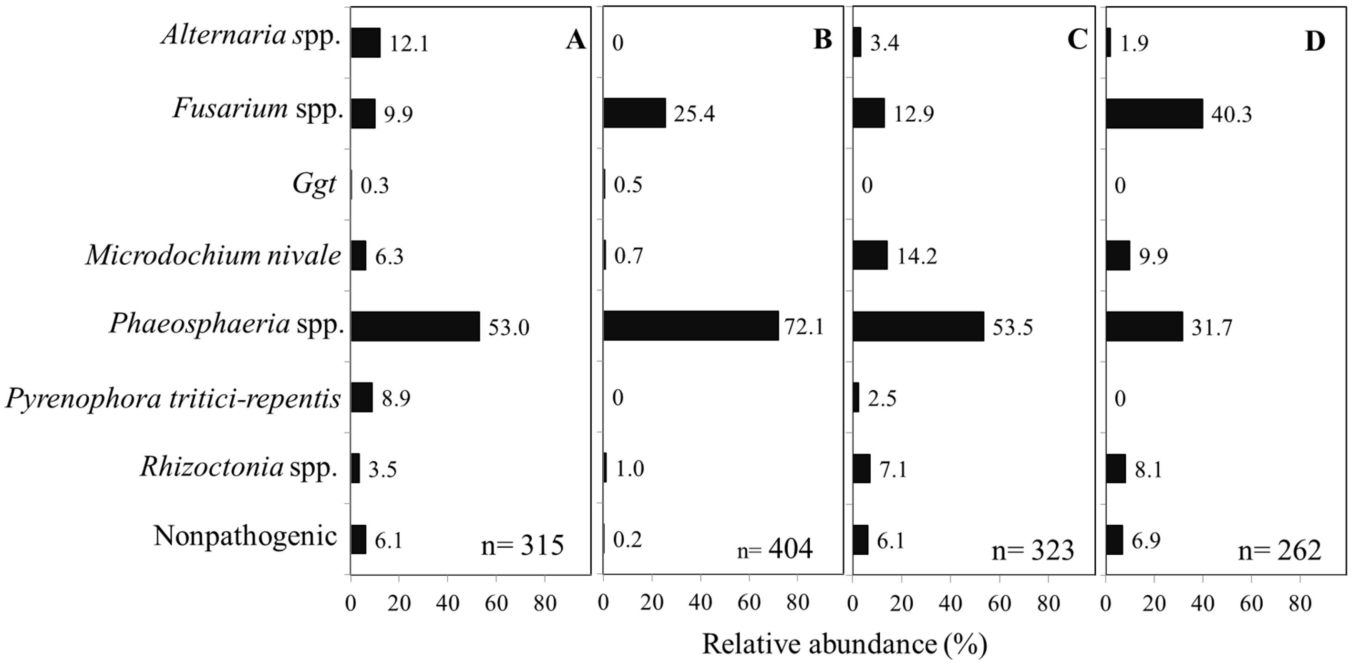

Figure 1. Relative abundance (%) of the fungal species isolated from the first internode of wheat culms on 20% acid PDA obtained from four intensively sampled fields in southern Chile during the grain-filling period: ((**A**): Potrero 11-Perquenco, (**B**): El Retén-Osorno, (**C**): Potrero 13-Perquenco, and (**D**): Cajón-Temuco). N = total isolates obtained from 24 sampling points collected by field. In each sampling point, 20 culms were selected to be analyzed. A sampling point corresponded to 1 linear m of whole wheat plants, including tillers and roots up to 25 cm deep, collected from each field within an 800 m transect.

Other recurrently isolated genera were *Alternaria* (4.35%) and *Microdochium nivale* (7.78%). Species of the genus *Rhizoctonia* were isolated from all the analyzed sites in percentages not exceeding 8% of relative abundance. Molecular analyses by amplification of the regions ITS allowed for identifying the presence of the species *Ceratobasidium cereale* D. Murray & L.L. Burpee (anamorph = *R. cerealis* van der Hoeven) and *Waitea circinata* Warcup & Talbot (anamorph = *R. oryzae* Ryker & Gooch) in the analyzed samples. *G. tritici* (Ggt), the causal agent of the take-all disease of wheat, was the pathogen isolated with the lowest frequency and the only positive samples were observed in fields sampled during the season 2012–2013 (Potrero 11-Perquenco and El Retén-Osorno). The frequency of Ggt was under 0.5%. Fungal genera generally considered to be non-pathogenic and saprophytic included *Acremonium*, *Chaetomium*, *Cladosporium*, *Cylindrocladium*, Mucorales, *Trichoderma*, *Ulocladium*, and *Zygorinchus*. Other nonsporulating fungi were isolated, but their importance was low, despite representing values close to 6% of the total isolated fungi. Sequences accessions of the fungal microorganisms identified in this research are available in the GenBank under numbers OM964671-OM964715.

