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Integración entre cepas de *Pseudomonas protegens* y fluquinconazole para el control de mal del pie en trigo.

Integration between strains of *Pseudomonas protegens* and fluquinconazole for the control of take-all in wheat.

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INTEGRACIÓN ENTRE CEPAS DE *PSEUDOMONAS PROTEGENS* Y FLUQUINCONAZOLE PARA EL CONTROL DE MAL DEL PIE EN TRIGO.

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INTRODUCCIÓN GENERAL

La enfermedad mal del pie causada por el hongo *Gaeumannomyces graminis* (Sacc.) Arx *et* Oliver var. *tritici* Walker (*Ggt*) es considerada en trigo como una de las más destructivas a nivel mundial (Cook, 2003; Gutteridge *et al*., 2003; Freeman y Ward, 2004; Andrade *et al.*, 2011; Kwak *et al*., 2012) generando desuniformidad en la emergencia, reducción de altura, menor producción de espigas, presencia de espigas blancas y masa radical necrótica, lo que se traduce en pérdidas considerables en el rendimiento de grano (Freeman y Ward, 2004; Latorre, 2004; Bockus *et al*., 2010). Si bien, el uso de fungicidas desinfectantes de semilla es la herramienta más utilizada, el mal del pie no cuenta con métodos de control completamente efectivos (Cook, 2003; Andrade, 2004). Bacterias como *Pseudomonas fluorescens* y *P. protegens*, han sido estudiadas como potenciales agentes de biocontrol y relacionadas directamente a la disminución de enfermedades e inhibición de patógenos radiculares como *Ggt* (De la Fuente *et al.*, 2006; Weller *et al*., 2007; Ramette *et al*., 2011; Mavrodi *et al*., 2012; Kwak *et al*., 2012). Se ha demostrado que uno de los principales mecanismos de protección es la producción de antibióticos como: 2,4-diacetilfloroglucinol (2,4-DAPG), pioluterina, ácido fenazina-1-carboxílico (PCA) y pirrolnitrina (Okubara y Bonsall, 2008; Siasou *et al*., 2009; Paulitz *et al*., 2010; Ramette *et al.*, 2011; Kwak *et al*., 2012). Sin embargo, en Chile, existen escasos antecedentes sobre estrategias de control biológico para el control de mal del pie (Andrade, 2013), constituyendo una interesante alternativa complementaria al uso de fungicidas.

HIPOTESIS

Cepas bacterianas de *Pseudomonas protegens* productoras de 2,4-diacetilfloroglucinol aplicadas a la semilla e integradas con un fungicida a base de fluquinconazole, disminuyen la incidencia y severidad del hongo *Gaeumannomyces graminis* var. *tritici* en cultivos de trigo.

OBJETIVO GENERAL

Evaluar el efecto de tratamientos de semillas de trigo con dos cepas bacterianas de *Pseudomonas protegens* asociadas a la producción de 2,4-diacetilfluoroglucinol (2,4-DAPG)

e integradas con un fungicida a base de fluquinconazole, sobre la enfermedad mal del pie en trigo, causada por el hongo *G. graminis* var. *tritici*, bajo condiciones de campo.

OBJETIVOS ESPECÍFICOS

1. Determinar el efecto individual y en mezcla de dos cepas de *Pseudomonas protegens* (2,4-DAPG), sobre características agronómicas y severidad de la enfermedad en plantas de trigo.
2. Determinar el efecto individual y en mezcla de dos cepas de *P. protegens* (2,4-DAPG), en combinación con un fungicida a base de fluquinconazole sobre características agronómicas y severidad de la enfermedad en plantas de trigo.
3. Cuantificar poblaciones bacterianas de *P. protegens* (2,4-DAPG), al ser tratadas en combinación con el fungicida fluquinconazole en semillas previo a la siembra.
4. Detectar y cuantificar poblaciones de bacterias *P. protegens* (2,4-DAPG) en raíces de trigo en distintos estados de desarrollo, mediante la técnica de PCR en tiempo real (qPCR).

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CHAPTER 1

Integration between strains of *Pseudomonas protegens* and fluquinconazole for the control of take-all in wheat.

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Highlights

Fluquinconazole and *Pseudomonas protegens* cotreatment did not affect bacterial concentration on wheat seeds.

Pseudomonas protegens strains ChB7 and Ca10 showed early root colonization protecting wheat crop during tillering.

Pseudomonas protegens strains ChB7 and Ca10 estabilized populations in roots during anthesis.

Pseudomonas protegens plus fluquinconazole seed treatment achieved marked inhibition of take-all disease symptoms.

Pseudomonas protegens plus fluquinconazole increased plant height and biomass.

Pseudomonas protegens plus fluquinconazole increased grain yield and test weigth.

Abstract

Mixing fungicides with bacteria that produce antimicrobial compounds is an integrated management tool that can reduce root rot pathogens such as *Gaeumannomyces graminis* var. *tritici* (*Ggt*), which causes take-all disease of wheat. This study analyzed the effect of wheat seed treatments on the survival of two strains of *Pseudomonas protegens*, when were applied to the seeds alone or integrated with fungicide a.i. fluquinconazole. Through three field experiments, under wheat monoculture and rotation, and inoculated with *Ggt*, the effect of bacterial combination with the fungicide seed treatment on yield components, above ground symptoms and root rot severity of take all disease was determined. In addition, bacterial populations were quantified using PCR and qPCR with *phlD*+-specific primers in wheat roots during tillering and anthesis during two seasons. *P. protegens* did not show marked effects on the evaluated parameters; however, when combined with fungicide, they increased yield, grain quality, test weight parameters, biomass, and plant heigh in early stage of plant development, decreasing also the severity of the disease. Initial populations of the bacteria were not affected when were co-treated with fluquinconazole, which was related with an early root colonization and protection in initial stages of development to stabilize their population during anthesis. Results suggest that integration of *P. protegens* strains with fluquinconazole is an alternative management strategie to reduce take-all disease in southern Chile.

Key words: Take-all; fungicide; bacteria; integration; antimicrobial compounds; fluquinconazole.

1. INTRODUCTION

Common wheat (*Triticum aestivum* L.) is the most widely seeded cereal in Chile, occupying an approximate area of 257,900 ha with average yields of 60.7 qq ha^{-1} (Merino and Traub, 2017). However, fungal diseases such as take-all disease, caused by the ascomycete fungus *Gaeumannomyces graminis* (Sacc.) Arx *et* Oliver var. *tritici* Walker (*Ggt*), is considered one of the most destructive in wheat worldwide (Cook, 2003; Andrade et al., 2011; Kwak et al., 2012; Yang et al., 2014; Durán et al., 2017; Vazvani et al., 2017; Wang et al., 2017; Zhang et al., 2017). In Chile, the disease mainly occurs from the region of O'Higgins to Los Lagos

(Andrade, 2004; Vera et al., 2014), an area where about 98% of wheat production is concentrated (Merino and Traub, 2017). Recent studies determined that *Gaeumannomyces* corresponds to the most prevalent and distributed root pathogen in the southern zone, where *G. graminis* var. *tritici* is the most common species (Moya-Elizondo et al., 2015). This area is precisely where this pathogen causes the greatest economic damage, since it occurs with greater severity and frequency (Andrade *et al*., 2011; Andrade, 2013; Madariaga, 2015; Duran et al., 2017). The disease is characterized by non uniform emergence, chlorosis, reduced kernel production, height reduction, presence of white spikes, and necrosis of the crown and roots (Cook, 2003; Freeman and Ward, 2004; Kwak et al., 2012). Although *Ggt* was identified more than 160 years ago, there is no adequate commercial control method including varietal resistance (Cook, 2003; Kwak et al., 2012; Vera et al., 2014; Vazvani et al., 2017) other than exploiting take-all decline (Weller et al., 2002; Cook, 2003; Andrade et al., 2011; Duran et al., 2017). Use of fungicide seed treatments is one of the most used alternatives to the atenuation of this disease, and different active ingredients have been evaluated (Cook et al., 2002; Andrade, 2004; Paulitz, 2010). Among them, the triazol fluquinconazole has allowed the diminishing or retarding of the development and spread of the take-all disease (Dawson and Bateman, 2000), and increase grain yield by 38% in wheat established in a Chilean andisol soil (Vera et al., 2014). However, the efficacy and effectiveness of these fungicide groups is variable (Andrade, 2004; Bailey et al., 2009; Vera et al., 2014; Madariaga, 2015) and the latent risk of the resistance emergence to fluquinconazole described in other phytopatho-systems (Mavroeidi and Shaw, 2005) requires the search for practices that help maintain their effectiveness. On the other hand, there are no products at the commercial level that can eradicate the disease (Paulitz et al., 2010; Liu et al; 2011; Wang et al., 2017). Then, the use of biocontrol agents that reduce disease and increase the yield when mixed with a triazole, such as i.a. fluquinconazole, is an alternative to improve the control of take-all disease.

The use of antagonistic *Pseudomonas* spp. has been deeply studied as an alternative of take-all disease control (Weller et al., 2002; Souza et al., 2003; De la Fuente et al., 2006; Mavrodi et al., 2007; Kwak et al., 2012; Yang et al., 2014; Martini et al., 2015; Yang et al., 2017).

