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ACTIVIDAD ANTAGÓNICA DE CEPAS CHILENAS DE *PSEUDOMONAS PROTEGENS* SOBRE AGENTES CAUSANTES DE PUDRICIONES RADICALES EN TRIGO (*Triticum aestivum* L.)

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ACTIVIDAD ANTAGÓNICA DE CEPAS CHILENAS DE *PSEUDOMONAS PROTEGENS* SOBRE AGENTES CAUSANTES DE PUDRICIONES RADICALES EN TRIGO (*TRITICUM AESTIVUM* L.)

ANTAGONISTIC ACTIVITY OF CHILEAN STRAINS OF *PSEUDOMONAS PROTEGENS* ON FUNGI CAUSING CROWN AND ROOT ROT IN WHEAT (*TRITICUM AESTIVUM* L.)

RESUMEN

Los tratamientos a la semilla con bacterias antagonistas pueden reducir la severidad de infección de patógenos como *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia cerealis* y *Fusarium culmorum*, que causan pudriciones radicales del trigo. Esta investigación evaluó el efecto del tratamiento de semillas de trigo primaveral cv. Pantera-INIA con un consorcio bacteriano de tres cepas chilenas de *Pseudomonas protegens* sobre tres importantes enfermedades radicales que afectan al cultivo, y su efecto sobre los componentes del rendimiento. Durante dos temporadas de experimentos de campo con inoculación artificial de los patógenos mencionados, se analizaron componentes fitosanitarios (incidencia y severidad de infección aérea y de raíces), y agronómicos como altura de plantas (cm), número de granos, espigas y granos/espiga m^{-1}, peso de mil granos (g), peso hectólitro (kg hL^{-1}), acumulación de biomasa m^{-1} (g), y rendimiento (qqm ha^{-1}). En paralelo, se cuantificó mediante qPCR la densidad poblacional de *P. protegens* presente en el inóculo inicial pre y post siembra, y las poblaciones bacterianas en la rizósfera de plantas de trigo en estado de antesis (Z.6). En ambas temporadas fue posible observar disminución en la severidad de infección de enfermedades en aquellas parcelas con aplicación de *P. protegens* a la siembra, además de una mejoría en la mayoría de los componentes del rendimiento, como espigas m^{-1} y número de granos por espiga. El rendimiento final incrementó ante el tratamiento de las semillas con las bacterias que poseen el gen 2,4-DAPG, principalmente en la primera temporada de experimentos, donde se observó hasta 33% de aumento en el

rendimiento (P < 0,05). En general, se observaron mayores diferencias entre parcelas con y sin *P. protegens* en el experimento 2016-2017 que en el 2017-2018, presuntamente asociado a las condiciones climáticas de menores precipitaciones que favorecieron su actividad antagónica. Los resultados sugieren que el tratamiento de semillas con *P. protegens* es una alternativa de manejo para la reducción de enfermedades radicales en el cultivo de trigo en zonas geográficas con menores precipitaciones.

SUMMARY

Seed treatments with antagonistic bacteria reduced the severity of crown and root rot pathogens in wheat in inoculated field trials. The effect of the seed treatment of spring wheat cv. Pantera-INIA with a bacterial consortium of three Chilean strains of *Pseudomonas protegens* on three important crown and root rot pathogens as *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia cerealis*, and *Fusarium culmorum* affecting this crop and its effect on the yield component were evaluated. During two seasons of field experiments with artificial inoculation of the aforementioned pathogens, phytosanitary (incidence and severity) and agronomic components plus population density of *P. protegens* in the rhizosphere of wheat plants in anthesis state (Z.6) were assessed. In both seasons an average decreases of 16.8% in the severity of infections in those plots inoculated with *P. protegens* was observed. Increase in yield components such as spikes m^{-1} and number of grains per spike was also determined. Total yield was increased in those plots where seeds with the antagonistic bacteria during the first experimental season, where up to 31% increase in yield was observed (P < 0.05). In general, greater differences between plots with and without *P. protegens* were observed in the 2016-2017 experiment than in the 2017-2018 experiment, presumably associated to climatic conditions of lower rainfall, which favored their antagonistic activity. Results suggest that the treatment of seed with *P. protegens* is a management alternative for reducing crown and root diseases in geographic areas with less rainfall.

CAPÍTULO 1

INTRODUCCIÓN GENERAL

El cultivo del trigo (*Triticum aestivum* L.) es severamente afectado por diversos hongos que son habitantes comunes de suelo y que causan enfermedades de diversa consideración. Estos patógenos necrosan tejidos de la zona de raíces y corona de la planta de trigo, interrumpiendo el normal transporte de agua y nutrientes, resultando en importantes pérdidas en el rendimiento (Daval et al., 2010). Las enfermedades desarrolladas por estos hongos son de difícil diagnóstico, pues ocurren bajo suelo y sus síntomas pueden confundirse con deficiencias nutricionales, hídricas, o cualquiera ocasionada por factores abióticos (Raaijmakers et al., 2009). Dentro de estos patógenos destacan especies de los géneros *Gaeumannomyces*, *Fusarium* y *Rhizoctonia*. En Chile, diversas especies de los géneros *Fusarium* y *Rhizoctonia* fueron aislados desde siembras comerciales de trigo en la zona Sur a partir del primer internudo, observándose distintos porcentajes de incidencia y severidad (Moya-Elizondo et al., 2015), mientras que *G. graminis* var. *tritici* se consideró el hongo fitopatógeno que más pérdidas genera en agricultores productores de trigo (Andrade et al., 2011; Vera et al., 2014)

Gaeumannomyces graminis var. *tritici*, causa la enfermedad denominada "Mal del pie" en trigo, que se asocia a daños en las raíces y base del tallo de la planta y que en ataques severos, merma fuertemente el rendimiento del cultivo (McMillan et al., 2014; Vera et al., 2014). Este hongo ascomycete sobrevive principalmente como micelio de manera saprofítica en restos de tallos y raíces de plantas de trigo u otras gramíneas susceptibles. Al entrar este inóculo en contacto con una nueva planta de trigo, el patógeno desarrolla hifas corredizas que crecen superficialmente en primera instancia, para luego cubrir y penetrar los tejidos de la zona radical, colonizando y destruyendo tejidos vasculares, interrumpiendo así el transporte de agua y nutrientes en la planta hospedera (Kwak y Weller, 2013; Quan et al., 2015). A consecuencia de esto, las plantas comienzan a expresar síntomas de clorosis en hojas, crecimiento reducido, formación de espigas blancas en período de floración, producción de grano chupado, o muerte de la planta. Actualmente no existen variedades resistentes a la patología, y el control químico aunque ha tenido cierto éxito, aun no logra controlar la enfermedad en su totalidad (Vera et al., 2014). Diversos autores (Lebreton et al., 2004; Daval et al., 2010) han estudiado la variabilidad genética

de *G. graminis* var. *tritici*, identificando dos grandes grupos genéticos capaces de co-existir, los cuales demuestran diferir en la severidad de infección en plantas de trigo, prevalencia en monocultivos de trigo, sensibilidad a fungicidas, entre otros. En complemento a lo anterior, muestreos realizados a 48 siembras de trigo comercial en la temporada 2011-2012 en las regiones de La Araucanía, Los Ríos y Los Lagos, demostraron la existencia de un tercer grupo genético para la especie, hasta el momento desconocido, capaz de generar infecciones severas en plantas de trigo (datos no publicados).

En relación al complejo de hongos del género *Fusarium*, este se compone por diversas especies, entre las que destacan *F. graminearum* Schwabe, *F. culmorum* (Smith) Sacc. y *F. pseudograminearum* Aoki y O'Donnell (Beccari et al., 2018), los cuales han sido reportados como importantes agentes patógenos del cultivo de trigo a nivel mundial, afectando tanto el rendimiento final como la calidad del grano (Scherm et al., 2013). Es importante mencionar que miembros del género *Fusarium* son capaces de producir micotoxinas como deoxynivalenol (DON) y zearalenona (ZEA), metabolitos secundarios que son capaces de contaminar láminas foliares, cañas, glumas y granos, suponiendo esto un riesgo para el consumo humano y animal (Rohweder et al., 2011). En Chile no ha sido reportada la especie *F. pseudograminearum*, mientras que *F. culmorum* sería la de más prevalencia en el sur de Chile, pues está adaptada a condiciones frías, a diferencia de *F. graminearum*, que afectaría durante el proceso de establecimiento del cultivo (Moya-Elizondo et al. 2015). No obstante lo anterior, existen pocos antecedentes sobre la importancia fitopatológica de *F. culmorum* bajo las condiciones de producción de trigo en el sur de Chile.

En cuanto a *Rhizoctonia*, las especies pertenecientes al género son hongos habitantes comunes del suelo que desarrollan pudriciones radicales en una serie de especies vegetales de importancia agrícola pertenecientes a las familias Fabaceae, Solanaceae, Brassicaceae, Asteraceae, Poaceae (Ajayi-Oyetunde y Bradley, 2018). Dentro de esta última, en el caso del trigo destacan *R. solani* Kuhn (AG 8), *R. oryzae* Ryker y Gooch, y *R.cerealis* E.P.Hoeven (Weller et al., 2016). Los miembros del complejo *Rhizoctonia* atacan raíces generando pudriciones a nivel del córtex, afectando al vigor de la planta, y en consecuencia, su rendimiento (Okubara et al., 2014). En Chile las tres especies han sido identificadas afectando trigo en la zona radical (Moya-Elizondo et

al., 2015; Doussoulin et al., 2016). No obstante lo anterior, existen pocos antecedentes del daño cuantitativo que estos causan en el cultivo de trigo bajo los agrosistemas del sur de Chile.