Due to the importance represented by the genus *Fusarium* in the first internode of wheat culms, identification at species level was conducted, based on morphological analyses using taxonomic keys and shape of both macroconidia and microconidia. Such diagnosis was supported by the amplification of specific primers of some *Fusarium* isolates that presented similar characteristics of color and growth on PDA medium. The presence of several species was determined (Table 2), where *F. dimerum* (8.1%), *F. culmorum* (2.6%), *F. avenaceum* (2.5%), *F. oxysporum* (1.9%), *F. acuminatum* (1.5%), *F. graminearum* (1.3%), and *F. solani* (0.4%) were highlighted. Other species isolated from the first internode that corresponded to rare or saprophytic species in wheat plants, such as *F. chlamidosporium*, *F. equiseti*, *F. lateridium*, *F. proliferatum*, *F. semitectum*, and *F. subglutinans,* were isolated. Other *Fusarium* species that could not be identified, despite averaging 3.9% of the total, had a low level of importance within the sampling performed in the studied fields.

Table 2. Percentage of *Fusarium* isolated from the first internode of wheat culms obtained from four intensively sampled fields in southern Chile during the grain-filling period.

Sampled Farm-Location (Season)	*Fusarium* spp. [1] (%)							
	Fac	**Fav**	**Fc**	**Fdim**	**Fg**	**Fo**	**Fs**	**F. spp**
Potrero 11-Perquenco (2012/13)	0.6 [2]	1.3	0.0	2.9	1.3	0.6	0.3	2.9
El Retén-Osorno (2012/13)	0.0	2.5	2.5	7.4	3.7	0.0	1.0	8.3
Cajón-Temuco (2013/14)	2.2	1.2	0.60	6.8	0.0	0.3	0.0	1.8
Potrero 13-Perquenco (2013/14)	3.1	5.0	7.3	15.3	0.0	6.5	0.4	2.7
Mean	1.5	2.5	2.6	8.1	1.3	1.9	0.4	3.9

[1] Species of *Fusarium* isolated: Fac = *F. acuminatum*; Fav = *F. avenaceum*; Fc = *F. culmorum*; Fdim = *F. dimerum*; Fg = *F. graminearum*; Fo = *F. oxysporum*; Fs =*F. solani* y F.spp = other species of *Fusarium* less frequent, saprophytic, or weak pathogens, such as *F. sporotrichioides*, *F. scirpi*, *F. equiseti*, *F. lateridium*, *F. proliferatum*, and *F. semictectum*, among others. [2] Percentages were determined from total fungal isolates which were obtained from 24 sampling points collected by field. In each sampling point, 20 culms were selected to be analyzed. A sampling point corresponded to 1 linear m of whole wheat plants, including tillers and roots up to 25 cm deep, collected from each field within an 800 m transect.

The diversity of the fungal community that colonized the first internode of the wheat plant and which was able to be cultured on aPDA was represented on the basis of the Shannon–Wiener diversity index and values of abundance and evenness of species (Table 3). The greatest diversity of fungus was presented in the Cajón-Temuco field, whereas that located in El Retén-Osorno had a lower diversity index value (2.33 and 1.39, respectively). The opposite results were observed in the abundance of species, where El Retén-Osorno presented the highest values with 404 isolates compared, for instance, with Potrero 13 Perquenco, which obtained the lowest values with 262 fungal isolates. On the other hand, the evenness was greater in geographically closer fields, such as Potrero 11 and 13 from Perquenco and Cajón-Temuco than that from El Retén-Osorno. Statistically significant differences between fields were only observed for the abundance according to the χ^2 ($p < 0.001$) test.

Both the incidence and severity of damage on the first internode of the stem assessed during the grain-filling period presented significant differences among the fields and between sampling years ($p < 0.05$) (Figure 2). The incidence of damage caused by phytopathogens in the first internode varied between 33% and 97%, whereas the severity of the disease (IDSI) presented a variation between 9% and 48%. Fields sampled during the season 2012/2013 presented a greater incidence of affected culms than those collected during the season 2013/2014.

Table 3. Indices of abundance and evenness of fungus associated with the crown or the first internode of wheat culms observed in four intensively sampled fields of southern Chile during the grain-filling period.

Sampled Farm-Location (Season)	Shannon–Wiener (H) Diversity Index	Abundance [1]	Evenness (E) [2]
Potrero 11-Perquenco (2012/13)	2.11	315	0.70
El Retén-Osorno (2012/13)	1.39	404	0.46
Cajón-Temuco (2013/14)	2.33	323	0.74
Potrero 13-Perquenco (2013/14)	1.81	262	0.59
Mean	1.91	326	0.62
χ^2 (*p*-value)	ns [3]	<0.001	ns

[1] Abundance represents the total number of isolates in each sector. [2] Evenness was calculated as H/LnS, with H = Shannon–Wiener diversity index and LnS = natural logarithm of species richness "S" (number of species of taxonomic of interest). [3] ns: nonsignificant.

