

## Article

# Efficient Biocontrol of *Gaeumannomyces graminis* var. *Tritici* in Wheat: Using Bacteria Isolated from Suppressive Soils

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**Citation:** Méndez, I.; Fallard, A.; Soto, I.; Tortella, G.; de la Luz Mora, M.; Valentine, A.J.; Barra, P.J.; Duran, P. Efficient Biocontrol of *Gaeumannomyces graminis* var. *Tritici* in Wheat: Using Bacteria Isolated from Suppressive Soils. *Agronomy* **2021**, *11*, 2008. <https://doi.org/10.3390/agronomy11102008>

Academic Editor: Francesco Calzarano

Received: 9 September 2021

Accepted: 27 September 2021

Published: 4 October 2021

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**Abstract:** “Take-all” disease is the most important biotic factor affecting cereal productivity, causing 30–50% of crop losses. The causal agent is the ascomycete soil-borne pathogen *Gaeumannomyces graminis* var. *tritici* (Ggt). Current control measures are ineffective, because Ggt can remain saprophytic in soils for long periods. Therefore, the study of the microbiome residing in suppressive soils (SS) is a promising niche of Ggt biocontrol. Here, we evaluated the efficiency of *Serratia* sp., *Bacillus* sp., and *Acinetobacter* sp. isolated from SS against the incidence of Ggt on wheat. Our results demonstrated that plants inoculated with the bacterial consortium in both greenhouse and field conditions were highly efficient in Ggt biocontrol, more so than individual strains. The disease reduction was evidenced by higher biomass production, fewer copies of the Ggt genome with a concomitant curtailment of blackening of roots, a decrease of lipid peroxidation, and an increase of superoxide dismutase activity. The ability of the microbial consortium over that of single strains could be attributable to interspecies communication as a strategy to biocontrol; i.e., higher chitinase activity. In conclusion, bacterial consortia from SS are an important niche of Ggt biocontrol, serving as a model for other soil-borne pathogens.

**Keywords:** take-all disease; microbial consortium; biological control; *Triticum aestivum*; soil-borne pathogen; specific suppression

## 1. Introduction

Wheat (*Triticum aestivum*) is a major staple crop, playing a fundamental role in the human diet, especially in developing countries [1]. In the last decade, the area cultivated with wheat has been tripled, and more than 218 million ha are covered by this crop [2]. However, wheat production is vulnerable to soil-borne pathogens, such as *Gaeumannomyces graminis* var. *tritici* (Ggt), causal agent of “take-all” disease, the most economically important biotic factor affecting wheat crops [3]. This pathogenic fungus is transmitted from the soil to the plant, as well from plant to plant, via runner hyphae growing through root bridges [4]. Moreover, their survival through perithecia in crop residues is the main strategy to infect the next generation of crops [5]. Thus, the severity of Ggt could cause losses of approximately 30% to 50% of wheat production [6,7].

Traditionally, the control of Ggt has been dependent on the use of soil fumigants such as methyl bromide and chloropicrin [3]. Novel fungicides such as 4-chlorocinnamaldehyde

thiosemicarbazide (PMDD), which is a laccase inhibitor, have also been proposed for control of Ggt [8]. Nonetheless, the intensive use of chemical treatments induces pathogen resistance, causing economic and environmental damage, and also leads to an increase in the production cost [9]. Alternatively, crop rotation with nonsusceptible crops appears to be the only current sustainable method of take-all prevention [10]. However, it is not appropriate for all production systems [11]. For example, in the case of Ggt, crop rotation decreases the possibility of use cereals as barley (*Hordeum vulgare*), rye (*Secale cereale*), and triticale (*Triticum secale*, wheat-rye hybrid), since they are also affected by take-all [9]. Other control measures that have also been proposed include the application of saponins from *Quillaja Saponaria* [12] or triterpene extracts [13]. However, the control of the incidence of Ggt is a major global agricultural issue that is far from being completely solved. Thus, it is crucial to establish economically viable methods to control this persistent disease [9].

Plants have developed strategies to defend themselves against pathogens through the recruitment of beneficial microorganisms from the surrounding soil, and their latter stimulation and support [14]. Many plant-associated microorganisms can protect their host plant, either directly by inhibiting pathogen growth, or indirectly by inducing mechanisms that confer resistance against pathogens or mixed-path antagonism (antibiotics, lytic enzymes, etc.). These mutually compatible mechanisms have been demonstrated against pathogenic bacteria [15–17], fungi [18–20], viruses [21], and insects and pests [22–24], in which microorganisms can act simultaneously or synergistically [19]. In the case of antibiotics, the potential of phenazine (Phz)-producing strains for fungal pathogens biocontrol has showed similar or even higher antifungal activity in comparison with commercial fungicides [25].