El control de estas enfermedades radicales no es sencillo, e implica la integración de métodos de control tanto culturales, como genéticos, químicos y biológicos (Scherm et al., 2013). Dentro de este contexto, el control biológico aparece como una alternativa sustentable para el manejo de enfermedades fúngicas del suelo, y dentro de éste el fenómeno de los suelos supresivos toma relevancia (Doussoulin y Moya-Elizondo, 2011; Expósito et al., 2017; Durán et al., 2018). Un suelo supresivo es aquel donde un patógeno no puede establecerse o persistir, y de establecerse, genera un bajo nivel de daño en las plantas afectadas (Durán et al., 2017). En este tipo de suelos, la presencia de bacterias del género *Pseudomonas* han sido descrita como agentes importante en el proceso de supresión de patógenos asociado a la producción de antibióticos, tales como 2,4-diacetilfloroglucinol (2,4-DAPG), fenazina-1-ácido carboxílico (PCA), pirrolnitrina, rhizoxina, pioluteorina, hidrógeno de cianuro (HCN), y 2-hexyl-5-propyl resorcinol (HPR) (Loper et al., 2012; Ramette et al., 2011). Entre ellas, *Pseudomonas protegens* se caracteriza por producir 2,4-DAPG, pirrolnitrina y pioluteorina, y han mostrado eficacia en el control de hongos como *G. graminis* var. *tritici* (González et al., 2018). Estas bacterias han sido detectadas en Chile recientemente, llegando a detectarse posibles interacciones entre éstas y hongos comunes de suelo que afectan raíces y cuello de la planta de trigo (Moya-Elizondo et al., 2013). Sin embargo, no se ha analizado en mayor profundidad cómo estas poblaciones bacterianas antagonistas regulan la incidencia y severidad de los distintos hongos fitopatógenos que afectan raíces y corona del trigo bajo la condición de un suelo Andisol en Chile.

Este tipo de suelos de origen volcánico es muy poco común, representando menos del 1% de los suelos cultivables a nivel mundial (Delmelle et al., 2015) y caracterizándose por su alta capacidad de fijación de fósforo (P), altos contenidos de materia orgánica (MO), niveles elevados de acidez que desencadenan fitotoxicidad a diversos compuestos minerales como aluminio y manganeso, además de ser en la mayoría de los casos deficitarios en nutrientes como fósforo (P) y calcio (Ca) (Borie et al., 2010).

El objetivo principal de este estudio fue evaluar el efecto que la adición a la semilla de bacterias *P. protegens* tiene en la reducción del daño causado por distintos grupos genéticos de *G. graminis* var. *tritici*, y por especies de *Fusarium* y *Rhizoctonia* en plantas de trigo en un suelo

Andisol del sur de Chile. A través de ensayos de campo con inoculación artificial de los patógenos mencionados y un consorcio de *P. protegens* aplicadas a la semilla se determinó el antagonismo que estas presentan sobre estos hongos radiculares y los efectos sobre distintas variables agronómicas en plantas de trigo. Además se estudió mediante herramientas moleculares el nivel de colonización que tienen las bacterias sobre la rizósfera de trigo.

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CAPÍTULO 2

Antagonistic activity of Chilean strains of *Pseudomonas protegens* on fungi causing crown and root rot in wheat (*Triticum aestivum* L.)

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ABSTRACT

Seed treatments with antagonistic bacteria reduced the severity of crown and root rot pathogens in wheat in inoculated field trials. The effect of the seed treatment of spring wheat cv. Pantera-INIA with a bacterial consortium of three Chilean strains of *Pseudomonas protegens* on three important crown and root rot pathogens as *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia cerealis*, and *Fusarium culmorum* affecting this crop and its effect on the yield component were evaluated. During two seasons of field experiments with artificial inoculation of the aforementioned pathogens, phytosanitary (incidence and severity) and agronomic components plus population density of *P. protegens* in the rhizosphere of wheat plants in anthesis state (Z.6) were assessed. In both seasons an average decreases of 16.8% in the severity of infections in those plots inoculated with *P. protegens* was observed. Increase in yield components such as

spikes m^{-1} and number of grains per spike was also determined. Total yield was increased in those plots where seeds with the antagonistic bacteria during the first experimental season, where up to 31% increase in yield was observed ($P < 0.05$). In general, greater differences between plots with and without *P. protegens* were observed in the 2016-2017 experiment than in the 2017-2018 experiment, presumably associated to climatic conditions of lower rainfall, which favored their antagonistic activity. Results suggest that the treatment of seed with *P. protegens* is a management alternative for reducing crown and root diseases in geographic areas with less rainfall.

Keywords: Fusarium crown rot, Take all, Sharp eyespot, *Pseudomonas protegens, Triticum aestivum*, 2,4-DAPG.

INTRODUCTION

Wheat cultivation (*Triticum aestivum*) is severely affected by various pathogenic fungi that are common inhabitants of the soil and that cause diseases of varying consideration. These pathogens cause necrosis from the root and crown areas of the wheat plant, interrupting the normal transport of water and nutrients, resulting in significant yield losses (Daval, et al. 2010). The diseases developed by these fungi are difficult diagnosed because they occur under the soil and may be confused with water or nutritional deficiencies, or any caused by abiotic factors (Raaijmakers et al. 2009). Among these pathogens, species of the genera *Gaeumannomyces, Fusarium*, and *Rhizoctonia* can be mentioned. In Chile, various species of *Fusarium* and *Rhizoctonia* were isolated from commercial wheat crops in southern Chile, being observed different percentages of incidence and severity (Moya-Elizondo et al. 2015), whereas *G. graminis* var. *tritici* has been considered the phytopathogenic fungus that generates greatest losses in Chilean wheat production (Andrade et al. 2011; Vera et al. 2014).

Gaeumannomyces graminis var. *tritici* causes the disease called “Take all”, which damages in roots, crown and culms of the plant and strongly reduces crop yield in severe infections (McMillan et al. 2014; Vera et al. 2014). This ascomycete fungus survives mainly as mycelium in a saprophytic manner in stems, crowns, and roots of wheat plants and other susceptible grasses. The pathogen develops sliding hyphae that grow superficially in first instance to then cover and

penetrate the tissues of the root area, colonizing and destroying vascular tissues, thus interrupting the transport of water and nutrients in the host plant (Kwak and Weller, 2013; Quan et al. 2015). As a consequence, the plants begin to express symptoms of chlorosis in leaves, reduced growth, formation of white spikes in flowering, production of sucked grain, or death of the plant. On the other hand, the genetic variability of *G. graminis* var. *tritici* identifies two large genetic groups capable of co-existing, which present differences in the severity of infections in wheat plants, prevalence in wheat monocultures, and sensitivity to fungicides (Lebreton et al. 2004; Daval et al. 2010). Samplings carried out on 48 commercial wheat crops in the 2011-2012 season in the Araucanía, Los Ríos and Los Lagos regions of Chile showed the existence of a third genetic group until now unknown, capable of causing severe infections in wheat plants (unpublished data).

The fungal complex of the genus *Fusarium* is composed of several species that affect grasses, including *F. graminearum* Schwabe, *F. culmorum* (Smith) Sacc. and *F. pseudograminearum* Aoki & O'Donnell (Beccari et al. 2018). These species have been reported as significant pathogens in wheat cultivation worldwide, affecting both final yield and grain quality (Scherm et al. 2013). Members of the genus *Fusarium* are capable of producing mycotoxins such as deoxynivalenol (DON) y zearalenone (ZEA), secondary metabolites that can contaminate leaf, spikes, glumes and grains, implying risks for human and animal, which consume these products (Rohweder et al. 2011). *F. culmorum* would be the most prevalent member of the fusaria group present in southern Chile, since it is adapted to colder conditions (Moya-Elizondo et al. 2015), but there are few antecedents about the phytopathological importance of *F. culmorum* under the conditions of wheat production in southern Chile.

In wheat, the species *R. solani* Kuhn (AG 8), *R. oryzae* Ryker & Gooch, and *R.cerealis* E.P.Hoeven are important soilborne pathogens (Mavrodi et al. 2012b; Weller et al. 2016) . Member of the *Rhizoctonia* complex attack roots, generating decay at the cortex level, affecting the vigor of the plant and the wheat production (Okubara et al. 2014). In Chile, the three species have been identified affecting wheat (Moya-Elizondo et al. 2015; Doussoulin et al. 2016). However, there is little background of the quantitative damage caused in wheat crops under the agrosystems of southern Chile.

The control of root diseases is not simple and involves the integration of cultural and genetic, chemical and biological control methods (Scherm et al. 2013). For example, currently, there are no resistant varieties to *G. graminis* var. *tritici* and although chemical control have had some success, the disease is not completely controlled (Vera et al. 2014). In this context, biological control appears as a sustainable alternative for the management of fungal soil diseases and within this, the phenomenon of suppressive soils becomes relevant (Doussoulin and Moya-Elizondo, 2011; Expósito et al. 2017; Durán et al. 2018). In this type of soils, the presence of bacteria of the genus *Pseudomonas* have been described as important agents in the process of suppression of pathogens, which is associated with the production of antibiotics such as 2.4-diacethylfloroglucinol (2,4-DAPG), phenazine-1-carboxylic acid (PCA), pyrrolnitrin, rhizoxin, pioluteorin, cyanide hydrogen (HCN), and 2-hexyl-5-propyl resorcinol (HPR) (Ramette et al. 2011; Loper et al. 2012). Among these species, *Pseudomonas protegens* is characterized by producing 2,4-DAPG, pyrrolnitrin and pioluteorin and this bacterium has recently been detected in Chile (Moya-Elizondo et al. 2013) and has shown efficacy in the control of fungi such as *G. graminis* var. *tritici* (Gonzalez et al. 2018).

Recent studies carried out in Chile have evaluated the effect of *P. protegens* on take-all disease, detecting possible interactions between soil fungi and bacteria producing 2,4-DAPG and PCA (Moya-Elizondo et al. 2013; González et al. 2018; Vera et al. 2019). However, it has not been analyzed in depth how these antagonistic bacteria regulate both incidence and severity of the different phytopathogen fungi that affect wheat roots and crown under the conditions of an andisol soil in Chile. This type of soils of volcanic origin is very rare in the world, representing less than 1% of the cultivable soils worldwide (Delmelle et al. 2015). These soils are also characterized by their high capacity of phosphorous (P) binding capacity, high contents of organic matter (OM), high levels of acidity that trigger phytotoxicity by aluminum and manganese. In addition, these characteristics cause phosphorous and calcium deficiencies (Borie et al. 2010).