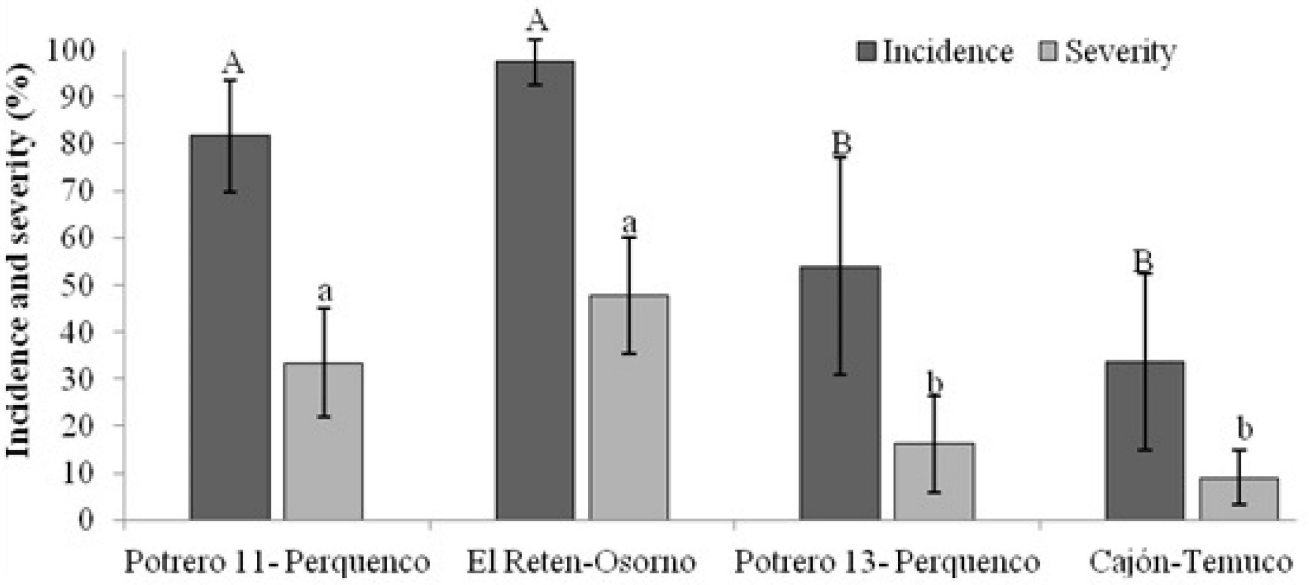

Figure 2. Incidence and severity of microorganisms causing culm-darkness of the first internode in wheat plants obtained from four intensively sampled fields in southern Chile during the grain-filling period. Letter differences on columns of the same color were significantly different, according to the Kruskal–Wallis test ($p < 0.05$).

3.1. Detection and Quantification of 2,4 DAPG and PCA-Producing Pseudomonas spp.

2,4-DAPG-producing *Pseudomonas* spp. were detected more frequently than PCA-producing *Pseudomonas* spp., where statistically significant differences between fields were observed for the frequency of positive sampling points to the presence of both antimicrobial genes (*phlD* and *phzCD*) ($p < 0.05$; Table 4). The number of sampling points positive for the presence of *phlD* did not present major variations during the grain-filling stage and harvesting, nevertheless, there were differences between fields ($p < 0.05$). However, in three of the fields under study (Potrero 11-Perquenco, Cajón-Temuco, and Potrero 13-Perquenco) a higher percentage of *phlD*-positive sampling points during the grain-filling stage was observed. Only in the field of Potrero 13-Perquenco was a higher frequency of *phlD*-positive sampling points during the harvest period (96%) observed.

Table 4. Percentage of sampling points [1] (n = 24) positive to bacterial populations with the genes *phlD* and *phzCD* determined through PCR from the rhizosphere of 20 wheat culms per sampling point obtained in four intensively sampled fields in southern Chile during the grain-filling and harvesting periods.

Sampled Farm-Location (Season)	*phlD*+ (%)		*phzCD*+ (%)	
	Grain Filling	Harvest	Grain Filling	Harvest
Potrero 11-Perquenco (2012/13)	50	38	83	50
El Retén-Osorno (2012/13)	38	29	0	12
Cajón-Temuco (2013/14)	42	38	4	0
Potrero 13-Perquenco (2013/14)	71	96	0	0
Mean	50	50	22	16
χ^2 (*p*-value)	<0.05	<0.001	<0.001	<0.001

[1] A sampling point corresponded to 1 linear m of whole wheat plants, including tillers and roots up to 25 cm deep, collected from each field within an 800 m transect.

PCA-producing *Pseudomonas* spp. were detected in three of the four fields under study. Overall, the average percentage of PCA-positive sampling points for the four fields were 22% and 16% in the grain-filling and harvesting periods, respectively (Table 4). The highest percentage of positive sampling points for *phzCD* was determined in Potrero 11-Perquenco, with 83% and 50% of positive samples in grain fill and harvest, respectively.

Total population densities of bacteria in samples of rhizospheres from the sampled fields were high, using the PCR endpoint assay the population of *phlD*-producing bacteria did not overpass 10^5 CFU g^{-1} during both sampling periods.