The discovery of suppressive soils opened new alternatives for environmentally friendly approaches for soil-borne disease biocontrol [6]. Soil suppression is defined as the ability of a natural soil to reduce or suppress the activity of plant pathogens as a consequence of soil microorganisms [3]. The natural presence of soil microorganisms increases the ecosystem resilience by creating redundancy in ecosystem services, making soil less vulnerable to short-term changes in the environment [26]. In this sense, general suppression is based on a general antagonistic effect exerted by the total soil microbial biomass against a broad spectrum of soil-borne pathogens [27], and antagonistic effects occur mainly in the bulk soil, being especially effective against pathogens with a saprotrophic phase (i.e., fungistasis) or influenced by bulk soil chemistry [28]; whereas specific suppression is limited to a particular pathogen and is mediated by one or a few specific microorganisms, being transferable to nonsuppressive soil or conducive [6]. However, this can occur in the soil rhizosphere and is influenced by the host plant (known as the rhizobiome). Specific suppression could be induced by monoculture practices by growing susceptible crops (host) in coexistence with an infective pathogen. For example, our group screened Ggt-suppressive soil occurrence in 16 locations managed by small farmers using monoculture for more than 10 years. Six of these soils were confirmed to be suppressive, since they reduced take-all disease in wheat plants. Suppressiveness was lost upon soil sterilization, and recovered by adding 1% of the natural soil, hence confirming that suppressiveness was closely associated with the soil microbiome community composition [6].

Despite that suppressive soils have been studied for more than 100 years, efficient techniques to take advantage of this important niche have not been developed. Therefore, the study and understanding of the microorganisms and the associated mechanisms involved in the suppression process have an attractive biotechnological potential for disease biocontrol. The use of a persistent control over time (over many seasons) could be induced and established, with wide-ranging applications for disease biocontrol. In this scenario, we proposed to evaluate the single and combined (as a consortium) effect of three bacterial strains, *Serratia* sp., *Bacillus* sp., and *Acinetobacter* sp., isolated from suppressive soils against the incidence of Ggt in wheat plants under both greenhouse and field conditions.

## 2. Materials and Methods

### 2.1. Microbial Traits of Selected Bacteria

Three bacterial strains isolated in previous studies from suppressive soils were selected according to their biocontrol capacity against *Gaeumannomyces graminis* var. *tritici* in an *in vitro* condition [6,20]. The strains were molecularly identified as *Serratia* sp. 126\_3 (MF449130) 4B, *Bacillus* sp. (MK138520), and *Acinetobacter* sp. E6.2 (KF561870). Additionally, a bacterial consortium formulated with the three bacterial strains also was evaluated. Prior to the formulation of the consortium, it was verified in agar Luria Bertani (LB) that there was no antagonistic effect between the bacterial strains.

#### 2.1.1. Plant Growth Promoting Traits

The capacity of utilized insoluble phosphorus (P) forms was examined based on the appearance of clear zones around the colonies on National Botanical Research Institute phosphate (NBRIP) growth medium supplemented with tricalcium phosphate ( $\text{Ca}_3[\text{PO}_4]_2$ ) [29], and halo formation was measured as follows: +++, very high capacity; ++, high capacity; +, normal capacity; and –, no capacity. The siderophore production was evaluated on agar plates supplemented with chrome azurol S (CAS) reagent [30].

#### 2.1.2. Determination of Phenazine (Phz) Presence in Bacterial Strains

To screen for the presence of phenazine in selected strains and the consortium, a PCR assay was conducted by using primer pair PHZ1 (5'-GGGGCGGGCCGTGGTGTATGGA-3') and PHZ2 (5'-YCCCGCSGCCTGYCTGGTCTG-3') [31]. Samples were exposed to a denaturation step at 94 °C for 120 s, followed by 25 cycles of 94 °C for 60 s, 56 °C for 45 s, and 72 °C for 105 s, then finishing at 72 °C for 60 s.

#### 2.1.3. Chitinase Activity

The crude extract from each bacterial strain and the consortium were used to measure the reducing sugar released from the colloidal chitin [32]. The bacterial cultures were centrifuged at 10,000 rpm for 15 min at 4 °C. Then, 150 µL of crude extract was added to a mixture containing 300 µL of colloidal chitin (0.1%) and 150 µL of phosphate buffer pH 7.0 (0.1 M). After incubation at 55 °C for 10 min, it was centrifuged at 10,000 rpm for 5 min at 4 °C. The resulting supernatant (200 µL) was mixed with 500 µL of distilled water and 1000 µL of Schales reagent (a solution of 0.5 M sodium carbonate and 0.5 g L<sup>-1</sup> potassium ferricyanide), then boiled for 10 min. After cooling, the absorbance was measured in UV-vis Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc, Osaka, Japan) at 420 nm. One unit of chitinase activity was defined as the amount of enzyme that produced 1 µmol of reducing sugar as equivalent of N-acetyl-D-glucosamine (GlcNAc) per minute [33].