The main objective of this study was to assess the effect seed treatments with the bacteria *P. protegens* has on the damage caused by different genetic groups of *G. graminis* var. *tritici*, and by species of *Fusarium* and *Rhizoctonia* in wheat plants in an andisol soil in southern Chile. Through field trials with artificial inoculation of the aforementioned pathogens and a consortium

of *P. protegens* applied through the seed, the antagonism presented by the bacteria on these fungi was determined. Likewise, the effects on different agronomic variables were also assessed. In addition, the level of colonization of the bacteria on the wheat rhizosphere was studied through molecular tools.

MATERIALS AND METHODS

Study area

Between 2016 and 2018, two field experiments were carried out in the plant-breeding area of the Santa Rosa Experimental Station, belonging to the Instituto de Investigaciones Agropecuarias (INIA), in the Centro Regional de Investigación Quilamapu, at 25 km from the city of Chillán, Ñuble Region, Chile (36°31'53'' S, 71°54'50.1'' O, 220 m.a.s.l). The soil corresponds to an Andisol (Typic Melanoxerands) of the Arrayán series that uses gravitational irrigation (Stolpe, 2006). Both experiments were established under a split plot design, with 10 treatments and four repetitions, which are described in Table 1. The experiments considered a factor that corresponds to the inoculation with four root and crown rot wheat pathogenic fungi, plus a control not inoculated with a fungus. On the other hand, a factor with or without application of antagonistic bacteria to the seed was also considered. Seeding of the first experiment was performed on August 8, 2016 and the second one, on August 16, 2017. The experimental unit corresponded to a plot with six rows, each of 2 m long and 0.2 m between rows (2 m^2), where spring wheat (*T. estivum*) cv. Pantera-INIA was seeded. In the case of rows inoculated with phytopathogenic fungi, oat grains inoculated with the pathogens were placed next to the wheat seed at the time of sowing, in a 1:3 proportion. Only the four central rows of each plot were inoculated.

Inoculum preparation of phytopathogenic fungi

The inoculum preparation for all phytopathogenic fungi was carried out by following the methodology described by Vera et al. (2014). Briefly, 500 mL Erlenmeyer flasks were filled with 200 g of clean oat and 100 mL of distilled water, allowing soaking during 24 h to be then autoclaved at 120°C for 15 min, at a pressure of 15 psi, twice in 24 h. Subsequently, pieces of agar from each of the Petri dishes with pathogenic isolates under study were cut and then

incorporated into the sterile flasks with oat for 15 to 30 days in order to uniformly colonize the kernels. These oat kernels were then seeded together with the wheat seeds in the field to inoculate the experiments.

Fungi under study were previously grown in APD medium during 7 days at 24 ± 2 °C. These corresponded to the following species: *G. graminis* var. *tritici* isolate GGT_G2-INIA, which belonged to the genetic group 2 of this pathogen, which is highly pathogenic (Vera et al. 2014); *G. graminis* var. *tritici* isolate Oso1, belonging to the proposed new genetic group 3 (GGT_3); *F. culmorum* isolate F_CULM: and *R. cerealis* isolate M31S. All these fungal isolates were obtained in a survey conducted on commercial wheat crop fields in southern Chile (Moya-Elizondo et al. 2015).

Antagonistic microorganisms

A mixture of bacteria composed of strains of *P. protegens* Ca10, Ca6, and ChB7 were used. These bacteria were isolated from the wheat rhizosphere from different Andisol soils located in southern Chile (Moya-Elizondo et al. 2013). Studies performed in our laboratory have demonstrated the presence of the *phlD* gene (associated with the production of 2, 4-DAPG) and metabolic mechanisms to promote growth in the strains used in this investigation, such as phosphorus solubilizing activity and IAA (indole acetic acid) production (unpublished data). These bacteria are stored at -80°C in King'B medium plus 20% glycerol, at the Phytopathology Laboratory of the Faculty of Agronomy at the Universidad de Concepción in Chillán Campus, Chile.

The ChB7 strain was obtained from a field located in the city of Chillán, whereas strains Ca10A and Ca6 were isolated from two fields located in Cajón at 8 km from the city of Temuco, in the Araucanía Region, Chile. Briefly, bacterial suspensions of the strains Ca10, Ca6 and ChB7 (~10^7 colony forming units [CFU] mL^{-1}) were centrifuged at 4,000 rpm for 8 min at 20°C. Then, the supernatant was eliminated and the samples were washed in a buffer (25 ml sodium chloride 0.9%). Subsequently, the samples were centrifuged again under the same conditions mentioned above for 5 min and the supernatant was removed. A total of 100 µL sterile KB medium and 3 mL of 0.5% carboxymethyl cellulose were added to improve the bacteria adherence to the seed. Finally, 3.1 mL of bacterial suspension were mixed at vortex to be applied to 1 kg of seed before

seeding. A dose of 50 g of seeds per experimental plot was used for treatments with and without the antagonistic bacteria.

Agronomic management and weather conditions

Seeeding was carried out manually, after soil preparation and the application of herbicide. The products used were Flufenacet+Flurtamone+Diflufenican (Bacara® Forte 360 SC, Bayer S.A.). A second application of herbicide was performed at tillering, growth stage Z2.3 (Zadoks and Schein, 1974), using Iodosulfuron-methyl-sodium + Mesosulfuron-methyl (Cossack 150 WG, Bayer S.A.). Fertilization broadcast was carried out 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 Zn sulfate ($ZnSO_4$) (3 kg ha^{-1}). Nitrogen fertilization was carried out using split applications , with 133 units of sodium nitrate ($NaNO_3$): 93 units (375 kg ha^{-1}) in main shoot and three tillers state (Z2.3 Zadoks scale) and 60 units (580 kg $ha^{-1)}$ at the end of the tillering state (Z2.9 Zadoks scale).

The average monthly rainfall corresponded to 24.6 mm during the 2016-2017 seasons and 77.9 in the 2017-2018 seasons (Fig. 1). Flood irrigation was used in both seasons, where irrigation were performed on October 11, November 9 and 21, 2016. The average monthly air temperature was quite similar between seasons, being registered as 14°C in both crop cycles. Climatic information was obtained from the agrometeorological station available at the INIA Quilamapu Santa Rosa Experimental Station.

Evaluations

In order to determinate *P. protegens* populations in the rhizosphere and evaluate their antagonistic effect of on phytopathogenic fungi of the root and crown under study, quantification of bacterial populations, and sanitary and agronomic evaluations were carried out during both crop seasons.

Determination of bacterial populations of *Pseudomonas protegens* present in the seed before and after seeding and in the rhizosphere of the plants in anthesis (Z.6)

The bacterial populations of *P. protegens* were quantified after treatment of the seeds with the bacterial inoculum (pre-seeding) and 24 h later, because this was the time that elapsed between the inoculation of the seeds and the sowing in the field. Determination of initial bacterial

inoculum in seeds was performed through serial dilutions, where a gram of seeds inoculated with the mix of bacteria was diluted in 9 ml of sterile distilled water (SDW) and vortexed by 25 s. Then, 100 µL were sowed in KB agar medium. Dishes were incubated at 25°C for 24 h to determine CFU g^{-1} seeds. In the anthesis state (Z.6), a random sample of roots from each plot was collected. These were cleaned by removing the surplus soil with sterile brushes. Samples were placed in 15 mL individual Falcon tubes in order to be stored in a freezer at -80°C until use. Both quantification and bacterial detection of samples collected in anthesis was carried out through the real-time quantitative PCR technique (qPCR), based on protocols described by Vera et al. (2019). Briefly, 1 g of roots was collected from each plot and 9 mL SDW were added and left to rest for 24 h. From this decanted sample, a DNA extraction was performed using the PowerSoil® kit (Qiagen N.V, Germany). Subsequently, an analysis of the presence of the phlD gene group A was also performed. This is associated to Pseudomonas producing 2,4-DAPG, belonging to Group A, which corresponds to the genetic Group of *P. protegens* strains ChB7, Ca10 and Ca6. StepOnePlus™ Real Time PCR Systems (Applied Biosystems) equipment was used. For the analysis, primers A_Up y A_Low (Mavrodi et al. 2007) and each qPCR reaction contained a mixture of 5 µL SYBR® Green (KAPA SYBR® FAST qPCR, KAPA Biosystems), 2 µL de ADN, 0.1 µM ROX (passive reference) and 0.5 µM of each primer to generate a final volume of 10 µL. The qPCR plate was loaded with three technical replicas of each sample.

Evaluation of damage by disease

After observing the first symptoms of root diseases in the experimental plots, visual estimates were performed weekly on the percentage of the area of the plots affected by symptoms associable to root rot, such as chlorotic patches, reduced plant growth, and presence of white spikes. Data obtained were used to calculate the area under the disease progress curve (AUDPC), which combines multiple observations of visual symptoms over time and expresses them in one single value (Simnko and Piepho, 2012). In each experimental season, the assessment of root diseases incidence was performed through plant sampling of 50 linear cm of the second row of each plot. Collection of wheat plants was conducted one day before harvest, where symptomatic stems were determined respect to the total number of stems. In addition, disease severity or IDSI (internodes discoloration severity index) was determined through a 6-digit visual scale, corresponding to the percentage of damage observed in the first internode, where 0 = 0% or

without symptoms, 1 = 1 a 25 %, 2 = 25 to 50 %, 3 = 50 to 75 % and 4 = 75 to 100 % of darkening of the tissue in the first internode (Moya-Elizondo et al. 2015). The IDSI for each field was then calculated as: [Σ (class value × frequency)/(total number of plants × the highest class value)] × 100 (Hogg et al. 2007), and it was evaluated in 80 stems of wheat plants one day after harvest. In addition, a sample of 10 symptomatic stems was taken for the re-isolation of pathogens from each experimental plot.