3.2. Genotypic Diversity of the Gene phlD+ in Sampling Sites

In this study, the presence of the genetic groups A, B, D, K, L, and P was detected by using genotype allele-specific PCR detection for the six genotypes of *phlD*. Variation in the genetic composition of the bacterial populations with *phlD*+ between fields and the phenological stage of wheat plants was observed (Figure 3). The Potrero 11-Perquenco field evidenced the presence of the genotypes A, B, D, K, and L, but only genotypes A and D were only identified during the period of grain fill and harvest, respectively. Similarly, the Potrero 13-Perquenco field presented the lowest variability of genetic groups, because only genotypes L and P were detected, while during the harvest period only the presence of genotype L was detected. The field from Cajón-Temuco evidenced the presence of all six genotypic groups in the grain-filling period, though genotype B was not detected during the harvest stage.

3.3. Relation between the Prevalence of Phytopathogens (Incidence and Severity) and Yield Variables in Sampling Points with and without Populations of 2,4 DAPG-Producing Pseudomonas

Variable relations were observed when the positive and negative sampling points to the presence of bacterial populations with *phlD* were considered. In general, by considering the total of samples collected (96 sampling points), high positive correlations between yield and incidence were observed (Spearman coefficient r = 0.59; $p < 0.001$) and the same was observed when the presence or absence of bacterial populations with *phlD* was considered (Table 5). Plant height presented negative correlations with the incidence in those negative-*phlD* sampling points for *phlD* during the grain-filling period (Spearman coefficient r = −0.39; $p = 0.006$), whereas no significant correlations were observed when positive sampling points were considered. These results were influenced by the effect of the sampling season, in which the yield presented negative correlations to incidence only during the season 2013/2014 (Spearman coefficient r = −0.39; $p = 0.006$), and this correlation was influenced by the presence of negative sites to *phlD* bacterial population

during the grain-filling period (Spearman coefficient r = −0–65; $p = 0.002$; Table 5). Plant height was correlated in a contrasting way depending on the season because during the season 2012/2013, negative correlations for all evaluations performed were found, whereas in the following season a positive correlation was observed when the total samples were considered (Spearman coefficient r = 0.42; $p = 0.003$), but when presence or absence of bacterial populations with *phlD* was considered this correlation was not significant (Table 5). Independent analysis per field showed a low correlation between incidence and the variables analyzed in the study (data not shown), probably associated with the reduced number of samples collected from each field (24 sampling points per field).

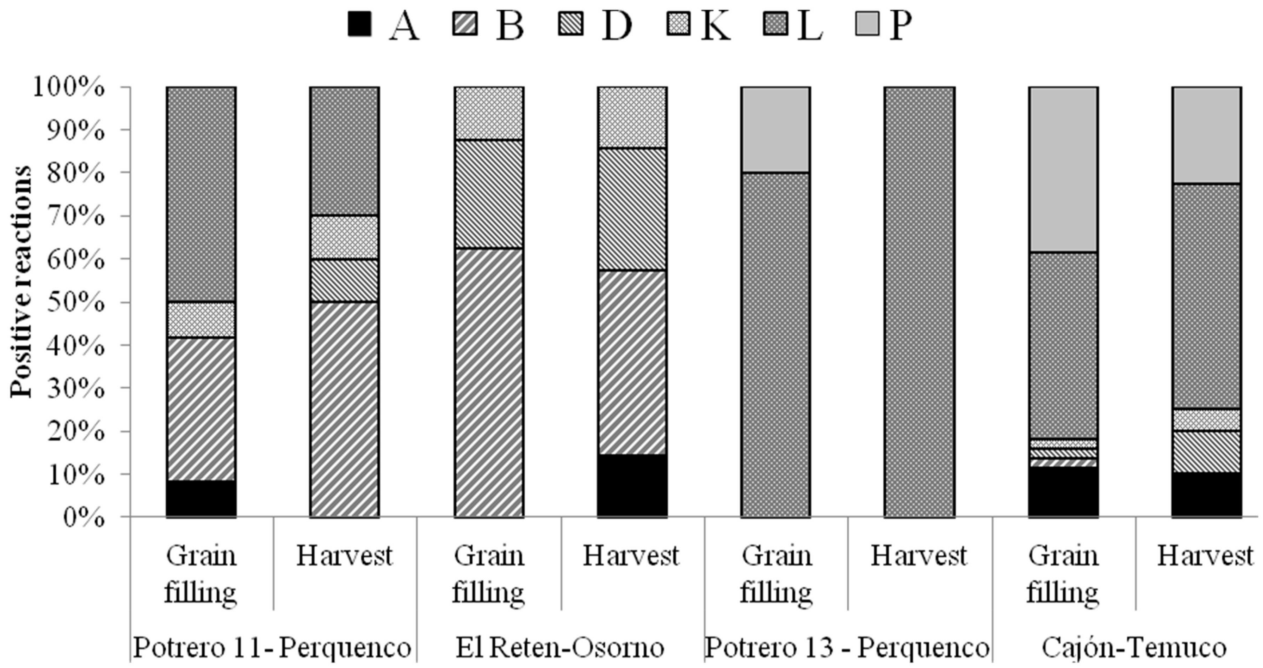

Figure 3. Frequency of genotypes of *phlD*+ identified in bacterial populations isolated from the rhizosphere of wheat plants obtained from sampling points sampled by amplification with specific primers, according to the protocol by De la Fuente et al. [18] in four intensively sampled fields of southern Chile during the grain-filling and harvesting periods.