Fluorescent *Pseudomonas* species, such as *P. fluorescens* and the novel specie *P. protegens*, have been described controlling phytopathogens by using induced systemic resistance in plants, competition with another microorganism, and production of antimicrobial compounds (Ramette et al., 2011; Martini et al., 2015). Fluorescent *Pseudomonas* are directly related to the decrease of take-all diseases, and they has been associated with inhibition of pathogens and soils suppressiveness properties (Take-all decline) (Weller et al., 2007; Ramette et al., 2011; Mavrodi et al., 2012; Yang et al., 2017; Durán et al., 2017). Production of antibiotics by these rhizosphere colonizing bacteria is considered to be the primary mode of action(Weller et al., 2007; Okubara and Bonsall, 2008) that allows for suppression and protection of the roots against take-all disease, due to the production of secondary antimicrobial metabolites, such as: 2,4-diacetylchloroglucinol (2,4-DAPG) and pyoluteorin by *P. protegens* species has been associated as the main mechanism mechanisms allowing suppression and protection of the roots against take-all disease (Kwak et al., 2009; Ramette et al., 2011; Mavrodi et al., 2012; Kwak et al., 2012; Yang et al., 2014; Martini et al., 2015). Integration of biological control agents with fungicide in seed treatments has resulted promising in the control of root rot diseases in wheat (Cook et al., 2002; Liu et al., 2011; Moya-Elizondo and Jacobsen, 2016; Wang et al., 2017). Notwithstanding the above, recently determination of pseudomonads 2,4-DAPG-producing bacteria in Chilean soils (Moya-Elizondo et al., 2013; Duran et al., 2016) and variable results of fungicide in controlling Ggt in this area (Andrade, 2013; Vera, et al., 2014), suggests that the integration of these bacteria as a complement to the use of fungicides could improve control strategies for take-all disease in Chilean wheat crops. Several studies show that the treatment of the seed with antagonistic pseudomonads significantly reduced take-all disease compared to the untreated control (Okubara and Bonsall, 2008; Kwak et al., 2012). In the same way, the integration of bacteria with fungicides has been an undeveloped strategy for the management of complex diseases such as take-all disease. Early work by Cook et al., (2002), conducted for several years, showed substantial increases in yield when using certain rhizobacteria combined with fungicides, although their response was always variable over the years under study. In the same line, Andrade (2013) demonstrated in andisoles soils of southern Chile that bacteria of

the *Pseudomonas* genus, when used individually or in mixture with triazoles, increase grain yield and reduce disease in a meaningful way. However, these studies did not clearly answer the effect that fungicides have on the populations of these antagonistic rhizobacteria on wheat roots.

The main objective of this study was to assess the control effect on wheat take-all disease of seed treatments with two bacterial strains of *P. protegens*, alone or integrated with a fungicide based on fluquinconazole, under field conditions. In order to accomplish this objective, seed treatment surviving of the combinations of these bacteria with fluquinconazole was determined on wheat seeds, such as effect of these treatments on agronomic variables and severity of the disease in wheat plants. In addition, colonization of wheat roots by *P. protegens* combined with fluquinconazole, seed treatments in different stages of the crop development was determined by using molecular methods.

2. MATERIALS AND METHODS

2.1. Study area

Field experiments were conducted in two fields in the Santa Rosa Experimental Station from the INIA-Quilamapu Agricultural Center, which is located in Chillán, Ñuble region, Chile (36°31'48" S, 71°55'13" O, 220 m.o.s.l). Three similar experiments were performed, one of them during 2014-2015 season and the other two in 2015-2016 season. Two experiments were seeded under wheat monoculture conditions, and they were coded as Monoculture_2014 and Monoculture_2015, in which the number corresponds to the year during which the experiment was seeded. The third experiment was coded as Rotation_2015, because it was performed during season 2015-2016 and where the season prior to the establishment of wheat experiment was sown with oat-vetch (*Vicia sativa* L.) as part of the crop rotation. The soil where the experiments were conducted corresponds to an andisol of the Arrayán series that uses gravitational irrigation (Stolpe, 2006).

Treatments assessed in the three field experiments are described in Table 1. Seeding was performed on June 13, 2014 for Monoculture_2014 and June 10, 2015 for the experiments Monoculture_2015 and Rotation_2015. The experimental units were plots of 2.4 m^2 with six rows of 2 m long by 0.2 m between rows, which were sown with 7.2 g of seed per row,

equivalent to a dose of 180 kg ha^{-1} of the winter wheat cv. 'Maxwell' (Jobet et al., 2017). Each row was manually seeded in a rate of three wheat seeds and one oat grain infested by *Ggt* into the furrow, which was the inoculum source in the inoculated treatments. Oat grains were placed together with the wheat seed in the sowing furrow. Inoculum in oat seeds (*Avena sativa* L.) was prepared previously in the lab with the highly virulent Ggt isolate 2010_04_G, following the procedure described by Vera et al. (2014).

2.2. Bacterial preparation

Pseudomonas protegens strains ChB7 and Ca10 were applied as a treatment to the seed. Both strains were isolated from wheat roots from andisol soils located in Chillán (Ñuble Region) and Cajón (Araucanía Region), respectively (Moya-Elizondo et al., 2013), and were stored at -80 °C in the Plant Pathology Lab of the Universidad de Concepción in Chillán, Ñuble Region, Chile. Both bacterial strains previously to be applied on the seed were assessed in the presence of the *phl*D gene associated with 2,4-DAPG and pyoluteorin biosynthesis by using PCR with *phlD*- specific primers B2BF and BPR4 (McSpadden et al., 2001) and the *pltB*-specific primers plt1 and plt2 (Yang et al., 2011), respectively.

In order to obtain the bacterial concentration, 40 µL of each bacterium was applied individually in a Falcon tube with 10 mL of KB broth, leaving it in agitation at 150 rpm during 24 h. Eight mL were transferred to a flask with 200 mL of KB broth, which was subsequently by rotary shaking at 250 rpm during 48 h. An aliquot of 40 mL of this bacterial culture was centrifuged (4,000 rpm, 20°C, 8 min) to remove the supernatant. The bacterial suspension that remained in the Falcon tube was re-suspended in 45 mL NaCl buffer (0.9%) and centrifuged again (4,000 rpm, 20°C, 5 min), removing supernatant components. This bacterial suspension was mixed with 100 µL of KB broth and 3 mL of 0.05% carboxymethylcellulase [CMC], and vortex during 25 s to be applied to the wheat seeds.

2.3. Seed treatment

Seeds were treated with the fungicide and the bacterial strains according each treatment described in Table 1. Fungicide based on fluquinconazole was dissolved in the equivalent of 10 mL of water per kg of seed, which was mixed and shaken with the seeds until achieving a uniform coverage. Treated seeds were dried in a laminar flow chamber. Subsequently, the

bacterial suspensions with CMC described abode were applied using the same procedure mentioned for the fungicide.

2.4. Bacterial quantification before and after seed treatment

Bacterial concentration of the suspention applied to the seed was determined through a bacterial serial dilutions (1: 9), starting with 100 µL of the mixture with the bacterial strains and KB broth in 900 µL of sterile distilled water (SDW). Then, from each dilution were seeded 3 micro-drops of 10 µL on a Petri dish with agar KB medium. These plates were incubated at 25°C during 24 h and colony forming units per mL (CFU mL^{-1}) for each dilution were counted. Bacterial concentration after seed treatment of each treatment was conducted by taken 1 g of the treated seeds, which were mixed with 9 mL of NaCl buffer (0.9%) and vortex three times during 1 min. From this mixture, 100 µL was taken and a serial dilution was conducted following the same procedure described above.

2.5. Experimental crop management practices and weather conditions

Weed control of the experiments consisted in applying herbicide based on the i.a. flufenacet, flurtamone and diflufenican (Bacara® Forte 360 SC, Bayer S.A, Santiago, Chile) product label at the time of sowing. Subsequent weed controls was performed manually. Plots were fertilized at seeding with FDA (260 kg ha^{-1}), potassium chloride (KCl) (60 kg ha^{-1}), sulpomag (200 kg ha^{-1}), boronatrocalcite (10 kg ha^{-1}) and sulphate of Zn ($ZnSO_4$) (3 kg ha^{-1}). Subsequently, wheat plants were fertilized with 153 units of N ($NaNO_3$) partialized in early tillering (375 kg ha-1) and when half of the tillers were formed (580 kg ha-1); growth stages Z2.2 and Z2.4, respectively (Zadoks and Schein, 1979).

The rainfall of the area, from sowing to harvest, in a normal year corresponds to 829.8 mm, in the 2014-2015 cycle corresponded to 778.1 mm (Monoculture_2014), and in the 2015-2016 cycle corresponded to 853.6 mm (Monoculture_2015 and Rotation_2015) (Fig. 1). Irrigation was considered in two times during stem elongation (Z3.0) and anthesis (Z6.0) growth stages. The average monthly air temperature did not present differences between the different crop cycles. Weather data were obtained from the agrometeorological station available in the Agricultural Experimental station Santa Rosa.

2.6. Disease assessments

2.6.1. Severity of symptoms of the disease. The percentage of plants with symptoms over the total population of plants in the field plots was visually determined during tillering (Z2.4), booting (Z4.1), and anthesis (Z6.9). Assessment was conducted by estimating visually the percentage of the area with symtoms associated with the disease on the plot. Additionally, white heads counting was conducted on each plot during early dough stage (Z8.3).