Agronomic evaluations

Ten days after seeding, plant emergence was evaluated by counting the number of plants present in a linear meter within the four central rows. After the emergence, the phenological crop stages were determined weekly using the Zadoks scale (Zadoks and Schein, 1974). In addition, the height of the plants were registered until they reached the harvest maturity (Z.9).

One day prior to harvest, a wheat plant samples were collected to determined yield components, such as number of spikes per m^2, number of grains per spike and the weight of 1,000 grains. The sample corresponded to 20 spikes, collected in a linear meter of the second row in each experimental plot, which were manually threshed. In addition, total biomass and harvest index were determined. The harvest of the first experiment was carried out on January 23, 2017 and the second one on January 26, 2018 using a Winterstager threshing equipment and considering only the four central rows of each experimental plot. With the sample of wheat obtained at harvest, hectoliter weight and grain yield per plot (ton ha^{-1}) were determined.

Statistical analysis

The results of each evaluation were subjected to an analysis of variance (ANOVA), after checking the assumptions of normality, homoscedasticity and independence of the data. When differences were found ($p < 0.05$), the Fisher's least significant difference (LSD) was used to determine differences between treatments. Percentages were adjusted through the square root transformation through the formula $y = \sqrt{(x + 0.5)}$, where x = percentage value to be transformed (Little and Hills, 1976). Pearson correlation analyzes were performed to determine the relations between pathogenicity of isolates and bacterial populations of *P. protegens* with respect to the variables evaluated. All analyzes were performed with SAS software version 8 (SAS, 1999).

RESULTS

Quantification of populations of P. protegens in seed and rhizosphere of wheat plants

Pre and post seeding quantification of the bacterial inoculum on the seeds ranged between 10^9 and 10^7 CFU g^{-1} of seed (data not shown). Bacterial populations present in the rhizosphere of wheat plants during the phenological state of anthesis (Z.6) showed differences between those treatments with and without application of *P. protegens* for both experiments ($P < 0.05$; Fig. 2). The inoculation of the bacteria increased the concentration of these in the rhizosphere, passing from an average of 10^3 CFU g^{-1} of root present in the treatments not inoculated with the bacteria to 10^5 CFU g-^{1} root in those inoculated with the bacteria in the 2016-2017 experiment, while in the second experiment this varied from 10^4 CFU g^{-1} in the not inoculated treatments to 10^6 CFU g^{-1} in those inoculated with the bacteria (Fig. 2).

The highest responses in bacterial population in the root for the seed inoculation with *P. protegens* were observed when plots were infested with the two isolates of *G. graminis* var. *tritici*, reaching more than 10^5 CFU g^{-1} of root in the measurements performed in both years. GGT_3 without inoculation with bacteria *P. protegens* in the 2016-2017 experiment presented no detection of these bacterial populations in the root during anthesis, which was due to the fact that the concentration of bacteria present in the sample was below the detection range of the qPCR equipment used.

Effect of the pathogens and their interactions with the bacterial consortium of P. protegens on the damage in foliage and roots of spring wheat

The integration of the percentage of symptoms observed on experimental plots along the crop development is expressed through the AUDPC obtained for each treatment (Table 2). In the 2016-2017 experiment, significant differences for the AUDPC were observed between plots with and without *P. protegens* in all treatments infested or not with some root rot pathogen, with the exception of *F. culmorum* (Table 2). In this season, both healthy controls (UTC+ and UTC-) and *F. culmorum* plus the addition of *P. protegens* (+Pp) showed the lowest damage index, not being different between them and presenting the healthy control (UTC) + Pp the lowest AUDPC value. Significant differences were obtained with the rest of the treatments. The control non-inoculated with the bacteria (-Pp) and *F. culmorum* + Pp and *R. cerealis* + Pp showed lower levels of visual

damage, whereas treatments GGT_2 + and -, GGT_3, and *R. cerealis* non-inoculated with bacteria (- Pp) had higher expression of symptoms associable to root damage at field level. The incidence assessment showed that there were no differences between both controls with respect to *F. culmorum*, *R. cerealis*, and GGT_3. GGT_2 reached the highest number of affected stems and differed from the other treatments, except *F. culmorum* + Pp and GGT_3 with and without Pp. In general, the addition of bacteria to the seed had no influence on the incidence of damages observed in stems. Regarding the severity of infection observed in the first internode of the wheat plants, it was observed that the lowest infection was obtained by healthy controls and treatments inoculated to the soil with *R. cerealis*, ranging from 7.1 to 8.6% (Table 2). Plots artificially infested with *R. cerealis* showed no significant differences between these inoculated or not with Pp, or with respect to both treatments inoculated with *F. culmorum*, which developed a mild to moderate infections, below 14% on the stem. In the case of GGT_2 + and – Pp, these showed the highest severity of infection (29-34%), being statistically different from the rest of treatments ($p < 0.0001$), whereas GGT_3 + and – Pp remained under 17% in wheat plants. IDSI presented no differences between plots with and without addition of bacterial consortium inoculated with the same pathogen. Moreover, during the 2016-2017 crop cycle, a positive significant correlation was observed between the values of AUDPC and the severity of the infection observed in the stems (Pearson: 0.30; $p = 0.0412$).

In the 2017-2018 experiment, treatments inoculated with bacteria in the control and *R. cerealis* were different in their AUDPC with respect to their untreated pairs with *P. protegens* in 30% and 40.7%, respectively ($P < 0.001$; Table 2). GGT_3 + Pp presented an AUDPC value 13% higher than its pair not inoculated with Pp, whereas those treatments inoculated with GGT_2 and *F. culmorum* presented no differences with their pairs with and without application of bacteria to the seed. Both controls together with the treatments infested with *F. culmorum* and *R. cerealis* and inoculated with Pp showed the lowest expression of foliage symptoms associated to crown and root rot diseases. On the other hand, GGT_2 + and – Pp, GGT_3 + and – Pp, and *R. cerealis* – Pp presented the highest observable damages on foliage of the experimental plots, reaching an average AUDPC value of 1,816.6 , which was 104.5% higher than the average of both controls. The incidence results followed an infection and control response on pathogens similar to that observed for AUDPC. The addition of bacteria to the seed markedly reduced the incidence of damage in the stems evaluated between the two controls (135% less). On the other hand,

treatments inoculated in the soil with the pathogens and treated with the bacterial consortium tended to have greater damage and this characteristic was significant for GGT_2 ($p < 0.0001$). The severity results showed the same patterns observed for the treatments in the incidence evaluation, except for the controls, where the addition of Pp to the seed had no effect on the percentage the damaged first internodes.

Effect of bacteria P. protegens on agronomic variables in spring wheat inoculated with different root patterns

Final plant height reached by each treatment (cm) in both crop seasons is shown in Table 3. In the 2016-2017 experiment, *F. culmorum* + Pp reached the highest final height of all treatments (94 cm), being equal to the healthy control +Pp and *R. cerealis* +Pp ($p = 0.0179$), which reached a value slightly greater than 91 cm. The addition of Pp to the seed implied an average increase of 5.3% among all treatments. Moreover, this difference is significant in the case of GGT_3 and *F. culmorum*. In general, the inoculation with the pathogens markedly reduced the growth of the plants, although treatments GGT_2 and GGT_3 without the addition of the bacterial consortium showed the lowest growth by the end of the season. In this case, plant heights were lower than 86 cm on average. On the other hand, in 2017-2018 season the treatments with highest final growth were the healthy control + Pp and *R. cerealis* + Pp, which reached 94 cm in height and were statistically different from to both treatments of GGT_2 and GGT_3.In both growing seasons, a negative correlation was observed between the final height reached by the plants and the determined AUDPC values (Pearson: -0.64, $p < 0.0001$ and Pearson: -0.52, $p = 0.0006$, respectively).

The number of stems, spikes and grains/spike evaluated on a linear meter of the plots during the 2016-2017 experiment presented significant differences in all parameters mentioned ($P < 0.05$; Table 4). In general, improvements of these foliage components were observed in those plots treated with *P. protegens* to the seed, in addition to the evident effect of the infection of GGT_2 and GGT_3 on these three parameters. In the case of GGT_3, the inoculation of *P. protegens* to the seed may be associated to the significant increase of 37 stems m^{-1}, whereas the addition of the bacterial consortium was significant and implied 30 spikes m^{-1} and 4 grains/spike m^{-1} more in the plots of GGT_2 +Pp. These were the treatments where significant differences were observed when treatments inoculated with bacteria were contrasted with their pair without the beneficial bacteria

(p <0.05). Inoculation with *F. culmorum* and *R. cerealis* showed no differences from the control when considering the evaluation of number of stems and spikes, nor when they were significantly influenced by the addition of antagonistic bacteria. However, in the measurement of grains/spike m^{-1}, these fungi had higher values than the healthy control without Pp.

In the 2017-2018 experiment, differences were observed in the number of stems and spike m^{-1} between treatments, where the inoculation of isolates of GGT_2 and GGT_3 strongly affected the performance of the wheat plants in these variables (p <0.05; Table 3). On the other hand, the inoculation with *F. culmorum* and *R. cerealis* had an erratic performance, although slightly more aggressive in the case of *Rhizoctonia*. In this growing season, a trend to the increase in the number of stems and spikes was again observed counted in the plots inoculated with the *phlD+* bacteria consortium, especially in the case of GGT_2, where the bacterial addition implied a significant increment of 15 stems per lineal meter, despite the high aggressiveness presented by this isolate. In the number of grains per spike, no differences were observed among treatments during the season (Table 4). In the same crop cycle, only a negative correlation between severity of the infection (ISDI) with respect to the number of spikes was obtained (Pearson: -0.34, p = 0.0308).