### 2.2. Inoculum Preparation

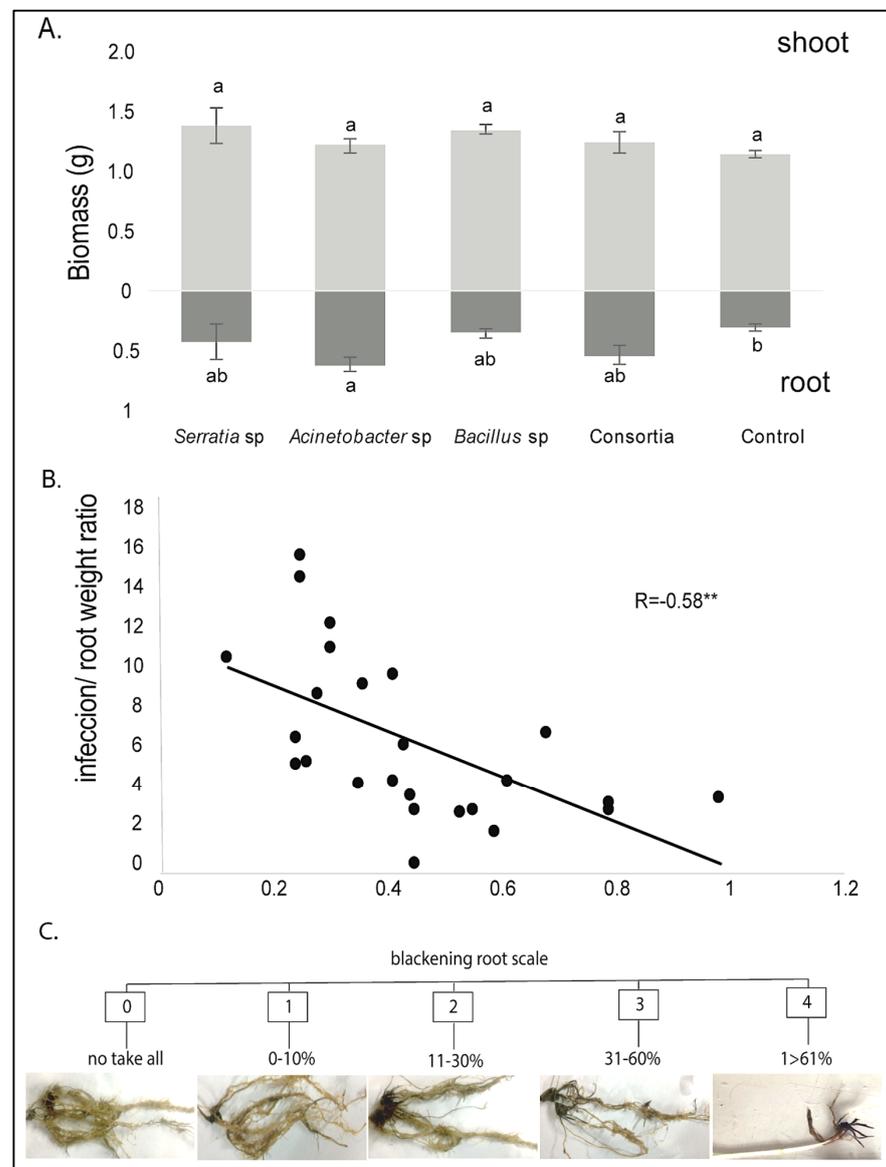
#### Bacterial Inoculation under Greenhouse Conditions

For bacterial inoculum preparation, each single strain and the consortium were grown overnight in 800 mL of LB media, at 30 °C for 24 h with continuous shaking (150 rpm). The consortium was formulated by mixing equal proportions of the three bacterial strains (1:1:1). Bacterial cells were collected by centrifugation at 10,000 × g for 10 min and rinsed twice with sterile saline solution (0.85% NaCl). Bacterial cells were resuspended in sterile distilled water for the greenhouse experiment, and 2 mL by pot was inoculated with ~10<sup>9</sup> CFU mL<sup>-1</sup> (estimated by agar plate-counting) as described by Barra et al. (2017). The best treatment to biocontrol take-all disease under greenhouse experiment was used in the field assay.