2.6.2. Root Severity. Fifteen days before harvest, one linear m from the first row of each plot were taken to assess the disease incidence (%) by counting the symptomatic stems with respect to the total number of stems. In addition, the severity damage affecting the first internode and the roots was determined by using a visual scale, where disease symptoms were categorized in: 0=0% (without symptoms), 1=1 to 25%, 2=25 to 50%, 3=50 to 75 % and 4=75 to 100% of darkening of roots and culm tissues. Then, a darkening severity index (DSI) was obtained according the following formula, which was adapted from Moya *et al.*, 2015:

$$\text{DSI}=\left(\frac{\sum(\text{Number of plants of each category x value of each category})}{\text{Total number of plants x number of categories}}\right)\text{x } 100$$

2.7. Detection and quantification of populations of *Pseudomonas* spp. from wheat roots

In the Monoculture_2014 experiment, four wheat plants were randomly collected per plot at tillering growth stage (Z2.5). Bulk soil was removed from the roots with sterile brushes to be stored -80°C in individual Falcon tubes of 15 mL until use. In order to perform the bacterial quantification, 1 g of roots were taken per plot, adding 9 mL SDW to be incubated for 24 h at lab temperature (±23°C). Samples were then vortexed four times during 25 s. Subsequently, 100 µL of each sample were diluted four times in 96-well cell culture plates, previously loaded with 200 µL of SDW. Then, 50 µL of the last three dilutions for each sample were taken and transferred to another micro cell culture plate with 200 µL medium 1/3 KB, enriched with the antibiotics ampicillin (40 mg L^{-1}), chloramphenicol (13 mg L^{-1}), and cyclohexamide (100 mg L^{-1}). Microplates were incubated in darkness (48 h at 25°C), and then quantified by spectrophotometry at an absorbance of 600 nm. Detection of the *phlD* gene associated with *Pseudomonas* populations was determined by PCR, using the specific

primers B2BF and BPR4 and the protocol described by McSpadden et al. (2001). Root samples from the field experiments Monoculture_2015 and Rotation_2015 were collected during tillering (Z2.5) and beginning of anthesis (Z6) and stored as described previously. In these experiments, DNA was extracted from 1 g of roots tissue after incubation for 24 h at room temperature, using PowerSoil® DNA Isolation Kit following the intruction of the manufacturer. The presence of *Pseudomonas* populations with the gene *phlD* was conducted by qPCR, using the specific-primers A-Up and A-Low associated with the genetic group A of *Pseudomonas*, considering that strains ChB7 and Ca10, belong to this genetic group. Quantitative PCR protocol was the same described by Mavrodi et al. (2007).

2.8. Development and crop production assessments

Different agronomic variable measurements in the three experiments were conducted during wheat growth and after harvest. Plant height was determined in three growth stages: tillering (Z2.3), anthesis (Z6.9), and ripening previous harvest (Z9). Yield components such as ears per square meter were determined the day before harvest. Number of grains per spike and the weight of 1,000 grains were assessed on 20 spikes collected from a linear m of the plot, which were manually threshed to obtain the kernels. Total aerial biomass (t ha^{-1}) was determined at harvest. The Monoculture_2014 experiment was harvested on January 20, 2015, with a Winterstager threshing machine, considering only the four central rows of each plot, while the Monoculture_2015 and Rotation_2015 experiments were harvested in the same manner on January 22, 2016. Subsequently, grains were cleaned and weighed manually to obtain grain yield (t ha^{-1}). Finally, wheat test weight (kg hL^{-1}) was determined by means of a Schopper balance of 250 cm^3, considering a grain moisture of 11±1% at harvest in each experiment.

2.9. Experimental design and statistical analysis

In the three experiments, treatments were established in an experimental randomized complete block design with four repetitions. Results obtained from each assessment were subjected to an analysis of variance (ANOVA). If there were significant differences, the mean separation test LSD (Less Significant Differences) was used to determine differences among treatments. Percentage data were adjusted to normal distribution by the formula $y = \sqrt{(x+0.5)}$,

where x = percentage value. CFU units were transformed at logarithm previous to conduct the analyses. Normality and homoscedasticity of the data were verified before ANOVAs. Correlation analyses between different disease and yield component variables were performed using the Pearson correlation analysis. All analyses were performed with the statistical software SAS, version 8 (SAS, 1999).

3. RESULTS

3.1. Bacterial populations before and after seed treatment.

The initial bacterial concentration of ChB7 and Ca10 strains to be applied to the seed was 1 x 10^7 and 9 x 10^6 CFU mL^{-1}, respectively. Two days after the seed was treated, the final concentration ranged between 4 x 10^3 and 4 x 10^4 CFU g $seeds^{-1}$. The highest bacterial population surviving to the seed treatments (4 x 10^4 CFU) was obtained by mixing both strains (ChB7+Ca10), and was significantly greater than the individual use of them ($P \leq 0.05$). Fluquinconazole incorporation to the seeds treated with the bacteria (ChB7+F and Ca10+F) did not affect the bacterial population, and was similar to the use of the bacterial strains individually or in mixture. However, bacterial population in the mixture of both strains plus the fungicide (ChB7+Ca10+F) was greater than in the fungicide mixed with each bacterium individually ($P \leq 0.05$).

3.2. Disease assessments

3.2.1. Disease incidence and severity of disease symptoms

Take-all symptoms were observed at virtually all of the development stages of wheat plants in the three experiments. There were not differences among treatments in the incidence of necrotic stems for each experiment (data not shown), but differences in the severity of the take-all symptoms on wheat foliage were observed (Table 2). First symptoms were observed from the beginning of tillering (Z2.4), where chlorotic basal leaves were observed. In the Rotation_2015 experiment, the use of bacterial strains in combination with fungicide (ChB7+F, Ca10+F and ChB7+Ca10+F) reduced the severity of the aforementioned symptoms between 70% (Ca10+F) and 85% (ChB7+F), compared to the inoculated control (UTC+) ($P \leq 0.05$; Table 2). However, in both monoculture experiments, the greatest effect was obtained only by combining the strain ChB7 with the fungicide, reducing symptoms in

66 and 60% (P≤0.05). Treatment ChB7 plus fungicide was similar to the control that was not inoculated with *Ggt* (UTC-) and to the treatment that only incorporated the fungicide (F) (Table 2). During booting (Z4.1), yellowish patches of stunted plants were observed within the plots. When comparing both untreated controls, a noticeable impact was observed in terms of the severity of disease symptoms (P≤0.05), with clear differences in Rotation_2015 during this growth stage (Table 2). However, in Monoculture_2014 and 2015, these differences were erratic.

In general, seed inoculation with the strains of *P. protegens*, alone or in mixture, did not decrease the level of the disease in any of the experiments (P>0.05) with respect to the control inoculated with *Ggt* (UTC+). However, only the treatment with Ca10 (Rotation_2015) was statistically different from the control inoculated with the fungus, reducing the severity of the disease by 25% at booting (Z4.1) (P≤0.05). The use of bacteria in combination with fungicide (ChB7+F, Ca10+F or ChB7+Ca10+F) caused the decrease of these symptoms at booting and anthesis, overcoming the control inoculated with *Ggt* in the three experiments (P≤0.05; Table 2). The greatest reduction in symtoms were observed during booting (Z4.1), corresponding to the treatments that integrated bacteria with the fungicide, in which the treatments ChB7+Ca10+F and Ca10+F from the monocultures 2014 and 2015 experiments reduced the disease in 42% and 71%, respectively (Table 2).

At early dough stage (Z8.3), the count of white spikes due to the premature ripening of the plants did not show significant differences among treatments in any of the experiments (data not shown).

3.2.2. Disease severity at the root level

Levels of root rot severity obtained in the three experiments are shown in Figure 2. In the Rotation_2015 experiment, treatments with bacteria, except for the mixture of both strains (ChB7+Ca10), significantly reduced wheat root rot between 34% (ChB7) and 58% (Ca10+F) in comparison to the control inoculated with *Ggt* (UTC+). The combinations of bacteria with the fungicide (ChB7+Ca10+F and ChB7+F) out ranked other treatments in terms of control, considering that they diminished the root rot severity by 49 and 56%, respectively (P≤0.05). The control inoculated with the fungus (UTC+) reached 92% more root rot compared to the

uninoculated control [UTC-] ($P \leq 0.05$). Meanwhile, in both experiments under wheat monoculture there were no differences between among treatments and plots corresponding to the uninoculated control showed high levels of take-all symptoms in the roots ($P > 0.05$, Figure 2). Despite this, the mixture of both strains of *P. protegens* plus the fungicide (ChB7+Ca10+F) reduced the severity at 22% on average with respect to the control inoculated with the fungus.

A positive strong correlation between radical necrosis and the severity levels observed on the foliage during tillering [Z2.4] ($p=2.6^{-6}$, $r=0.69$), booting [Z4.1] ($p=2.3^{-3}$, $r=0.49$), and anthesis [Z6.9] ($p=3.9^{-7}$, $r=0.73$) in the Rotation_2015 experiment were determined. However, in Monoculture_2015, this direct correlation was reflected only during booting [Z4.1] ($p=0.04$, $r=0.35$) and anthesis [Z6.9] ($p=2.1^{-3}$; $r=0.50$), while in the Monoculture_2014 experiments this relationship was determined only during flowering [Z6.9] ($p=0.02$, $r=0.38$).

3.3. Detection and quantification of *Pseudomonas protegens* populations in roots

In the Monoculture_2014 experiment, populations of these bacteria were detected by PCR in all treatments, even in those that were not inoculated with the strains under study. *phlD*+ bacterial population fluctuated between 10^3 to 10^5 CFU g of root^{-1} and the percentage of positive samples reached higher values in those treatments where bacterial strains were inoculated (data not shown).

During the 2015 season, qPCR was used to detect and quantify these populations (Table 3). Fifty percent of the control plots, as well as the treatment with chemical disinfection (F) and without bacteria, presented indigenous populations of pseudomonads *phlD*+ bacteria. Bacterial populations with the *phlD* gene were only detected in 50% of the plots of the untreated controls, while all the treatments that were inoculated with *P. protegens*, either alone, in mixture or in combination with fungicide, were different from both controls in terms of the presence of bacterial populations during tillering at the Rotation_2015 experiment and from the untreated control no inoculated with Ggt (UTC-) at the Monoculture experiment ($P \leq 0.05$). During anthesis (Z6.9), there was no difference among treatments and *phlD*+ populations were stabilized between 4.2×10^5 and 2.8×10^7 CFU g^{-1} root in the Rotation_2015 experiment and between 1.9×10^5 and 3.0×10^6 CFU g^{-1} root in

Monoculture_2015.