Results of total biomass had coherence with the severity of damage observed in those plots inoculated with GGT_2. In this treatment, the biomass showed 39.3% less biomass respect to the average of the controls. This was statistically different from all treatments (p = 0.05), with the exception of *R. cerealis* -Pp with respect to GGT_2 -Pp. When grain biomass was assessed, the same previous performance was observed, with GGT_2 registering the lowest values. Likewise, GGT_2 +Pp were different to all other treatments (p < 0.0001). The control without inoculation and with treatment of *P. protegens* registered the highest value in this last component, reaching 182 g m^{-1}, value 130% higher to the 79 g m^{-1} of biomass of grains obtained in plots inoculated with GGT_2 +Pp. In general, with the exception of GGT_2, an improvement is observed in the plots in which the bacterial consortium of *P. protegens* (+Pp) with respect to the grain biomass which is also reflected in the harvest index (HI), where control +Pp could be associated to the increase from 43.6 to 63.5 in this index (45%, p = 0.005). In the 2016-2017 experiment, it was again observed that all plots inoculated with strains of *G. graminis* var. *tritici* reached the lowest accumulation of biomass (p > 0.0001), where in the total weight, both GGT_2 are shown different

from the rest of the treatments. On the other hand, the addition of Pp caused no effect on this variable in front of this genetic group. On the other hand, the addition of Pp in the healthy control implied an increase of almost 30 g per lineal m in grain biomass. Likewise, in the treatment inoculated with GGT_3 the consortium increased its yield in 26.2 g per lineal m, not being different from the healthy control not treated with bacteria ($p < 0.05$). Those plots inoculated with *F. culmorum* and *R. cerealis* where less aggressive and on average were on average 12% lower than the healthy control +Pp and similar to the healthy control without bacteria.

Harvest index (HI) in this spring wheat crop cycle is in accordance with previous results, where the healthy control +Pp present highest HI, obtaining on average a value 12.7 % higher than that of the plots inoculated with GGT isolates. In the second crop cycle, total biomass and grains m^{-1} with respect to the severity of infection showed a significant negative correlation (Pearson: -0.49, $p = 0.0012$, and Pearson: -0.52, $p = 0.0005$ respectively).

Grain test weight (kg hL^{-1}) for both experiments at harvest is shown in Table 6. In the 2016-2017 experiment in GGT2 + and – Pp and GGT3 + and - Pp the lowest hectoliter weights were registered, presenting the healthy control differences with respect to both treatments ($p < 0.0001$). Same differences were observed in those plots infested with *F. culmorum* and *R. cerealis* with respect to the plots infested by the two genetic variants of *G. graminis* var. *tritici*. Controls and those infested with *F. culmorum* and *R. cerealis* reached an average near to 82 kg hL^{-1}, while treatments of GGT_2 and GGT_3 showed no differences between them and registered weights below 79.6 kg hL^{-1}. Similar performance was observed in the experiment of the following season, where both treatments of GGT_2 and GGT_3 reached the lowest hectoliter weights (81-83 kg hL^{-1}) and were significantly different from the rest of the treatments ($p < 0.0001$). On the other hand, healthy controls, *F. culmorum* and *R. cerealis* showed homogeneous weight around 85 kg hL^{1}. In general, the addition Pp had no major influence on increasing the hectoliter weight. Regarding the weight of thousand grains (g) during both seasons of experimentation, this ranged from 50 and 53 g among treatments, without being observed an impact on the pathogens. Likewise, the addition of bacteria had no impact on the obtained values (data not shown).

Finally, grain yield (ton ha^{-1}) in the 2016-2017 experiment showed that both GGT_2 and GGT_3 – Pp were those treatments in which the grain production was most affected (-35% respect to control +Pp) and where the inoculation to the seed of *P. protegens* caused the yield to increase

significantly from 5.5 to 7.2 ton ha^{-1} for GGT_2 (31% increase) and fluctuated in 1.2 ton ha^{-1} for GGT_3 (17.6% increase; $p > 0.05$). A second group of treatments that were similar to the healthy control -Pp was that integrated by GGT_3 +Pp, *R. cerealis* (-Pp and +Pp), and *F. culmorum* -Pp, where between 8.0 and 9.5 ton ha^{-1} were reached. Finally, treatments with *F. culmorum* +Pp and healthy control + Pp achieved 20% higher yields than the rest, with an average of 10.6 ton ha^{-1}. During this crop cycle, a negative and significant correlation was observed between grain yield and the severity of infection and visual symptoms observed per plot (AUDPC) (Pearson: -0.37, $p = 0.0183$ y Pearson: -0.87, $p< 0.0001$, respectively). On the other hand, positive correlations were observed between the final yield and plant height (Pearson: 0.74, $p <0.0001$), number of stems m^{-1} (Pearson: 0.57, $p < 0.0001$), spikes m^{-1} (Pearson: 0.59 , 0.0001), total biomass (Pearson: 0.69, $p < 0.0001$), biomass of grains (Pearson: 0.72, $p < 0.0001$), harvest index (Pearson: 0.40, $p = 0.0103$) and weight of thousand grains (Pearson: 0.43,$p = 0.0059$).

In the 2017-2018 experiment, the two strains of *G. graminis* var. *tritici* caused the highest yield losses among the analyzed treatments, averaging 6.1 ton ha^{-1} and being significantly different from the rest of the treatments ($p > 0.05$; Table 6). Treatments inoculated with *R. cerealis* and *F. culmorum* caused no major effect on the yield and only the inoculation of *Rhizoctonia* without bacteria was different from both controls. Healthy controls presented no differences between them and the addition of the bacterial consortium caused no effect on productivity, and only differences in the treatments inoculated with *R. cerealis* ($P <0.01$) were observed. Seed treatment with bacteria showed an increase of 6.5% in the final productivity in those plots infested with *R. cerealis*, while, in the case of GGT_3 a scarce 1.5% increase was observed in the final yield ($p < 0.05$) (Table 6). In this crop season, significant negative correlations were observed between the final yield and incidence and severity of infection (Pearson: -0.73, $p <0.0001$ and Pearson: -0.75, $p <0.0001$, respectively). On the other hand, positive correlations were observed respect to yield components, such as number of stems m-1 (Pearson: 0.43 , $p = 0.0051$), spikes m-1 (Pearson: 0.51 , $p = 0.0007$), total biomass (Pearson: 0.73, $p <0.0001$), grain biomass (Pearson: 0.72, $p < 0.0001$) and harvest index (Pearson: 0.32, $p = 0.0414$).

DISCUSSION

Currently in Chile, crown and root rot diseases affecting wheat crops can generate considerable losses in yield. Notwithstanding the above, to date there is not an adequate control method for

these plant pathogens (De Cornick et al. 2015, Vera et al. 2019). The use of chemical fungicides as seed treatment such as fluquiconazole and others, although it has achieved certain success it is not capable to completely control some of this phytopathogens (Vera et al. 2014), demonstrating variables levels of efficacy and efficiency. Therefore, the antagonistic effect of Chilean strains of *P. protegens* on important crown and root rot pathologies such as *G. graminis* var. *tritici*, *F. culmorum* and *R. cerealis* was evaluated during two consecutive years in this research carried out on a spring wheat under field conditions. Inoculation at a concentration of 10^8 CFU g^{-1} seeds of *P. protegens* plus carboxymethyl cellulose reached a population density of approximately 10^5 CFU g^{-1} roots in the anthesis state (Z6) in both years of experiments. This value is in accordance with the minimum population density that has been reported as the required to trigger the phenomenon of take-all decline in suppressive soils (Kwak et al. 2009; Yang et al. 2014). The quantification of bacterial populations based on the presence of the *+phlD* gene, associated to the production of the antimicrobial compound 2,4-DAPG, in both years showed that seed inoculation markedly increased the natural concentration of bacteria in the rhizosphere. These populations went from 20,000 CFU g^{-1} average roots observed in plots not inoculated with *P. protegens*, to an average of 40,000 CFU g^{-1} roots in plots with inoculation treatment, reaching a population maximum of approximately 1,700,000 CFU g^{-1} roots during the 2017-2018 experiment. This demonstrates that the inoculation of the seed is an adequate tool to favor the colonization of the rhizosphere by antagonistic bacteria such as *P. protegens*. This has also been reported for other species such as *P. fluorescens* (Fox et al. 2016; Imperiali et al. 2017).

During the 2016-2017 experiment, the quantification of bacterial populations of *P. protegens* per root gram was 2.6 times lower than in the 2017-2018 experiment, where there were about 190,000 CFU g^{-1} roots of difference between both seasons. The above could be related to the climatic conditions of each season, where the variation in the accumulated precipitation in both crop cycles had a difference of 320 mm, being the season 2017-2018 markedly rainier than 2017-2018 season. This important difference in the precipitation regimes could have affected the colonization of microorganisms, which has been reported by Mavrodi et al. (2012a) and Paulsen et al. (2018). These authors pointed out that *Pseudomonas* with *phlD* gene associated to rhizospheres of wheat plants under conditions of higher soil moisture are favored in comparison to bacteria of the same genus, but without the presence of this gene. On the other hand, in those studies was determined that those phenazine producing *Pseudomonas* are more recurrent under

conditions of lower water availability. This suggests that within a context of climate change associated with lower rainfall, that those producing *Pseudomonas* could be affected and predominate over populations of 2, 4-DAPG producing *Pseudomonas*.

With respect to the inoculated phytopathogenic fungi, they behaved similarly in both experiments, where *G. graminis* var. *tritici* of the genetic groups 2 and 3 (GGT_2 y GGT_3) were the most pathogenic. These genetic groups evidenced a high aggressiveness of the attack observed in roots and first internode of wheat plants, decrease total and grain biomass, decrease in the accumulation of total and grain biomass, and grain yield. On the other hand, *R. cerealis* and *F. culmorum* presented an erratic and weak pathogenicity, showing in some cases similar values to the controls without inoculation in some of the assessed parameters on wheat plants. Notwithstanding the above, phytopathogenicity tests showed that all pathogens could be re-isolated from infected plant material, confirming that the fungi were capable to enter and established into the host, developing different levels of infection (data not shown).