Likewise, the severity was positively correlated to the yield by considering the total samples (96 sampling points) as a function of summing positive and negative sampling points to the presence of bacterial population with *phlD* (Spearman coefficient r = 0.61; $p < 0.001$). However, by analyzing the results per sampling year no significant correlations were observed between severity and yield during the season 2012/2013, while during the seasons 2013/2014, a negative correlation was observed when the absence of bacteria with *phlD* was considered (Spearman coefficient r = 0.57: $p = 0.007$; Table 5). Those sampling points, in which the presence of populations of *Pseudomonas* complex with *phlD* was determined, evidenced no significant correlations with yield during both seasons. The plant height evidenced a negative correlation in those sampling points without the presence of bacterial populations with *phlD* (r = −0.33; $p = 0.021$; Table 5). Significant negative correlations between disease severity and plant height were only observed during the season 2012/2013 for sites with the presence and absence of bacterial populations with *phlD* because during season 2013/2014, correlations were not significant. Analogous to the results observed in the relationships of incidence, the independent analysis by field demonstrated a low correlation between severity versus the analyzed variables (data not shown).

Table 5. Spearman correlation coefficients for the incidence and severity of damage versus yield (ton ha^{-1}) and plant height (cm) observed in four intensively sampled fields from southern Chile, analyzing the total collected samples (n = 96), sampling year (2012/2013; n = 48 and 2013/2014; n = 48), and considering those sampled points [1] that presented positive bacterial populations (only+) and negative (only−) to the gene *phlD*.

Factor	Spearman Correlation Coefficient			
	Grain Yield		Plant Height	
	phlD+	***phlD−***	***phlD+***	***phlD−***
	Incidence			
Total samples	0.58 (<0.001)	0.63 (<0.001)	−0.04 (ns)	−0.39 (0.006)
2012/2013	−0.17 (ns [2])	0.20 (ns)	−0.73 (<0.001)	−0.58 (0.002)
2013/2014	−0.07 (ns)	−0.65 (0.002)	0.34 (ns)	0.40 (ns)
	Severity			
Total samples	0.64 (<0.001)	0.62 (<0.001)	−0.01 (ns)	−0.33 (0.021)
2012/2013	−0.11 (ns)	0.19 (ns)	−0.46 (0.035)	−0.42 (0.027)
2013/2014	0.29 (ns)	−0.57 (0.007)	−0.24 (ns)	0.33 (ns)

[1] A sampling point corresponded to 1 linear m of whole wheat plants, including tillers and roots up to 25 cm deep, collected from each field within an 800 m transect. Data of incidence, severity, and *phlD+* bacterial populations were obtained from 24 sampling points collected by field. In each sampling point, 20 culms and their root systems were selected to be analyzed. [2] ns: No significant correlation ($p > 0.05$).

3.4. Relation between Prevalence of Phytopathogens (Incidence and Severity) and Diversity of Fungi in Sampling Points with and without Populations of 2,4 DAPG Producing Pseudomonas

Data for the incidence and severity of fungal microorganisms obtained from each sampling point in the wheat culms tillers were correlated to each other and with the diversity indices of Shannon–Wiener, observing a negative correlation between incidence and diversity (Spearman coefficient r = −0.339: $p > 0.001$) and between severity and diversity (Spearman coefficient r = −0.372: $p > 0.001$). This correlation was maintained by considering those sampling points with *phlD+* (Spearman coefficient$_{incidence}$ r = −0.470: $p < 0.001$ and Spearman coefficient$_{severity}$ r = −0.483: $p < 0.001$), while this correlation was not observed for those sampling points negative to *phlD*. These results suggested that by increasing the diversity of fungi in the wheat crown in presence of *phlD+* bacteria (Spearman coefficient$_{incidence}$ r = −0.141: $p = 0.338$ and Spearman coefficient$_{severity}$ r = −0.189: $p = 0.199$), a decrease in the incidence and severity of the damage in the first internode of the wheat plants was produced.

Considering that the field Potrero 11-Perquenco presented a high prevalence of sites positive to the phenazine gene (*phzCD*), correlations between parameters of prevalence and diversity were performed, where similar trends to those for *phdlD* were observed (positive sampling points, Spearman coefficient$_{incidence}$ r = −0.50: $p = 0.03$ and Spearman coefficient$_{severity}$ r = −0.63: $p = 0.003$).