3.4. Effect of inoculation with *Pseudomonas protegens* and their integration with fungicides in the development and production of the wheat crop.

Effect of treatments on wheat plant heights in each experiment are described in Table 4. In Monoculture_2014, there were no differences among treatments in any of the assessment dates for plant height (P>0.05). Meanwhile, in Rotation_2015 experiment, during tillering and booting, there were differences when strains were combined with the fungicide ($P \leq 0.05$), raising the height between 29% (ChB7+F) to 34% (ChB7+Ca10+F) with respect untreated control inoculated with Ggt (UTC+). In Monoculture_2015 experiment, those two treatments increased plant height in 21% and 24%, respectively (Table 5). The use of individual bacterial strains stood out in the experiment with Rotation, achieving 20% (Ca10) and 31% (ChB7) increase in this parameter ($P \leq 0.05$) with respect to the control inoculated with the fungus. During tillering (Z2.3), when comparing both untreated controls, a significant plant height reduction was observed, detecting differences of 24% (Monoculture_2015) and 41% (Rotation_2015) ($P \leq 0.05$) against the UTC+.

In the assessment of yield components (Tables 5 and 6; Fig. 3), the best effect on the number of spikes m^{-2}, was obtained by combining both bacterial strains with the fungicide (ChB7+Ca10+F) in the Monoculture_2014 experiment, since this presented 28% more spikes (109 spikes more per m^{-2}) in comparison to the untreated control inoculated with *Ggt* ($P \leq 0.05$; Table 6). The use of both bacteria alone (ChB7, Ca10) and the combination of ChB7 with the fungicide were different from the untreated control [UTC+] ($P \leq 0.05$). Other treatments were not different from the control inoculated with *Ggt*, but they were on average 16% higher than UTC+. In Rotation_2015 and Monoculture_2015 experiments no treatment was differentiated from the inoculated untreated control [UTC+] (P>0.05; Table 6).

The weight of 1000 grains was not affected by the disease and treatments with bacteria did not have an effect on this parameter in any of the experiments. Only the mixture between strain Ca10 and the fungicide (Ca10+F) achieved statistical differences with respect both untreated controls in the Monoculture_2015 experiment ($P \leq 0.05$), increasing this parameter by 21% (8.6 g) with respect to the inoculated control (Table 6). The number of grains per

spike did not show statistical differences between treatments, in any of the experiments (data not shown).

The biomass analysis, wich was only performed in the experiments from the 2015-2016 crop cycle, showed that the biomass was not different among treatments in the experiment with rotation, despite of reduction levels was 2.5 t ha^{-1} (25%) lower in the untreated control inoculated with Ggt (UTC+) with respect the uninculated untreated control (UTC-). Bacteria-fungicide combinations did not differentiate clearly from the both untreated controls (Fig. 3). In the Monoculture_2015 experiment, no differences in biomass were detected between both untreated controls, but they were different from those treatments that integrated fluquinconazole with one of the *P. protegens* strains ($P \leq 0.05$; Fig. 3). Biomass reached between 3.4 to 4.2 t ha^{-1}, which is 31 to 38% higher than the values obtained by the untreated control (Fig. 3). When evaluating biomass, the individual use of Ca10 was different from the control inoculated with *Ggt* ($P>0.05$), while the use of both bacterial strains (ChB7+Ca10) increased at 19% the aerial biomass ($P\leq0.05$), being similar to the use of the fungicide alone or in mixture. In addition, a direct relationship was obtained between plant height reached up to harvest maturity (Z9) and the biomass (Monoculture_2015: $p<0.001$-$r = 0.55$; Rotation_2015: $p=0.04$-$r=0.34$).

Yield reduction levels achieved under conditions of artificial inoculation with the fungus were on average 52% in Rotation_2015, equivalent to 2.3 t ha^{-1} less than that obtained in the uninoculated control ($P\leq0.05$; Table 6). In Monoculture_2014 and 2015 experiments, differences of 14% were obtained, equivalent to 0.4 and 0.5 t ha^{-1}, respectively ($P>0.05$). In the present study, the fluquinconazole use in Rotation_2015 allowed to increase at 0.9 t ha^{-1} (21%) of grain yield, while in Monoculture_2014 and 2015 between 0.5 and 1.2 t ha^{-1} (18-34%) respectively was incresed the yield, compared to the untreated control inoculated with *Ggt* [UTC+] ($P\leq0.05$). The mixture of fungicide with *P. protegens*, in Monoculture_2014 and 2015, using the mixture ChB7+Ca10+F allowed to raise this parameter between 0.9-1.7 t ha^{-1} (33-47%); Ca10 + F between 0.7-1.9 t ha^{-1} (25-54%), and ChB7+F between 0.7-1.6 t ha^{-1} (25 - 46%). In the case of the experiment under the rotation condition, just the integration of the fungicide with both bacteria (ChB7+Ca10+F) increased grain yield significantly by

1.5 t ha^{-1} (34%), since all the other treatments were similar to the inoculated untreated control (UTC+).

Grain quality expressed as wheat test weigh (W), in the treatment that used individual or mix of both strains with the fungicide as a seed treatments, were similar to the untreated control and better to use the fungicide alone in Rotation_2015 experiment ($P>0.05$; Table 6). In the same experiment, the use of bacterial strains together with fluquinconazole, either ChB7+F, Ca10+F or ChB7+Ca10+F, allowed to increased test weight with respect to the control inoculated with *Ggt.* These treatments were similar to the non-inoculated control ($P\leq0.05$), generating in Rotation_2015 and Monoculture_2015, average increases of 1.1 to 1.8 units in W values (Table 6). Although, in Monoculture_2014, these treatments were not statistically differentiated from the inoculated untreated control, they increased W from 1 to 1.6 units.

4. DISCUSSION

The presence of the *phlD* and *plt* genes, associated with the production of 2,4-DAPG and pyoluteorin in strains ChB7 and Ca10 of *P. protegens* isolated in Chile (not published data), suggests that these strains could be producing antimicrobial compounds that have an antagonistic effect on *Ggt*, allowing protection and suppression against the wheat take-all disease in these field experiements (Freedman and Ward, 2004; Mavrodi et al., 2007; Ramette et al., 2011). Therefore, the feasibility of integrating them with fungicides is important to improve the control of this radicular disease that severely affects wheat crops in Chile.

The population density inoculated to the seed, which started with 10^3 and 10^4 CFU g^{-1} of seed, was not affected when co-treated with fluquinconazole and increased in tillering (Z2.5), fluctuating between $3x10^4$ - $6x10^6$ (Rotation_2015) and $1x10^4$-$5x10^5$ CFU g^{-1} root (Monoculture_2015). Similarly, seed treatment with the beneficial bacteria alone or in a mixture with the fungicide reached bacterial concentrations in the seed that were similar to the population densities detected by Okubara and Bonsall (2008), who determined fluctuations of 10^4 to 10^6 CFU g^{-1} root and that when inoculated to the seed reached concentrations of 10^2 to 10^5 CFU g^{-1} seed. In the same way, these bacterial populations could establish themselves in the root system of wheat plants under field conditions, observing an

increase even in tillering stage (Z2.5), which suggested early root colonization and probable protective effects in early stages of crop development. During anthesis (Z6.9), these populations stabilized, so that the application of the beneficial bacteria to the seed was consistent with the bacterial quantification performed on roots sampled in the field. Concentration of antagonistic bacteria detected under conditions of wheat monoculture should be greater than that determined under rotation (De Souza, et al. 2003; Yang et al., 2011). The latter is contrary to the data presented, because bacterial seed treatments showed higher concentrations in rotation conditions, obtaining the highest difference when mixing both strains, either alone or with the fungicide, which indicates that seed treatment with strains Ca10 and ChB7 should increase the beneficial populations in the rhizosphere of wheat plants, especially when a field is managed with crop rotation. Previous studies show that this disease's suppression is correlated with the concentration of PCA and *phlD*+-pseudomonas (Weller et al., 2007; Yang et al., 2011; Kwak et al., 2012; Yang et al., 2014), where the threshold density required for the decline of take-all in the wheat rhizosphere is 10^5 CFU g^{-1} root (De Souza et al., 2003; Kwak et al., 2009; Weller, 2014), which was reached in tillering under rotation with the seed treatments with the strains assessed in this study, and in anthesis under monoculture. Nevertheless, other studies have quantified populations above the threshold density of 10^6 and 10^7 CFU g^{-1} root; then stabilizing its population at 10^8 g^{-1} root at seedling growth stage (De Souza et al., 2003; Weller et al., 2007; Kwak et al., 2012), bacterial densities near to those values were reached by our treatments in anthesis. On the other hand, bacterial seed treatments did not affect seed germination, expressed as the number of plants per linear m (data not shown), which suggests that metabolites production by bacterial populations that were stablished in the root from seeds treatment did not affect the normal development of the wheat seedling as has been reported (Okubara and Bonsall, 2008). Due to the low mobility of this fungus during the crop cycle (Bailey et al., 2009), the artificial inoculation of the experiments allowed to ensure a more homogeneous distribution and infection of Ggt in the experimental sites. Through the assessments of disease severity, was confirmed the high virulence of the '*Ggt* 2010_04_G' isolate, which belongs to the genetic group G_2, described as the most aggressive and pathogenic of this fungus species (Daval et

al., 2010). All three experiments showed a positive correlation between the levels of root severity and the foliar symptoms caused by the take-all disease. During tillering, the severity levels of disease symptoms in the foliage were higher in the experiment under rotation than those obtained in experiments with wheat monoculture. Nevertheless, in later assessments this tendency was not reflected, which can be explained by a greater antagonistic activity of the bacteria between emergence to tillering in the soils under monoculture compared to the activity occurred in anthesis, which favored the infection by *Ggt* in the experiments under this management condition.