Low phytopathogenicity could be related to the geographical origin of the isolates, since both *R. cerealis* and *F. culmorum* used in the experiments were obtained from the rhizosphere of commercial wheat crops sampled during the 2011-2012 season, in the central valley located between Araucanía and Los Lagos Regions (Moya-Elizondo et al. 2015). In this area, accumulated average rainfall that fluctuate between 1,050 and 1,560 mm per year is recorded regularly, as well as air and soil temperatures that vary between 11°C and 13°C, respectively. During the two experiments in the Chillán area, 307 mm of average rainfall was registered during the crop cycles, whereas air and soil temperatures were around 14°C. These differences in climatic conditions between the origin area of the isolates and the study area possibly reduced the pathogenic capacities of the isolates by being eventually adapted to higher precipitation regimes and lower air and soil temperatures. Notwithstanding the above, this is contradictory with Moya-Elizondo (2013), who mentions that infection by *Fusarium* is favored under drought conditions. This situation may also explain the higher rates of AUDPC, incidence of symptomatic stems and damage in the first internode and roots observed in the first season, which was drier for the treatments inoculated with *F. culmorum*. In the case of *Rhizoctonia*, results are, also contradictory, since that this wheat pathogen is commonly observe causing problems in geographic areas with annual rainfall lower than 400 mm, such in the case of the Pacific

Northwest, USA (Weller et al. 2016; Yin et al. 2013). In our research, greater visual symptoms over the foliage (AUDPC) were observed in plots inoculated with *R. cerealis* in the first season, which was markedly less rainy that the second one. The fact that these fungi are secondary pathogens that colonize spring wheat plants should be taken into account, although they are not capable to cause appreciable damage. In this sense, it is important to consider levels of pathogenicity in future experiments with artificial inoculation of pathogens in the field. Screening through pot trials with artificial inoculation of the isolates previous seeding would allow evaluating and determining their levels of aggressiveness. Therefore, the potential effect on plants in the field, in function of the damage generated in roots and first internode of the plants in the early stages of crop development, could be previously known. However, no greater effect of these fungi was observed in the emergence of the crop, which would indicate that they are weak pathogens that are more aggressive depending on the environmental conditions presented. The fact that the cv. Pantera-INIA presents resistance to these phytopathogenic fungi can not be ruled out.

Despite a smaller quantification of bacterial populations in the first experiment, the beneficial effects on the vegetative development and productivity of the bacteria with *phlD* gene on the crop inoculated with root pathogens were more evident in this season than in the second experiment. In the first experiment, there was a marked decrease of aerial symptoms associated to these crown and root rots pathogens, as well as significant increases in the number of stems and spikes m^{-1}, in the biomass accumulation, and finally in the grain yield. These results are coincident with those reported by Rubin et al. (2017), who mentioned that the effectiveness of PGPR (Plant Grow Promoting Rhizobacteria) is greater under conditions of lower soil moisture. In the study of Rubin et al. (2017), field experiments with artificial seed inoculation of bacteria increases up to 22% the root volume , 50% mass of secondary roots and 110% in yield in various grasses, C4 plants and legumes. On the other hand, Mäder et al. (2011) reported an increase of 31% in grain yield when wheat seeds were artificially inoculating with two strains of *Pseudomonas fluorescens* in field experiments. In our experiments in spring wheat, and in accordance with the previously mentioned authors, were observed up to 31% of increase in yield, 18% in total biomass and 42% in grain biomass in plots with artificial seed inoculation.

Results obtained in the present research suggest that the bacteria *P. protegens* have growth

promoting activity as that reported for PGPR microorganisms. Directly, PGPR organisms use mechanisms such as the production of growth regulators, nitrogen fixation and phosphorous biosolubilization to benefit plants, whereas indirectly they are capable to generate biocontrol on phytopathogens through the production of antimicrobial compounds, lytic enzymes, competition for resources, niche occupation (Ramette et al. 2011; Tabassum et al. 2017; Sahu et al. 2018) and induction of resistance (Maketon et al. 2012). On the other hand, *P. protegens* is characterized by the production of the antimicrobial compounds 2, 4-DAPG, pioluterin and pyrrolnitrin associated to the strains Ca6 y Ca10 and ChB7 used in this study. In addition, the ability of these strains to solubilize phosphorous and produce indole acetic acid has also been described (unpublished data). This would explain the reduction of symptoms associated to crown and root rot and its impact on growth and yield crop of spring wheat crop. Both the colonization achieved by strains of *P. protegens* in the roots through inoculation to the seed and the reduction of damage caused by these pathogens, plus the effect on development and productivity of the wheat plant could have practical implications for the future use of these bacteria strains in productive systems of geographical areas with low rainfall. The agricultural production in andisol soils between Ñuble (36º37'00''S 71º57'00''W) and Los Lagos regions (41º28'18'' S 72º56'12''W) in Chile is mainly characterized by being conducted under dryland conditions, associated in many cases to small agricultural producers (Durán et al. 2017). On the other hand, the genera *Gaeumannomyces*, *Fusarium* and *Rhizoctonia* are recurrently found in this type of soil (Moya-Elizondo et al, 2015). Andisol soils are characterized by their high capacity to fix phosphorus and its acidic pH soil reaction, so that the use of bacteria that promote phosphorous solubilization is a good tool to improve the productivity of these areas. This is even more relevant in a scenario of climate change, where the possibility of using plant growth promoting microorganisms to reduce the harmful effects of water stress in plants could mean not only the protection of crop, but also the conservation or better use of water resources. As in most countries, strong impacts on agricultural production have also being predicted in Chile, mainly related to changes in precipitation regimes. This has been especially noticeable in the last decade, where long and consecutive drought periods have been registered in the country from 2010, with rainfall deficits between 25% and 45%. This phenomenon has been considered as a "mega drought" (Garreaud et al. 2017). As a result and according to models of future simulation, it is estimated that for 2050 the yield of wheat in the country will decrease between 15% and 20% (Meza and Silva, 2009).

Spring wheat production in andisol soils is facing risks of water deficits, so having determined that *P. protegens* has a biocontrol effect on different crown and root rot pathogens and is able to promote significant growth and yield under conditions of lower rainfall could indicate that its use may be a good strategy to be considered in the future. In this regard, several authors have reported that *Pseudomonas* allows decreasing the harmful effects associated to the activity of ethylene as a molecule that accelerates senescence in water stress episodes (Arshad et al. 2008; Kanwal et al. 2017). This can be counteracted by using antagonistic agents that secrete enzymes such as ACC-deaminase, which hydrolyzes the ethylene precursor (1-aminopropane-1-carboxyllic acid, ACC), blocking the biosynthesis of this phytohormone. This ability has been reported in species of *Pseudomonas* by the authors mentioned above. Therefore, the study of this ability in strains of *P. protegens* should be considered in light of the results observed in this study.

During the second year experiment, a lower control and growth promoter effect was observed when seeds were inoculated with bacteria, although greater populations of *P. protegens* were observed in the rhizosphere of spring wheat plants. Clearly, the environmental condition of higher rainfall could have influence the expression of the diseases caused by the inoculated pathogens, but it does not explain the non-effect of the addition of bacteria in showing clear differences in crop development variables and yield components. A possible explanation would be to consider the energy and/or metabolic costs associated with the triggering of processes of inducing resistance in plants associated with the presence of beneficial microorganisms. In this context, several authors (Denancé et al. 2013; Vos et al. 2013) consider that plants that are found under infective episodes can activate induced resistance phenomenon. In order to achieve this, defense mechanisms are preferably allocated in detriment of other physiological processes, resulting in less growth and development. The induction of resistance in wheat plants by microorganisms such as *P. fluorescens* has been associated with the production of 2, 4-DAPG (Sahu et al. 2018; Maketon et al. 2012). The *phlD* gene associated with the production of 2,4-DAPG is present in the strains evaluated in this research, which allows assuming that high populations of bacteria could produce this compound in the rhizosphere of wheat plants under conditions of greater soil moisture. Therefore, the induction of systemic resistance in the plants is promoted, but this condition could have generated a metabolic cost. To the above, a greater environmental condition that favored the soil pathogens, added to a greater population of bacteria

P. protegens could favor this phenomenon of induced resistance, increasing the metabolic cost phenomenon in plants. This can be reflected in what was observed with *F. culmorum* in the second year experiment, where in plots inoculated with *P. protegens* to the seed, an average of 8% of decrease in the accumulation of grain biomass and grain yield to harvest was recorded ($p < 0.05$).

Results obtained in this research suggest the importance of using PGPR microorganisms inoculated to the seed for the stimulation of plant development and protection against various phytopathogens that cause crown and root rot diseases in spring wheat. This is particularly valid for the conditions of low precipitation in the Chillán area, in the Ñuble Region, Chile. Considering a scenario of imminent climate change, where drought periods will continue or will be more recurrent, as well as the decline in wheat crop yields could reach 20% in the near future. Therefore, the use of beneficial bacteria such as *P. protegens* becomes relevant and may become a possible sustainable strategy to reduce the negative impact of biotic and abiotic factors on wheat crops.

NOTES

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COMPLIANCE WITH ETHICAL STANDARDS

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

HUMAN AND ANIMAL RIGHTS

Research do not involve Human Participants or Animals. And all authors informed consent.

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TABLES

Table 1. Seed treatments with antagonistic bacteria and artificial inoculation with crown and root rot pathogens used in field experiments with spring wheat cv. Pantera.

Table 2. Area under the disease-progress curve (AUDPC) on foliage, percentage of incidence and crown and root rot severity index (IDSI) observed in experimental plots seed treated with a consortium of *Pseudomonas protegens* and infested with different crown and root rot pathogens on spring wheat cv. Pantera-INIA during two crop seasons.

Table 3. Final average plant height (cm) observed in experimental plots seed treated with a consortium of *Pseudomonas protegens* and infested with different crown and root rot pathogens on spring wheat cv. Pantera-INIA, during two crop seasons.

Table 4. Average number of stems and spikes m^{-1} and number of grains/spikes observed in experimental plots seed treated with a consortium of *Pseudomonas protegens* and infested with different crown and root rot pathogens on spring wheat cv. Pantera-INIA, during two crop seasons.

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Table 6. Grain test weigth (kg hL^{-1}) and grain yield averages (ton ha^{-1}) at harvest with different crown and root rot pathogens on spring wheat cv. Pantera-INIA, during observed in experimental plots seed treated with a consortium of *Pseudomonas protegens* and infested with different crown and root rot pathogens, during two crop seasons.