Principal components analysis (Figure 4) revealed that the fields presented different compositions of fungal populations and only one grouping for fields Potrero 11 and 13 from the Perquenco area was observed in those sites with bacterial populations of *phlD+*. In addition, a negative relationship between populations of *Fusarium* and *Rhizoctonia* was observed in sampling points without populations of *Pseudomonas* with *phlD*, despite the fact that coexistence in sampling points with the presence of the gene was also observed. These results suggested the existence of an interaction between both populations of wheat pathogens, which may be regulated by the presence of these beneficial bacteria. An inversely proportional relationship was observed between saprophytic or nonpathogenic fungi and *G. tritici* (Ggt, Figure 4B), whereas this relation was not observed when bacterial population with *phlD* was present (Figure 4A).

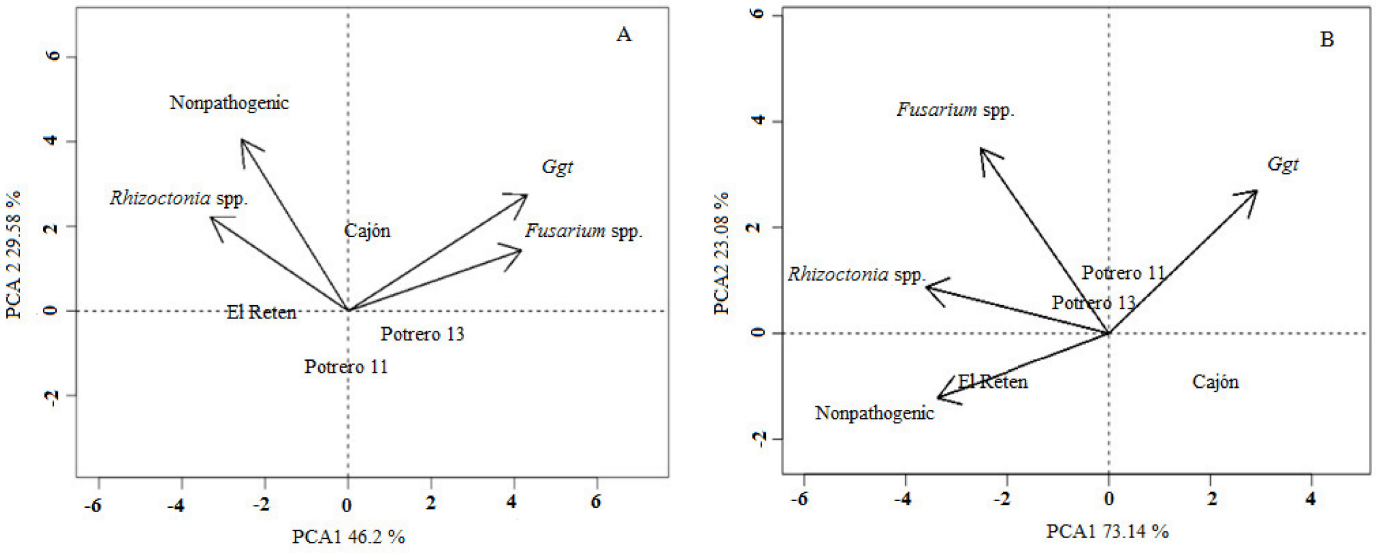

Figure 4. Principal components analyses for frequency of fungi present in four intensively sampled field in southern Chile during the grain-filling period. Relationships obtained for fungal populations in sites that presented negative bacterial populations (**A**) and positive populations with *phlD* (**B**).

4. Discussion

The fungal diversity associated with roots, crown, and first internodes of wheat plants in commercial fields has not been frequently studied, because the objective has been to determine the frequency of phytopathogenic fungi in different wheat production areas of global importance [2,31,32,41–45], as well as in Chile [28]. Additionally, this research corresponds to the first study that relates the presence of 2,4-DAPG and PCA-producing bacteria in the roots with the diversity of fungal populations that inhabit the base of the first internode of the wheat plant under field conditions. For this reason, this research provides relevant information concerning the situation presented in southern Chile.

Most genera and species of fungi found in this study have already been associated with the root and crown of wheat in other studies [2,28,32,42–45]. It should be noted that fungi of the genus *Phaeosphaeria* (*P. nodorum* and *P. pontiformis*) were the most frequently isolated in the four fields (72% of incidence in the culms analyzed in Osorno, El Retén). *Fusarium* species, such as *F. dimerum*, *F. oxysporum*, *F. acuminatum*, and *F. solani*, considered to be saprophytic or weak pathogens and pathogenic, such as *F. culmorum*, *F. avenaceum*, and *F. graminearum*, associated with Fusarium crown or root rot of wheat were commonly isolated. The presence of *Ceratobasidium cereale* (syn. *Rhizoctonia cerealis*) and *Waitea circinata* (Syn. *R oryzae*) associated with the first internode of wheat stems was also determined. *Gaeumannomyces tritici* was observed only in the two fields studied during the season 2012/2013, although has been reported as one of the most important and common pathogens to be found in wheat crops around the world [46] and in southern Chile [27,47,48]. Environmental conditions could also be influencing the colonization of wheat plant pathogens. Higher temperatures and lower rainfall observed during the crop season 2013/2014 in southern Chile, when the presence of *G. tritici* was not found, could allow for species adapted to drier conditions to predominate, such as the *Fusarium* species. In addition, 2,4 DAPG-producing bacteria have been associated with the suppression of take-all disease [3,6,9,12,18,27]. In our research, the presence of these bacteria in the sampled fields was determined, a fact that could also explain the low incidence of *G. tritici*.