During crop cycle, in all evaluations of disease severity affecting foliage, regardless of crop management (rotation or wheat monoculture), the use of bacterial strains in combination with fungicide (ChB7+F, Ca10+F and ChB7+Ca10+F) allowed the reduction of severity of the disease symptoms compared to the untreated control inoculated with *Ggt* (UTC+). This tendency was constant until the anthesis assessment (Z6.9). The use of the fungicide alone showed a visual appearance of the symptoms observed on the plots that was similar to using it in a mixture with bacteria. This situation suggests that the integration of these beneficial microorganisms did not influence a greater or lesser expression of the disease on wheat crops. Under rotation, a reduction in disease general appearance of the plot was observed when bacteria were mixed with the fungicide. Similarly, in this experiment, although the bacterial seed treatments were not different from UTC+, they showed a tendency to reduce disease severity that was significant for Ca10 in the booting stage (Z4.1). This variability in disease control of biocontrol agents is consistent with results obtained in other phytopatho-systems where biocontrol agents have been integrated with fungicides (Kiewnick et al., 2001; Cook et al., 2002; Moya-Elizondo and Jacobsen, 2016; Wang et al., 2017).

Bacterial seed treatments, alone or or integrated with fluquinconazole, allowed to reduce the severity of crown and root rot in Rotation_2015 experiment, where take-all symptoms reached a 92% difference when comparing both untreated controls. Crown and root damages under rotating conditions were significantly different when they using the strains individually than when using both strains as mixture. Furthermore, the integration of the bacteria with the fungicide was not significantly increased, although there was a tendency to decrease root

damage when mixtures of the fungicide were used with each individual strain.

In the experiments under monoculture, the levels of root and foliage severity did not show major differences between both untreated controls, which is attributable to the fact that in the experimental site the pathogen was established naturally, considering that Ggt was not artificially inoculated in one of the untreated controls (UTC-). Both experiments under monoculture were performed in the same field, but in contiguous experimental sites, using a site where studies with Ggt have been conducted for more than a decade with continuous wheat seeding. Moreover, the non-increase of the disease observed in the control inoculated with *Ggt*, added to the presence of indigenous populations of bacterial populations with the *phlD* gene in the root samples of both controls allowed us to presume a possible phenomenon of take-all decline (TAD) associated with wheat monoculture (Cook, 2003; Andrade, 2004; Paulitz et al., 2010; Weller, 2014). Continuous wheat cultivation over time, a situation that has occurred at the site where the monoculture experiments were performed, can lead to disease suppression due to the presence of microorganism populations that inhibit the take-all disease (De Souza et al., 2003; Martini et al., 2015; Durán et al., 2017). The andisoles soils of Arrayan series would naturally have indigenous populations of bacteria with the *phlD* gene, considering that over 50% of the samples taken from the control plots showed bacterial populations with this gene. It should be noted that the *P. protegens* strain ChB7 was isolated from a field located 25 km from the site where these experimental studies were conducted, which suggests that this bacteria population could be found established naturally in the field and adapted to the physical-chemical and biological conditions of the soils of the south-central and southern zone of Chile. The TAD phenomenon was reported for the first time in Chile by Andrade et al. (2011) in 20 fields between Biobío and Los Lagos regions, presenting five of them with highly suppressive properties associated with several years of wheat monoculture or short rotations of wheat and prairies. In addition, studies associated with the microbiological characterization of soils with suppressive properties against root rot wheat diseases have corroborated the biological nature of this phenomenon in Chile (Andrade, 2013; Moya-Elizondo et al., 2013; Durán et al., 2017). At the same way, suppression has been attributed to Pseudomonas bacteria with the phlD gene (Moya-Elizondo et al., 2013),

which have presented an inhibition of mycelial growth of *Ggt* of 54 and 63% for *P. protegens* strains ChB7 and Ca10 under *in vitro* conditions, respectively (Unpublished data).

The suppressive activity in soils are correlated with the composition of the microbial community and not necessarily with the chemical or geographical origin (Durán et al., 2017), although TAD expression varied between fields and growing seasons. (Kwak et al., 2012) and the production of this antifungal metabolite depends on the genotypes of both the plant and the bacterial strain involved in the interaction (Bakker et al., 2013). In the present study, the severity levels of both disease symptoms in the foliage and in the roots decreased during the second season (2015); for example, when both untreated control inoculated with *Ggt* (UTC+) were compared, the decrease in the root damage severity was 23.3%, for trials under monoculture. Furthermore, in those cases in which *P. protegens* strains were incorporated into the wheat crop through seed treatment, either individually or in combination with the fungicide, there was a marked reduction in foliage and root disease severity symptoms. These results are in concordance with those reported by Okubara and Bonsall (2008), who determined a significant suppression of radical damage generated by *Ggt* when treating the seed with *Pseudomonas-phlD+*. In the case of the experiment under rotation, in the uninoculated untreated control plots (UTC-), it was not possible to verify the presence of take-all disease symptoms or signs in the sampled plants, unlike what was observed in the same control treatment in the monoculture experiments. Levels of radical necrosis severity and foliage damage achieved in UTC+ under Rotation were similar to that obtained in the first year of study during the Monoculture_2014 and slightly higher than that obtained during the same season in the study under monoculture, so that the artificial inoculation of the *Ggt* pathogen affected the experiment under rotation more than those under the monoculture. What would have been the results if these *P. protegens* bacteria integration with fungicide experiments had been performed under a monoculture condition without inoculum of the pathogen? it is a question that future research should attempt to answer, since it is likely that there will be a greater synergy when increasing populations of 2,4-DAPG-producing bacteria were established in the rhizosphere of the wheat plants through seed treatment, increasing probably the expression of take-all disease suppression phenomenon.

The action of antagonistic microflora, especially the presence of *Pseudomonas* spp. genus, have been described as the main factor responsible for the TAD phenomenon (Mavrodi et al., 2012; Kwak et al., 2012; Bakker et al., 2013; Weller, 2014; Martini et al., 2015), increasing microbial biomass and allowing suppression of the take-all disease (Weller et al., 2002; Kwak et al., 2012). The incorporation of the bacteria through the seed allowed an increase in the microbial populations in the rhizosphere of wheat plants, which was clearly reflected in the quantifications made by qPCR in this study. In this research, the incorporation of the antagonistic bacteria to the seed stabilized bacterial populations with the *phlD* gene into the plots within the treatments that contained them, considering that in both untreated control treatments and in those treated only with fungicide, especially under monoculture, indigenous bacteria populations with the aforementioned gene were lower or were not detected in some of the plots. The stabilization of the *phlD*+ bacteria populations was increased at tillering under rotational conditions and was slightly lower under monoculture conditions.

Weather conditions that prevailed during the experiments were favorable for pathogen establishment and development due to high levels of humidity at the beginning of the crop season, but toward the end of tillering the weather did not favor the pathogen, due to the evident drought conditions in both seasons. It has been determined that drought stress is also an important factor that forms the rhizosphere's microbiome, as does the adaptive strategy of the plant to environmental limitations (Bakker et al., 2013). However, this factor can be ruled out since these experiments were kept under gravitational irrigation.

During tillering (Z2.3), in two of the experiments, a significant reduction in plant height was determined, a characteristic symptom of the disease (Paulitz, 2010; Madariaga, 2015), detecting differences in 24% in plant height (Monoculture_2015) and 41% (Rotation_2015) when comparing both untreated controls. Despite this, the average potential heights of cv. 'Maxwell', expressed as that registered from the non-inoculated untreated control (UTC-) at maturity stage (Z9), reached the growth capacity that it normally records, since its plant height at ripening stage fluctuates between 70 to 95 cm (Jobet et al., 2017). During tillering, major differences between both untreated controls were detected when crop rotation was

performed (Rotation_2015). In monoculture experiments, the differences between the untreated controls were low except for the experiment performed during the 2015 season, in which there was less expression of the disease, influenced probably by the natural presence of pseudomonads that induced the natural decline of the disease. There were treatments in which *P. protegens* incorporated into the rhizosphere by seed treatment showed a tendency to promote growth in height in advanced stages of the crop, although the results were erratic when comparing experiments. Differences in plant height induced by the use of bacteria was most evident during early stages of crop development (tillering), which could be associated with higher levels of control in early stages of plant development, considering that concentration of antimicrobial compounds produced by antagonistic bacteria to take-all has been reported to be greater after the first weeks of seeding, for being decreased in the rhizosphere when the crop is grown (Kwak et al., 2012).

The initial effect of the inoculation with beneficial bacteria on plant height could influence crop biomass or yield components. Effect on biomass, which was only evaluated in experiments of the second season, was observed in Monoculture_2015, since under rotation there were no differences. Integration of *P. protegens* strains with the fungicide resulted in a higher aerial biomass when using only bacteria compared to the untreated control inoculated with *Ggt* (UTC+). This situation may be associated with a growth promotion effects, since studies performed in our laboratory (Unpublished data) have shown the presence of metabolic mechanisms to promote growth in strains Ca10 and ChB7, associated with phosphorus solubilizing activity and IAA (indole acetic acid) production. Our research determined that these *P. protegens* strains solubilize 70% more phosphorus (mg of P_2O4 L^{-1}) than that reported with other strains of the *Pseudomonas* genus (Kumar et al., 2014), and IAA production levels, which can influence plants' hormonal growth (Govindasamy et al., 2009). In the same way, biomass increased had a direct relationship with the height reached until harvest (Z9), which could be correlated to this growth promoting activity.