FIGURES

Fig. 1. Monthly accumulated rainfall (mm) in Chillán, Ñuble Region Chile, between August (seeding) to January (harvest) of crop cycles 2016–2017 and 2017–2018.

Fig. 2. Bacterial populations *phlD*+ gene (CFU per g of root) obtained by qPCR from wheat roots of cv. Pantera-INIA at anthesis stage (Z.6) in experimental plots that were seed treated with or without a consortium of *Pseudomonas protegens* (+ or - Pp) and infested with different crown and root rot pathogens (two aisolates of *Gaeumannomyces graminis* var. *tritici* [GGT]; *Fusarium culmorum* and *R. cerealis*), during two crop seasons.

TABLES

Table 1. Seed treatments with antagonistic bacteria and artificial inoculation with crown and root rot pathogens used in field experiments with spring wheat cv. Pantera.

	Treatments	Isolate name	*P. protegens* factor	Inoculum factor
1	Untreated control		+	-
2	Untreated control		-	-
3	*G. graminis* var. *tritici* G2 [a]	GGT_G2 (INIA)	+	+
4	*G. graminis* var. *tritici* G2 [a]		-	+
5	*G. graminis* var. *tritici* G3 [b]	Oso1	+	+
6	*G. graminis* var. *tritici* G3 [b]		-	+
7	*Fusarium culmorum* [c]	F_CULM	+	+
8	*Fusarium culmorum* [c]		-	+
9	*Rhizoctonia cerealis* [d]	M31S	+	+
10	*Rhizoctonia cerealis* [d]		-	+

Source: Own elaboration.

Isolate are described for the following authors: [a] Vera et al. (2014), [b] Gonzáles et al. (2018), [c] and [d] Moya-Elizondo et al. (2015).

Table 2. Area under the disease-progress curve (AUDPC) on foliage, percentage of incidence and crown and root rot severity index (IDSI)[a] observed in experimental plots seed treated with a consortium of *Pseudomonas protegens* and infested with different crown and root rot pathogens on spring wheat cv. Pantera-INIA during two crop seasons.

Treatments		Season 2016-2017						Season 2017-2018					
Pathogens factor	*P.protegens* factor	AUDPC		Incidence (%)		IDSI		AUDPC		Incidence (%)		IDSI	
Untreated control	+	1236,7	e	35,0	bc	8,0	d	800,0	f	13,1	d	3,1	c
Untreated control	-	1632,8	cd	34,3	bc	8,6	d	1040,8	e	30,9	c	4,8	c
G. graminis var. *tritici* group 2 (GGT_2)	+	2426,5	b	64,0	a	34,0	a	2064,7	a	68,5	a	34,5	a
G. graminis var. *tritici* group 2 (GGT_2)	-	3042,4	a	57,8	a	29,8	a	1966,8	ab	54,7	b	21,3	b
G. graminis var. *tritici* group 3 (GGT_3)	+	1969,8	c	44,4	abc	15,8	bc	1800,7	bc	51,3	b	18,1	b
G. graminis var. *tritici* group 3 (GGT_3)	-	2499,5	b	49,3	abc	16,8	b	1592,8	d	45,9	b	17,6	b
Fusarium culmorum	+	1521,7	de	53,1	ab	13,6	bcd	1004,4	e	28,7	c	4,0	c
Fusarium culmorum	-	1618,0	cd	34,3	c	10,5	cd	981,9	ef	21,3	cd	4,3	c
Rhizoctonia cerealis	+	1945,4	C	32,8	c	8,3	d	1149,3	e	32,2	c	6,5	c
Rhizoctonia cerealis	-	2407,1	B	29,3	c	7,1	d	1617,6	cd	32,6	c	5,9	c
CV [b]		12,01		18,2		20,12		9,65		9,49		22,4	
P value [c]		<0,0001		0,0164		<0,0001		<0,0001		<0,0001		<0,0001	

Source: Own elaboration.

[a] IDSI: Internodes discoloration severity index, where the scale included six classes (0-5), where 0 = no infected internode, 1 = < 25% of infected internode, 2 = 25%-50% of infected internode, 3 = 50%-75% of infected internode, 4 = 75%-100% of infected internode, and 5 = > 100% or infection in upper internodes. The IDSI for each field was then calculated as: IDSI = [Σ (class value × frequency)/(total number of plants × the highest class value)] × 100 (Hogg et al. 2007), and it was evaluated in 80 stems of wheat plants 1 day after harvest.

[b] CV: Coefficient of variation.

[c] Different letters in the same column showed significant differences among treatments according to the LSD comparison mean test (α = 0.05).

Table 3. Final average plant height (cm) observed in experimental plots seed treated with a consortium of *Pseudomonas protegens* and infested with different crown and root rot pathogens on spring wheat cv. Pantera-INIA, during two crop seasons.

Treatments		Final plant height (cm)			
Pathogens factor	***P.protegens* factor**	**Season 2016-2017**		**Season 2017-2018**	
Untreated control	+	91,8	ab	94,0	a
Untreated control	-	88,5	bcde	91,2	abcd
G.graminis var. *tritici* group 2	+	88,8	bcde	87,7	cd
G.graminis var. tritici group 2	-	85,5	de	88,7	bcd
G.graminis var. *tritici* group 3	+	90,5	abcd	87,5	d
G.graminis var. *tritici* group 3	-	85,3	e	88,7	bcd
Fusarium culmorum	+	94,3	a	92,7	abcd
Fusarium culmorum	-	86,5	cde	91,7	abc
Rhizoctonia cerealis	+	90,8	abcd	94,2	a
Rhizoctonia cerealis	-	87,0	bcde	91,5	abcd
CV [a]		3,86		3,14	
P value [b]		0,0179		0,0138	

Source: Own elaboration.

[a] CV: Coefficient of variation.

[b] Different letters in the same column showed significant differences among treatments according to the LSD comparison mean test (α = 0.05).

Table 4. Average number of stems and spikes m^{-1} and number of grains/spikes observed in experimental plots seed treated with a consortium of *Pseudomonas protegens* and infested with different crown and root rot pathogens on spring wheat cv. Pantera-INIA, during two crop seasons.

Treatments		Season 2016-2017						Season 2017-2018					
Pathogens factor	*P.protegens factor*	Nº stems/m^{-1}		Nº spikes/m^{-1}		Nº grains/spikes		Nº stems/m^{-1}		Nº spikes/m^{-1}		Nº grains/spikes	
Untreated control	+	122	ab	122	a	42	cd	112	a	109	a	50	n.s
Untreated control	-	104	bc	92	b	38	e	109	abc	107	ab	47	
G.graminis var. *tritici* group 2	+	111	abc	93	b	44	abc	87	d	87	cd	51	
G.graminis var. tritici group 2	-	94	cd	90	b	39	e	72	e	72	d	53	
G.graminis var. *tritici* group 3	+	116	abc	111	ab	40	de	97	bcd	95	abc	52	
G.graminis var. *tritici* group 3	-	79	d	79	ab	41	de	89	d	87	cd	52	
Fusarium culmorum	+	127	a	124	a	46	a	111	ab	106	ab	49	
Fusarium culmorum	-	118	ab	118	a	46	ab	97	bcd	96	abc	51	
Rhizoctonia cerealis	+	122	ab	118	a	43	cd	95	cd	93	bcd	50	
Rhizoctonia cerealis	-	114	abc	111	ab	43	cd	95	cd	91	cd	50	
CV [a]		14,4		14,7		4,58		10,5		10		7,89	
P value [b]		0,0089		0,0255		<0,0001		0,0003		0,0032		0,6001	

Source: Own elaboration.

[a] CV: Coefficient of variation.

[b] Different letters in the same column showed significant differences among treatments according to the LSD comparison mean test (α = 0.05).

Table 5. Total and grain biomass (g) and Harvest index (HI) observed in experimental plots seed treated with a consortium of *Pseudomonas protegens* and infested with different crown and root rot pathogens on spring wheat cv. Pantera-INIA, during two crop seasons.

Treatments		Season 2016-2017						Season 2017-2018					
Pathogens factor	*P.protegens* factor	Total biomass (g)		Grains biomass (g)		HI		Total biomass (g)		Grains biomass (g)		HI	
Untreated control	+	289,1	b	182,3	a	63,5	a	475,0	a	190,3	a	40,2	ab
Untreated control	-	294,7	b	128,4	cde	43,6	bcd	433,8	ab	161,1	bc	37,4	bc
G.graminis var. *tritici* group 2	+	196,0	d	76,6	f	39,5	cd	315,3	de	100,2	d	31,7	d
G.graminis var. tritici group 2	-	222,9	cd	115,0	de	52,7	ab	293,3	e	102,6	d	34,8	cd
G.graminis var. *tritici* group 3	+	307,0	b	139,5	cd	45,6	bcd	395,2	bc	146,0	c	36,9	bc
G.graminis var. *tritici* group 3	-	315,7	ab	108,7	e	35,7	d	348,9	cd	119,8	d	34,5	cd
Fusarium culmorum	+	366,6	a	177,9	ab	48,4	bc	432,5	ab	157,8	bc	36,8	bc
Fusarium culmorum	-	311,0	ab	150,9	bc	48,8	bc	407,5	b	170,6	ab	42,8	a
Rhizoctonia cerealis	+	298,5	b	152,7	abc	51,0	bc	416,3	b	156,4	bc	37,6	bc
Rhizoctonia cerealis	-	275,9	bc	133,2	cde	48,3	bc	420,0	b	150,6	bc	35,9	bcd
CV [a]		13,4		15,31		8,1		9,4		9,7		8,7	
P value [b]		0,0002		<0,0001		0,0055		<0,0001		<0,0001		0,0055	

Source: Own elaboration.

[a] CV: Coefficient of variation.

[b] Different letters in the same column showed significant differences among treatments according to the LSD comparison mean test (α = 0.05).

Table 6. Grain test weight (kg hL^{-1}) and grain yield averages (ton ha^{-1}) at harvest with different crown and root rot pathogens on spring wheat cv. Pantera-INIA, during observed in experimental plots seed treated with a consortium of *Pseudomonas protegens* and infested with different crown and root rot pathogens, during two crop seasons.