The difference determined by principal component analysis of the interaction between populations of *Fusarium* and *Rhizoctonia* in sampling points with and without the presence of bacterial populations with *phlD*+ suggested that there is a relationship between both populations of wheat pathogens that may be regulated by the presence of these beneficial bacteria. To determine this relationship will require conducting interaction studies that

analyze how these bacterial populations regulate the interaction between phytopathogens that compete for the same ecological niche. This is important, especially considering previous interaction studies between *F. pseudograminearum* and *Bipolaris sorokiniana*, which proved that different levels of one or another phytopathogen prevent the infection of the other in the first internode of the wheat plant. Nevertheless, *F. pseudograminearum* is capable of colonizing the first internode and reducing the populations of *B. sorokiniana* when both are co-inoculated [49]. How this dynamic occurs between species of *Fusarium* and *Rhizoctonia* will also need to be explored.

The ecological analysis indicated that the fungal diversity level in the base of the first internode presented normal values in fields Potrero 11-Perquenco and Cajón-Temuco, and low values in Potrero 13-Perquenco and El Retén-Osorno (Table 3), considering that Shannon–Wiener diversity index values range from 0 to 5 and usual ranging from 1.5 to 3.5. These values of diversity are higher than those described in soils with wheat crops in Australia and close to those observed in culms, leaves, and roots by Vujanovic [2].

The presence in the studied fields of bacteria populations with *phlD+*, which is associated mainly with the production of 2,4 DAPG in the rhizosphere of wheat plants, could explain, in part, the previous results. In the fields in which the presence of these bacteria was determined more frequently, a lower incidence and severity of damage in the first internode of wheat plants was observed. On the other hand, these relations were not observed in those sampling points in which the presence of *phlD* was not determined, indicating that in order for the positive effect of the diversity of species to be effective, the presence of antagonistic bacteria or other microorganisms that colonize the roots or crown of wheat plants is required. These results support those reported by Penton et al. [31], who proposed the need for including broad studies of the communities of fungi and bacteria associated with the suppression and the development of the crown and root rot diseases in order to obtain a complete comprehension of this phenomenon. Other authors have indicated that the suppression of diseases in the soil is determined by a change in the composition of the bacterial populations [4,31,50,51]. For instance, Schreiner et al. [50] working on barley in soils with take-all decline determined by means of molecular techniques (TRFLP and microarrays), found that the dynamics of the bacterial population varied according to the crop cycle, such that *Actinobacteria* prevailed during the first vegetative cycle of the crop, followed by populations of *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, and *Acidobacteria* at the time in which the disease was more severe, and finishing with a prevalence of *Protobacteria* during the declining cycle of the disease. In the present work, where only *Pseudomonas* that have genes associated with the production of antibiotics, such as 2,4 DAPG and PCA, were analyzed. Nevertheless, it is necessary to appreciate that these bacterial populations and compounds are among the most important antimicrobial compounds involved in the biological control of soil pathogens in wheat [18,52].

Bacteria with genes associated with the production of 2,4 DAPG were detected more frequently than PCA, suggesting that in the soils of southern Chile there is a predominance of 2,4 DAPG-producing *Pseudomonas*. This is similar to that reported by Raaijmakers et al. [40], who determined that 2,4 DAPG-producing bacteria were present in all soils with a take-all decline in the United States, but not phenazine-producing bacteria. PCA-producing bacteria associated with suppressive soils have been found with more frequent prevalence in soils from China [53] and the United States [54], suggesting that different patterns associated with the origin and conditions of these suppressive soils are associated with the populations of microorganisms that form them. It should be noted that the Chilean sampled fields had no history of soils suppressive to *G. tritici*, although experiments under controlled conditions carried out in our lab have demonstrated the capacity of inducing the phenomenon of take-all decline in these soils (data not published). These results also support the hypothesis that PCA-producing *Pseudomonas* spp. are adapted only to grow and survive in the rhizosphere of wheat plants under water-stress conditions, being that soil humidity is an important abiotic factor that regulates the development of PCA-producing bacteria [30,54].

The *phlD*-producing bacteria populations observed in our study (10^5 CFU g of roots^{-1}) were similar to those reported in other studies that used the same counting methodology as used in this research [18,30]. Most of the soils in southern Chile, just as those sampled in this study, correspond to allophanic Andisol soils that present a high content of organic matter, which promotes the population of microorganisms within them. Moreover, allophonic Andisol soils present low pH, associated with the high rainfall of the area and their origin from volcanic ashes [25]. The acidic reaction of the soil could also explain the predominance of *Pseudomonas* with *phlD+*, considering that when the pH of the soil is decreased, the abundance of these bacteria is increased [21].