Considering results of biomass assessment, under monoculture conditions around 3 to 8 t ha^{-1} of straw should be accumulate at the end of the crop, considering that between 1 to 2 kg of straw per 1 kg of grain is produced (Paulitz et al., 2010). This situation would imply that if

those plant residues are incorporated into the soil, the disease severity is increased under monoculture, since the fungus survives saprophytically for several years into the soil (Paulitz et al., 2010; Andrade, 2014; Madariaga, 2015). Therefore, avoiding wheat monoculture and establishing an adequate rotation with non-susceptible species is considered the most efficient control strategy to prevent take-all disease (Cook, 2003; Freeman and Ward, 2004; Mellado, 2007; Paulitz et al., 2010; Andrade, 2014). In the Rotation_2015, during the season prior to establishment, oat-vetch was used as part of the rotation, which are plant species not susceptible to *Ggt*, allowing the interruption of the biological cycle of this pathogen and reducing the amount of viable inoculum (Freeman and Ward, 2004; Paulitz et al., 2010; Madariaga, 2015). Despite this, in farms where rotation alternatives are scarce or practically null and there is not a continuous rotation of crops, seed treatments of *P. protegens* plus a triazol such as fluquinconazole would allow to reduce and/or maintain low infection and severity levels of the disease, allowing to reduce yield losses between seasons of monoculture.

In relation to the yield components, the seed treatment with the bacterial strains was erratic for the number of grains per spike and the weight of 1,000 grains among experiments. For example, in the experiment under Monoculture_2014 could be observed that strain Ca10 and both strains in mix the fungicide (ChB7+Ca10+F) increased in 30 and 34% the number of spikes m^{-2}, respectively. On the other hand, the strain Ca10 had the one of the lowest values of the weight of 1,000 grains, while its combination with the fungicide (Ca10+F), in the experiment under monoculture of 2015, reached the higher valued for this yield component, differentiating significantly from both untreated controls in over 21% of 1,000 grain weight. Wheat test weight (W) is related to the percentage of flour that can be obtained from the grains, which is why it is considered a key factor in determining the quality of wheat flour in Chile. In this variable of yield, under rotation, an average value of 76.7 kg hL^{-1} was obtained in the uninoculated untreated control (UTC-), value that is within the expected range for the cv. 'Maxwell', whose W fluctuates between 76 and 81 kg hL^{-1} (Jobet et al., 2017), while under monoculture conditions the values obtained were 4.5 and 2 units below the normal minimum. Interestingly, seed treatment of bacteria strains in combination with fungicide allowed to

increase of the quality of the grain in the rotation experiment compared to the untreated control inoculated with *Ggt* (UTC+), while under monoculture did not showed major differences.

Regarding the variables discussed above, grain yield is finally what defines the success of a wheat production at farmer level. Grain yield showed in this study that the inoculation with *Ggt* caused reduction ~ 13% under monoculture, and almost three times more losses in the experiment under rotation (33.8% loss), when compared both controls. Rotation_2015 experiment was the trial where the untreated control without *Ggt* inoculum (UTC-) obtained the best average yield in this study. The levels of yield reduction obtained in the experiments are within the range determined in previous studies with inoculation with *Ggt* in Chile (Andrade, 2004; Vera et al., 2014). This high difference in yield between the rotation conditions compared to monoculture was given by *Ggt* presence in the plots of the uninoculated untreated control as mentioned above. Environmental conditions of the season should not have influenced the yield result, but monoculture practice promoted a significant decrease in grain yield in our study, as been reported in other researches (Cook, 2003; Kwak et al., 2012; Vera et al., 2014).

Finally, the differences in the levels of control and effects on yield variables associated with the use of strains ChB7 and Ca10 of *P. protegens* could be linked to differences in the ability to produce antimicrobial compounds and rhizospheric competition, both for colonization and persistance in the soil (Validov et al., 2005; Okubara and Bonsall, 2008; Kwak et al., 2012). Strain effectiveness on the evaluated parameters could be favored by factors such as competence, mean life and aggressiveness, in addition to the time that these bacteria remain in the rhizosphere and the *Ggt* sensitivity to the antibiotics that they produce (Okubara and Bonsall, 2008; Kwak et al., 2012). However, it is normal to detect differences between strains, related to levels suppression generated and population variability of these (Kwak et al., 2012). Recent studies of wheat phytobiomas show the complexity existing in the bacterial community of soils, which is influenced by proximity to the plant (rhizosphere or 'bulk soil') or organ of the plant that these populations of bacteria colonize, in addition to the place, year or management practices (Gdnetz and Trail, 2017; Yin et al., 2017). Then, fungicide

incorporation for the control of diseases affecting wheat roots must influence microflora activity of a soil, at the same way that this bacterial community should influence incorporation of agents of biological control, such as *P. protegens* bacteria, in the rizhosphere of wheat plants.

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FIGURES AND TABLES

Figure 1. Monthly accumulated rainfall (mm) occurred in Chillán, Ñuble Region, Chile between June (seeding) to January (harvest) of crop cycles 2014-2015 and 2015-2016.

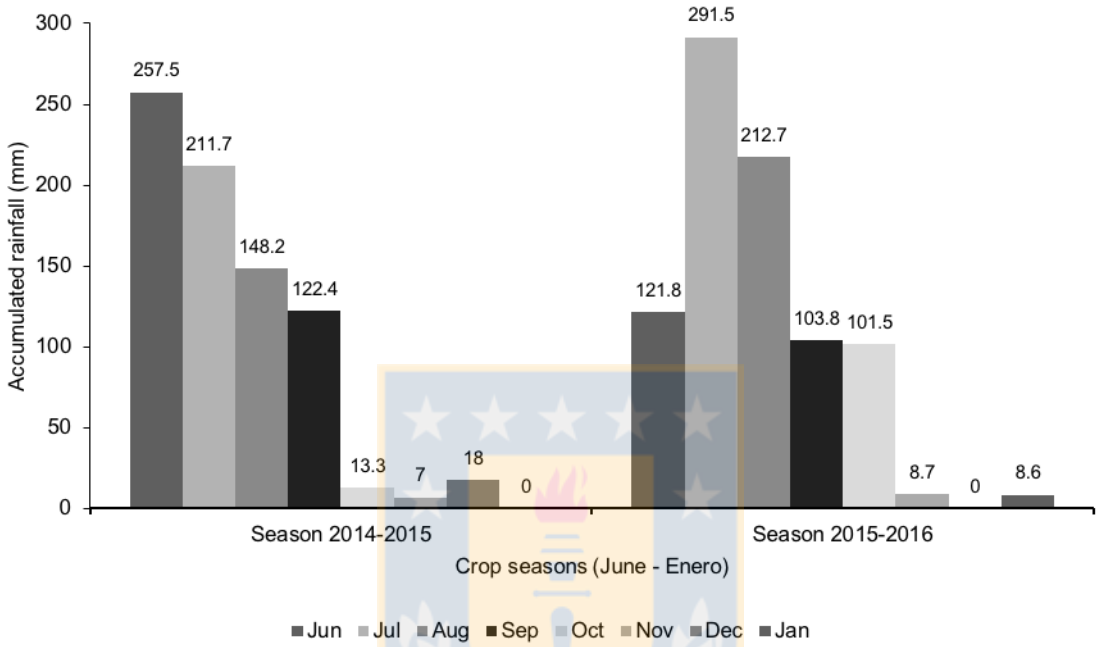
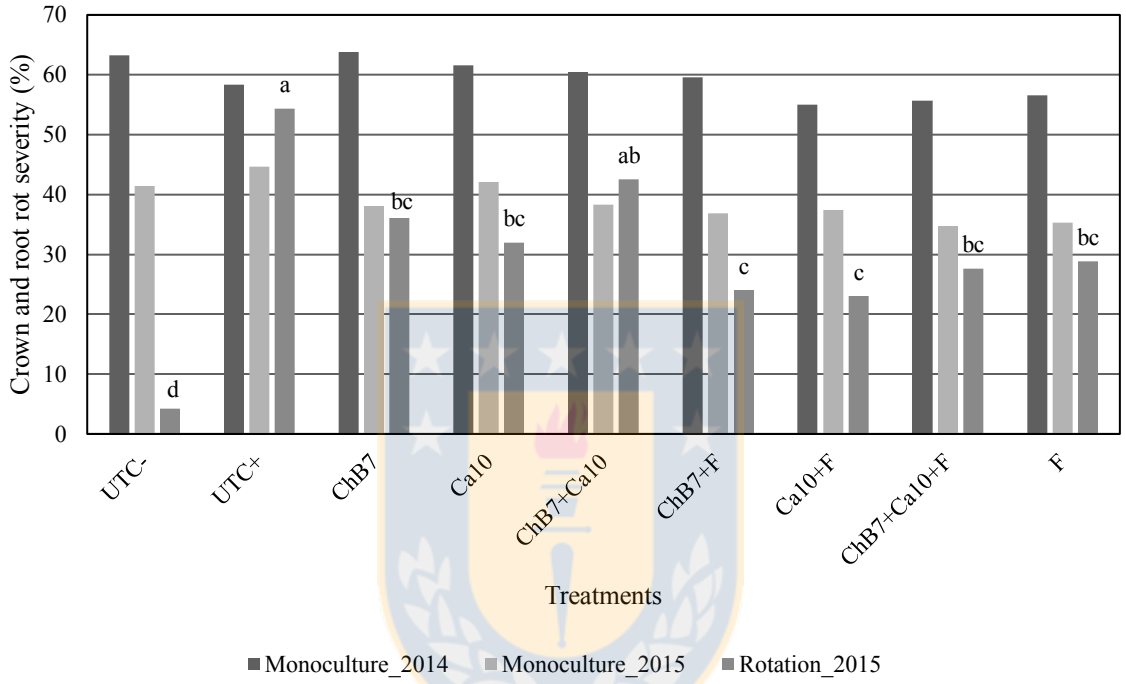