Treatments		Season 2016-2017				Season 2017-2018			
Pathogens factor	*P.protegens* factor	Grain test weight (kg hL^{-1})		Grain yield (ton ha^{-1})		Grain test weight (kg hL^{-1})		Grain yield (ton ha^{-1})	
Untreated control	+	82,0	a	10,5	ab	85,2	a	9,9	ab
Untreated control	-	81,2	a	8,6	cd	85,1	a	10,4	a
G.graminis var. *tritici* group 2	+	77,6	c	7,2	ef	81,6	c	5,3	f
G.graminis var. tritici group 2	-	78,5	bc	5,5	g	83,1	b	5,9	ef
G.graminis var. *tritici* group 3	+	79,6	b	8,0	de	83,1	b	6,7	d
G.graminis var. *tritici* group 3	-	79,5	b	6,8	f	83,0	b	6,6	de
Fusarium culmorum	+	81,8	a	10,7	a	85,5	a	9,5	bc
Fusarium culmorum	-	81,1	a	9,2	c	85,3	a	10,3	a
Rhizoctonia cerealis	+	82,1	a	9,5	bc	85,5	a	9,8	ab
Rhizoctonia cerealis	-	81,1	a	8,8	cd	85,4	a	9,2	c
CV [a]		1,05		8,65		0,92		5,99	
P value [b]		<0,0001		<0,0001		<0,0001		<0,0001	

Source: Own elaboration.

[a] CV: Coefficient of variation.

[b] Different letters in the same column showed significant differences among treatments according to the LSD comparison mean test (α = 0.05).

FIGURES

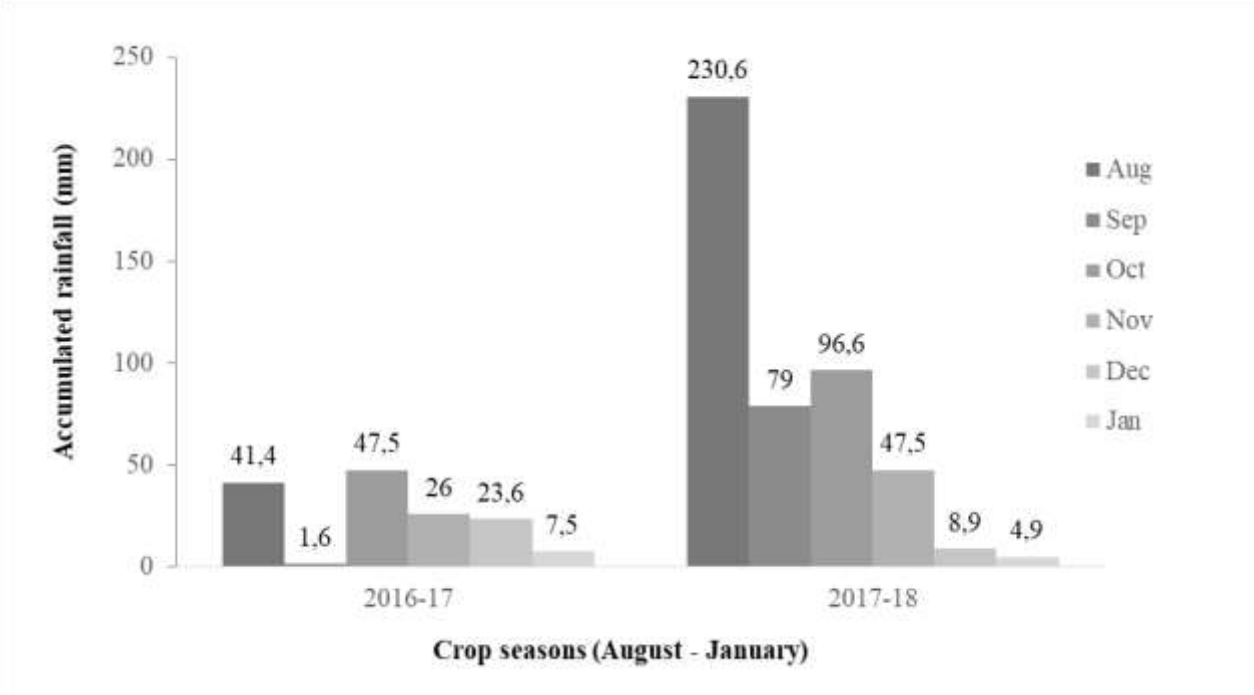

Fig. 1. Monthly accumulated rainfall (mm) in Chillán, Ñuble Region Chile, between August (seeding) to January (harvest) of crop cycles 2016–2017 and 2017–2018.

Source: Own elaboration.

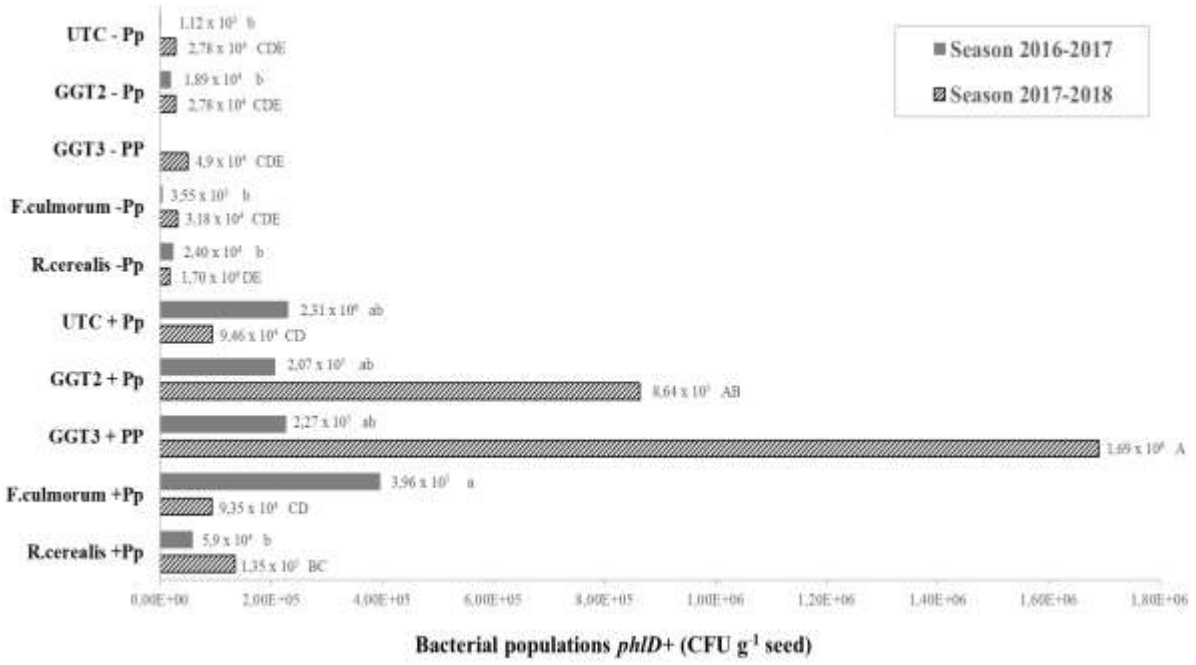

Fig. 2. Bacterial populations *phlD*+ gene (CFU per g of root) obtained by qPCR from wheat roots of cv. Pantera-INIA at anthesis stage (Z.6) in experimental plots that were seed treated with or without a consortium of *Pseudomonas protegens* (+ or - Pp) and infested with different crown and root rot pathogens (two aisolates of *Gaeumannomyces graminis* var. *tritici* [GGT]; *Fusarium culmorum* and *R. cerealis*), during two crop seasons.

Source: Own elaboration.

UTC: Untreated control.

Different lowercase letters on bars indicate significant differences among treatments during 2016-2017 season, while different capital letters indicate differences among treatments of the 2017-2018 season, according to comparison mean LSD test (α = 0.05).

CAPÍTULO 3

CONCLUSIONES GENERALES

La inoculación a la semilla con el consorcio bacteriano de cepas chilenas de *P. protegens* aumentó la concentración natural de bacterias de la rizósfera en estado de antesis (Z.6) significativamente, observándose claras diferencias en la cuantificación de UFC g^{-1} raíces entre parcelas con y sin aplicación de bacterias a la semilla previa siembra en ambas temporadas de experimentos.

Por su parte, la patogenicidad de aislados chilenos de *G. graminis* var. *tritici* varió en función de su variabilidad genética, siendo aquellos pertenecientes al grupo genético 2 ($GGT_{2,}$ aislado GGT_G2-INIA) los que mostraron mayor agresividad sobre plantas de trigo primaveral Pantera-INIA. GGT del grupo genético 3 ($GGT_{3,}$ aislado Oso1), aunque menos agresivo, al igual a GGT_2 desarrolló infecciones que afectaron fuertemente el rendimiento del cultivo. *Fusarium culmorum* (aislado F_CULM) y *Rhizoctonia cerealis* (aislado M31S) se comportaron como patógenos débiles sobre plantas de trigo, observándose infecciones leves sobre los tejidos.

Aun cuando los resultados más evidentes se observaron durante el experimento realizado en 2016-2017, en general se registró una disminución de la sintomatología foliar asociable a pudriciones radicales, así como también en la incidencia y severidad en raíces y primer internudo, mejoras en los componentes del rendimiento como el número de espigas m^{-1} y numero de granos por espiga, y rendimiento final a cosecha en ciertos tratamientos con aplicación de *P. protegens* a la semilla. Las condiciones climáticas de cada temporada pueden haber influido en la patogenicidad de los hongos en estudio, y en la actividad antagónica y promotora de crecimiento de *P. protegens* en las raíces de trigo. Estos resultados sugieren que el uso de cepas chilenas de *P. protegens* como tratamiento a la semilla podría ser una alternativa de manejo de enfermedades radicales y promoción del crecimiento en plantas de trigo primaveral, principalmente bajo condiciones de baja precipitación.