Genetic diversity of populations of 2,4 DAPG-producing *Pseudomonas* spp. has been previously described on wheat [40,55]. In this study, the presence of bacterial populations of the genotypes A, B, D, K, L, and P was determined during the grain-filling and harvesting period, which is the first study about the genetic groups of 2,4 DAPG-producing bacteria in Chile. Differences in the genetic composition of the fields analyzed in this study were observed, which is commonly noted in suppressive soils where the presence of multiple genotypes has been determined, and generally with the presence of a predominant genotype [16,18,29,56]. In the present study, genotype B was the most common to be found during the season 2012/2013, and genotype L was the most common during the season 2013/2014. It has been determined that several genotypes can be present in the soil in a given place, though generally there are one or two predominant genotypes in that soil [57]. Our results differ from those observed in studies conducted in the United States, where the main genotype found in suppressive soils is D [40,57]. It is clear that crop management factors, such as species used in rotation [16] or the wheat cultivar used [56,58], can influence the genetic composition of the populations of 2,4 DAPG-producing *Pseudomonas*. Fields analyzed presented differences in terms of rotations and genotypes of wheat used (Table 1), which could explain the differences in the observed genetic groups; for example, genotype B predominated in the field Retén-Osorno, whereas genotype L occurred in Cajón-Temuco. In addition, a greater genetic diversity was observed in the latter. Rotations performed in the fields sampled in this study correspond to those commonly used in the area and generally include several years of different cereals (wheat, oat, barley, and triticale), naturalized pastures, leguminous species, such as lupine or even Brassicas, in which the canola crop (*Brassica napus* var. *oleifera*) is commonly used.

It is important to note that the species of crops that grow in a given field enriches certain genotypes of 2,4-DAPG producing *Pseudomonas* [24,57], and these enrichments can be interrupted according to the species or crop used in the rotation [59]. Genotypes B and L have been frequently associated with wheat [55], though genotype B has also been associated with pea [24]. These results could explain the high frequency of these genotypes in the analyzed fields, though it has been determined that these genotypes present a lower capacity in order to compete in the rhizosphere than genotype D [24] so that wheat monoculture in southern Chile soils could produce an increase in the frequency of the genotype D. While genotype A is widely distributed worldwide, presence of genotype K is very uncommon and this genotype has been only described in soils from Ireland [18]. Interestingly, Irish soils are originated from volcanic ashes, such as the allophanic Andisol soils from southern Chile, which suggests that this uncommon genotype could be more prevalent in this type of soil.

The incidence and severity negatively affected parameters of plant height and yield in those sampling points without the presence of populations of *phlD+* bacteria. However, some of these correlations were not observed when *phlD+* sampling points were considered (Table 5), suggesting that the presence of bacteria with this gene in the wheat rhizosphere present a positive effect on the protection of the crop by increasing plant height and yield.

Weather conditions could have an influence on the correlations observed among incidence, severity, and parameters of yield and plant heights, because some of the significant results observed by analyzing the whole set of data were not matched by separating results per sampling year; i.e., correlations of incidence and severity with yield were only signifi-

cant during the season 2013/2014. In addition, by analyzing results per field, a positive and significant correlation was observed in the Retén-Osorno field (data unshown). This was also the field where the lowest frequency of bacteria *Pseudomonas phlD+* was observed, though it was also observed in Potrero 13-Perquenco when negative sites to the presence of bacterial populations with the gene were correlated with incidence and severity. Notwithstanding the above, the relationships observed in this research can be influenced by being dependent on the different wheat cultivars used, or by the different agricultural practices used by farmers in each field. Therefore, further research is necessary to explain some of the relationships observed in this study.

Investigations on microbial communities in suppressive soils have been focused mainly on the study of the fungi and bacteria of the soil [31,50,60]. Relevant information on fungal diversity associated with the base of the internode of wheat, as well as the genotypes of *Pseudomonas* with *phlD+* present in southern Chile, are provided in this study, which is the first evidence for South America about the diversity of such groups associated with wheat. On the other hand, relationships between the diversity of fungi of the first internode and the presence of *phlD+* bacteria were established. These relationships suggest a positive effect on the reduction in both the incidence and severity of wheat pathogens. Comprehension of the biodiversity of these relationships is key to the development of biological control strategies that utilize a mixture of compatible antagonists for the suppression of the main fungal diseases that affect the crown and roots of wheat.

5. Conclusions

Bacteria with genes associated with the production of 2,4-DAPG presented a higher frequency of isolation from wheat plants grown in the allophanic Andisol soils of southern Chile.

Pseudomonas bacteria of the genetic groups A, B, D, K, L., and P were detected in fields with commercial wheat crops established in allophanic Andisols soils in southern Chile, observing a variation in the genetic composition between fields and sampling years and plant stages.

The presence of 2,4 DAPG bacteria in the rhizosphere of wheat plants had a positive effect on the health of the crop, avoiding decreases in yield and height of plants caused by an increase in the incidence and severity of microorganisms in the crown, also a synergistic effect with microorganisms' diversity was observed. A lower incidence and severity were observed in those fields that presented a greater diversity of crown fungi and frequency of positive sites for 2,4-DAPG-producing bacteria.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12030710/s1, Table S1: List of PCR primers used in this study.

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