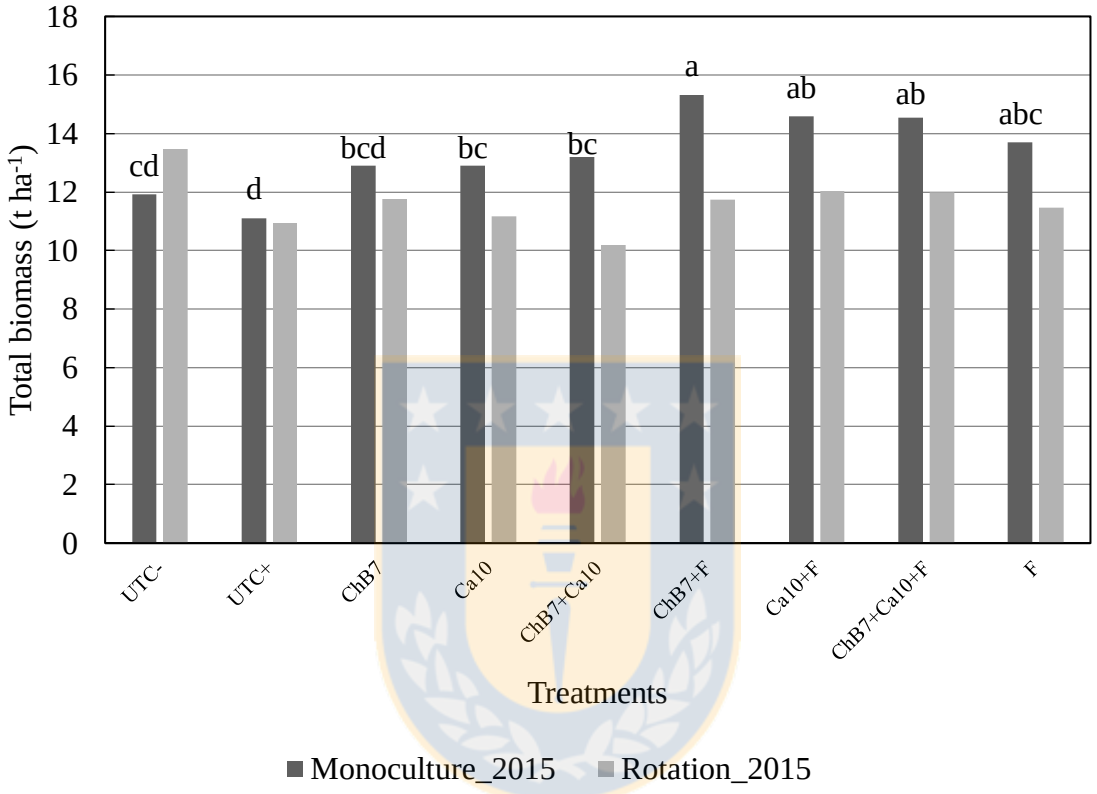
Figure 2. Averages crown and root rot severity (%) caused by take-all disease in winter wheat cv. 'Maxwell' at harvest, obtained by evaluating seed treatments with *Pseudomonas protegens* strains ChB7 and Ca10, applied individually or in combinations with fluquinconazole fungicide (F), under monoculture (M_2014 and M_2015) or rotation (R_2015) conditions during crop seasons 2014-2015 and 2015-2016.



Treatments code: (UTC-): Untreated control non inoculated with *Ggt*; (UTC+): Untreated control inoculated with *Ggt*; (ChB7): *Pseudomonas protegens* ChB7 strain, inoculated to the seed; (Ca10): *P. protegens* Ca10 strain, inoculated to the seed; (F): Fluquinconazole, active fungicide ingredient, applied to the seed in concetration of 87.5 g a.i. for 100 kg of seed. Bacterial strains were applied to the seed in a dose of 1.7 L (10^6 CFU) 100 kg^{-1} of seed.

Letter different in column of similar color showed significantly differences among treatments according to LSD comparison mean test ($\alpha = 0.05$).

Figure 3. Total Biomass (t ha^{-1}) at harvest in plots of winther wheat cv. 'Maxwell' inoculated with *Gaeumannomyces graminis var. tritici* and seed treateds with *Pseudomonas protegens* strains ChB7 and Ca10, applied individually or in combinations with fluquinconazole fungicide (F), under monoculture or rotation conditions in crop season 2015-2016.



Treatments code: (UTC-): Control not inoculated with *Ggt*; (UTC+): Control inoculated with *Ggt*; (ChB7): *Pseudomonas protegens* ChB7 strain, inoculated to the seed; (Ca10): *P. protegens* Ca10 strain, inoculated to the seed; (F): Fluquinconazole, active ingredient of fungicide, applied to the seed in concetration of 87.5 g a.i. for 100 kg of seed. Bacterial strains were applied to the seed in a dose of 1.7 L (10^6 CFU) 100 kg^{-1} of seed. Letter different in column of similar color showed significantly differences among treatments according to LSD comparison mean test ($\alpha = 0.05$).

Table 1. Seed treatments and artificial inoculation with *Gaeumannomyces graminis var. tritici* (*Ggt*) used in experiments with winter wheat cv. 'Maxwell'.

Treatments	Code	*Ggt* Inoculation
Untreated Control 1	UTC-	Without
Untreated Control 2	UTC+	With
Pseudomonas protegens ChB7 strain[1]	ChB7	With
Pseudomonas protegens Ca10 strain[2]	Ca10	With
P. protegens ChB7 strain + Ca10 strain	ChB7+Ca10	With
P. protegens ChB7 strain + Fluquinconazole[3]	ChB7+F	With
P. protegens Ca10 strain + Fluquinconazole	Ca10+F	With
P. protegens ChB7 strain + Ca10 strain + Fluquinconazole	ChB7+Ca10+F	With
Fluquinconazole	F	With

[1]ChB7: corresponding to *Pseudomonas protegens* ChB7 strain, applied to the seed in a dose of 1.7 L (10^6 CFU) 100 kg^{-1} of seed. This strain was isolated from wheat plant in Chillán, Ñuble Region, Chile.

[2]Ca10: corresponding to a *P. protegens* Ca10 strain, applied to the seed in a dose of 1.7 L (10^6 CFU) 100 kg^{-1} of seed. This strain was isolated from wheat plant in Cajón, Araucanía Region, Chile.

[3]Fluquinconazole (F): Active ingredient (a.i) of fungicide (87.5 g a.i.) corresponding to 175 mL of chemical product Galmano® 500 FS (Bayer S.A., Chile) for 100 kg of seed.

Table 2. Severity averages of take-all disease symptoms (%) observed on wheat foliage in three growth stages of the experimental plots seed treated with *Pseudomonas protegens* strains ChB7 and Ca10, applied individually or in combinations with fungicide based on fluquinconazole (F) in winter wheat cv. 'Maxwell', under monoculture conditions (M_2014 and M_2015) or rotation (R_2015) during crop seasons 2014-2015 and 2015-2016.

Treatments[1]	Wheat growth stages								
	Tillering (Z2.4)			Booting (Z4.1)			Anthesis (Z6.9)		
	M_2014	M_2015	R_2015	M_2014	M_2015	R_2015	M_2014	M_2015	R_2015
UTC-	11.3[3]	6.3 d	5.0 c	42.5 bc	42.5 abc	17.5 d	60.0 b	57.5 ab	6.3 c
UTC+	18.8	27.5 ab	57.5 a	60.0 a	60.0 a	52.5 a	70.0 ab	67.5 a	70.0 a
ChB7	27.5	15.0 abc	50.0 a	60.0 a	47.5 ab	35.0 ab	67.5 ab	50.0 abc	62.5 a
Ca10	16.3	27.5 a	40.0 a	50.0 ab	47.5 abc	30.0 bc	67.5 ab	50.0 abc	55.0 a
ChB7+Ca10	22.5	22.5 abc	45.0 a	60.0 a	42.5 abcd	40.0 ab	80.0 a	50.0 abc	70.0 a
ChB7+F	6.3	11.3 cd	15.0 b	42.5 bc	22.5 de	15.0 d	57.5 b	26.3 d	20.0 b
Ca10+F	13.8	13.8 bcd	22.5 b	42.5 bc	17.5 e	12.5 d	57.5 b	17.5 d	25.0 b
Chb7+Ca10+F	12.5	17.5 abc	18.8 b	35.0 c	27.5 bcde	12.5 d	52.5 b	30.0 bcd	17.5 b
F	7.5	16.3 abc	21.3 b	42.5 bc	27.5 cde	17.5 cd	55.0 b	30.0 cd	22.5 b
CV[2]	28 %	27%	20%	11%	20%	20 %	10%	22%	14%
P-value	0.072	0.010	<0.0001	0.010	0.007	<0.0001	0.029	0.004	<0.0001

[1] Treatments code: (UTC-): Control not inoculated with *Ggt*; (UTC+): Control inoculated with *Ggt*; (ChB7): *Pseudomonas protegens* ChB7 strain, inoculated to the seed; (Ca10): *P. protegens* Ca10 strain, inoculated to the seed; (F): Fluquinconazole, active ingredient of fungicide, applied to the seed in concetration of 87.5 g a.i. for 100 kg of seed. Bacterial strains were applied to the seed in a dose of 1.7 L (10^6 CFU) 100 kg^{-1} of seed.

[2] CV: Coefficien of variation.

[3] Letter different in a same column showed significantly differences among treatments according to LSD comparison mean test ($\alpha = 0.05$).

Table 3. Bacterial populations with the *phlD* gene obtained by qPCR[1] in wheat roots of cultivar 'Maxwell' in two growth stages for seed treatments with *Pseudomonas protegens* strains ChB7 and Ca10, applied individually or in combinations with the fungicide fluquinconazole (F), in experiments under conditions of Rotation_2015 and Monoculture_2015 during season 2015-2016.

Treatments[2]	Rotation_2015		Monoculture_2015	
	Tillering (Z2.5)	Anthesis (Z6.9)	Tillering (Z2.5)	Anthesis (Z6.9)
UTC-	4.8×10^3 b[4]	1.5×10^6	3.5×10^2 c	7.5×10^5
UTC+	1.8×10^5 b	1.8×10^6	9.4×10^3 b	8.9×10^5
ChB7	2.3×10^6 a	6.4×10^6	1.0×10^4 ab	2.8×10^6
Ca10	1.8×10^6 a	1.1×10^6	1.2×10^4 ab	4.5×10^5
ChB7+Ca10	5.6×10^6 a	5.6×10^6	6.7×10^4 ab	1.6×10^6
ChB7+F	1.9×10^6 a	2.5×10^6	7.5×10^4 ab	2.4×10^6
Ca10+F	4.8×10^5 a	4.2×10^5	3.0×10^4 ab	1.9×10^5
ChB7+Ca10+F	1.9×10^6 a	2.8×10^7	4.6×10^5 a	3.0×10^6
F	2.6×10^4 b	1.2×10^6	1.3×10^4 ab	7.1×10^5
CV[3]	37.6%	30.6%	29.3%	20.4%
P-value	0.003	0.613	0.004	0.247

[1] Results of CFU per g of root determined by qPCR were adjusted to the normal distribution by the formula y=Log(x+0.5).

[2] Treatments code: (UTC-): Control not inoculated with *Ggt*; (UTC+): Control inoculated with *Ggt*; (ChB7): *Pseudomonas protegens* ChB7 strain, inoculated to the seed; (Ca10): *P. protegens* Ca10 strain, inoculated to the seed; (F): Fluquinconazole, active ingredient of fungicide, applied to the seed in concetration of 87.5 g a.i. for 100 kg of seed. Bacterial strains were applied to the seed in a dose of 1.7 L (10^6 CFU) 100 kg^{-1} of seed.

[3] CV: Coefficien of variation.

[4] Letter different in a same column showed significantly differences among treatments according to LSD comparison mean test ($\alpha = 0.05$).

Table 4. Average plant heightss (cm) observed in winter wheat cv. 'Maxwell' in tillering (Z2.3), anthesis (Z6.9) and rippening (Z9.0) growth stages for seed treatments with *Pseudomonas protegens* strains ChB7 and Ca10, applied individually or in combinations with the fungicide fluquinconazole (F), under conditions of monoculture or rotation in two crop seasons.

Treatments[1]	Experiment condition and wheat growth stages								
	Monoculture_2014			Monoculture_2015			Rotation_2015		
	Z2.3	Z6.9	Z9.0	Z2.3	Z6.9	Z9.0	Z2.3	Z6.9	Z9.0
UTC-	22.8	69.9	72.5	22.3 ab[2]	77.6	80.0	21.4 a	78.8 a	75.8
UTC+	20.2	63.6	70.9	18.0 d	71.8	77.5	15.2 e	72.4 bc	72.8
ChB7	18.7	63.5	67.6	17.3 d	75.8	81.3	19.9 ab	71.9 bc	73.3
Ca10	21.6	63.0	67.8	19.5 cd	78.0	83.8	18.2 bcd	70.8 cd	69.8
ChB7+Ca10	20.6	63.3	66.0	20.8 bc	76.3	82.0	17.4 cde	65.9 d	73.9
ChB7+F	21.3	68.4	70.8	21.8 abc	79.1	83.3	19.6 abc	77.3 ab	74.3
Ca10+F	21.6	68.4	70.1	21.0 bc	82.1	87.5	16.8 de	72.4 bc	71.3
ChB7+Ca10+F	21.8	71.3	74.8	22.3 ab	76.5	85.0	20.3 a	78.8 a	73.6
F	21.0	68.5	72.1	23.8 a	77.1	83.8	19.9 ab	75.5 abc	75.0
CV[3]	11.4%	6.3%	6.9%	8.5%	5.7%	4.9%	8.3%	5.5%	4.8%
P[2] value	0.394	0.121	0.410	0.0003	0.089	0.125	0.001	0.008	0.421

[1] Treatments code: (UTC-): Control not inoculated with *Ggt*; (UTC+): Control inoculated with *Ggt*; (ChB7): *Pseudomonas protegens* ChB7 strain, inoculated to the seed; (Ca10): *P. protegens* Ca10 strain, inoculated to the seed; (F): Fluquinconazole, active ingredient of fungicide, applied to the seed in concetration of 87.5 g a.i. for 100 kg of seed. Bacterial strains were applied to the seed in a dose of 1.7 L (10^6 CFU) 100 kg^{-1} of seed.

[2] Letter different in a same column showed significantly differences among treatments according to LSD comparison mean test ($\alpha = 0.05$).

[3] CV: Coefficien of variation.

Table 5. Average number of spikes per m^2 and weight of 1,000 grains obtained at harvest in plots of winter wheat cv. 'Maxwell' inoculated with *Gaeumannomyces graminis var. tritici* and seed treateds with *Pseudomonas protegens* strains ChB7 and Ca10, applied individually or in combinations with fluquinconazole fungicide (F), under monoculture (M_2014 and M_2015) or rotation (R_2015) conditions in two crop seasons.

Treatments[1]	Number of spikes m^{-2}			1,000 grains weight (g)		
	M_2014	M_2015	R_2015	M_2014	M_2015	R_2015
UTC-	389 abc[3]	404	437	33.7 c	41.7 bc	51.5
UTC+	320 c	416	419	38.9 ab	41.3 bc	51.3
ChB7	398 ab	345	348	35.9 bc	39.1 c	47.6
Ca10	417 a	420	320	33.9 c	39.8 c	47.0
ChB7+Ca10	341 bc	368	429	37.3 abc	40.0 c	48.6
ChB7+F	398 ab	440	431	37.0 abc	43.9 abc	51.4
Ca10+F	381 abc	449	411	37.3 abc	49.9 a	49.8
ChB7+Ca10+F	429 a	390	390	38.9 ab	44.0 abc	51.3
F	369 abc	434	402	40.6 a	46.8 ab	50.3
CV[2]	13.7%	14.4%	17.0%	8.4%	9.6%	4.9%
P[2] value	0.022	0.095	0.229	0.019	0.015	0.053

[1] Treatments code: (UTC-): Control not inoculated with *Ggt*; (UTC+): Control inoculated with *Ggt*; (ChB7): *Pseudomonas protegens* ChB7 strain, inoculated to the seed; (Ca10): *P. protegens* Ca10 strain, inoculated to the seed; (F): Fluquinconazole, active ingredient of fungicide, applied to the seed in concetration of 87.5 g a.i. for 100 kg of seed. Bacterial strains were applied to the seed in a dose of 1.7 L (10^6 CFU) 100 kg^{-1} of seed.

[2] CV: Coefficien of variation.

[3] Letter different in a same column showed significantly differences among treatments according to LSD comparison mean test ($\alpha = 0.05$).

Table 6. Grain yield averages (t ha^{-1}) and grain test weight (kg hL^{-1}) at harvest in plots of winter wheat cv. 'Maxwell' inoculated with *Gaeumannomyces graminis var. tritici* and seed treateds with *Pseudomonas protegens* strains ChB7 and Ca10, applied individually or in combinations with fluquinconazole fungicide (F), under monoculture (M_2014 and M_2015) or rotation (R_2015) conditions in two crop seasons.

Treatments[1]	Grain yield			Grain test weight		
	M_2014	M_2015	R_2015	M_2014	M_2015	R_2015
UTC-	3.0 abcd	4.0 de	6.8 a	71.5 cd	74.1[3]	76.7 a
UTC+	2.6 bcd	3.5 e	4.5 cd	72.1 abcd	73.1	75.1 c
ChB7	2.3 d	4.3 bcde	5.0 bcd	71.8 bcd	74.3	75.3 c
Ca10	2.4 cd	4.2 bcde	4.8 bcd	71.8 bcd	73.8	75.2 c
ChB7+Ca10	2.3 d	4.2 cde	4.4 d	71.2 d	73.7	75.7 bc
ChB7+F	3.3 ab	5.1 abc	5.1 bcd	73.2 abc	74.5	76.2 ab
Ca10+F	3.3 ab	5.4 a	5.6 abc	73.6 ab	74.9	76.3 ab
ChB7+Ca10+F	3.5 a	5.1 ab	6.0 ab	73.7 a	74.2	76.7 a
F	3.1 abc	4.7 abcd	5.4 bcd	72.9 abcd	74.4	75.6 bc
CV[2]	18.2%	14.3%	16.3%	1.7%	1.0%	0.6%
P[2] value	0.005	0.002	0.013	0.008	0.099	<0.001

[1] Treatments code: (UTC-): Control not inoculated with *Ggt*; (UTC+): Control inoculated with *Ggt*; (ChB7): *Pseudomonas protegens* ChB7 strain, inoculated to the seed; (Ca10): *P. protegens* Ca10 strain, inoculated to the seed; (F): Fluquinconazole, active ingredient of fungicide, applied to the seed in concetration of 87.5 g a.i. for 100 kg of seed. Bacterial strains were applied to the seed in a dose of 1.7 L (10^6 CFU) 100 kg^{-1} of seed.

[2] CV: Coefficien of variation.

[3] Letter different in a same column showed significantly differences among treatments according to LSD comparison mean test ($\alpha = 0.05$).