### 2.3. Greenhouse Experiment

Wheat seeds (Otto cv) were surface sterilized (15% ethanol plus 1% sodium hypochlorite for 2 min), and 5 seeds were grown in pots containing 500 g of soil from the Maquehue

locality (Freire series, Table 1). The Ggt inoculum (as powder inoculum) was applied at 1% in relation to soil weight (5 g) [6]. After 10 days, the bacteria inoculum (5 mL) was directly injected in the rhizosphere of the wheat plants. The plants were watered every 3 days, and Taylor and Foyd nutrient solution [34] was applied each 15 days. The experimental design included: (1) uninoculated control plants; and plants inoculated with (2) *Serratia* sp., (3) *Acinetobacter* sp, (4) *Bacillus* sp., and (5) the bacterial consortium. All treatments were subjected or not (control) to Ggt inoculation. Each treatment was performed in quintuplicate. After 40 days, plants were carefully removed from the soil and weighted, and the blackening root percentage was determined. Each plant was assessed on a scale of 0 to 4 [35], where 0: no take-all; 1: 1% to 10%; 2: 11% to 30%; 3: 31% to 60%; and 4: 61% to 100% of the root system was affected (Figure 1C). In order to determine dry weight, shoots and roots were placed into individual paper envelopes and dried at 70 °C for 72 h. The most efficient treatment to diminish the incidence of take-all disease was validated under field conditions, and same analyses were carried out.



**Figure 1.** (A) Shoot and root biomass (g); (B) infection measured by blackening of roots 40 days after inoculation with 1% of Ggt and root biomass ratio; and (C) infection scale. Tukey's test was used to compare treatment means; values followed by the same letter did not differ at  $p < 0.05$  ( $n = 5$ ). \*\* Denotes significant differences at  $p < 0.01$ .

**Table 1.** The chemical parameters of the Maquehue soil (Freire series).

Variable	Values
N (mg kg <sup>-1</sup> )	29
P (mg kg <sup>-1</sup> )	27
K (mg kg <sup>-1</sup> )	344
pH (water 1:5, w:v)	5.75
Organic matter (%)	17
K (cmol <sup>+</sup> kg <sup>-1</sup> )	0.88
Na (cmol <sup>+</sup> kg <sup>-1</sup> )	0.15
Ca (cmol <sup>+</sup> kg <sup>-1</sup> )	7.61
Mg (cmol <sup>+</sup> kg <sup>-1</sup> )	1.77
Al (cmol <sup>+</sup> kg <sup>-1</sup> )	0.05
Al sat (%)	0.48
CICE (cmol <sup>+</sup> kg <sup>-1</sup> )	10.46
Bas. sat (cmol <sup>+</sup> kg <sup>-1</sup> )	10.41

#### 2.4. *Gaeumannomyces graminis* var. *Tritici* Detection and Quantification in Wheat Roots

To confirm Ggt infection, total DNA from fresh samples obtained from wheat root tissue were extracted with a soil DNA Isolation Kit (UltraClean<sup>®</sup>, Mo-Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The Ggt DNA was quantified by quantitative real-time PCR (qPCR). Standard curve was prepared in triplicate from 10-fold serial dilutions of Ggt genomic DNA from 0.8 to 8 × 10<sup>-5</sup> ng μL<sup>-1</sup> (obtained from a Ggt pure culture in PDA). A specific Ggt DNA fragment was amplified using GGT2F/GGT168R primer sets [7]. The reaction mixture was carried out in a final volume of 12 μL, containing Brilliant II SYBR, Green QPCR master mix (Stratagene, Agilent Technologies Company, Cedar Creek, TX, USA), 1 μL 1:10 Ggt DNA dilution (to determine standard curve) or 1 μL sample DNA, and 600 nM of each primer. The real-time PCR reaction was performed in an Applied Biosystems Step One™ Real-Time PCR System under the following conditions: an initial denaturing step at 95 °C for 10 min and 35 cycles at 95 °C for 15 s, 58.4 °C for 20 s, and 72 °C for 40 s. To determine the copy number of Ggt DNA in the soil samples, the following formula was used:

$$\frac{\text{DNA Ggt sample } \left( \frac{\text{ng}}{\mu\text{L}} \right) * 1 \times 10^{-9}}{\text{m(g)genome}} \div 13$$

where 13 represents the number of copies of the amplified fragment in the Ggt genome, and:

$$\text{m(g)genome} : \frac{\text{Ggt genome weight (43.768.664 bp)} \times \text{average MW double } \left( 660 \frac{\text{g}}{\text{mol}} \right)}{n^{\circ} \text{ avogadros}}$$

Then, roots were individually assessed for infection, and the percentage of the blackening of roots was measured on a 0–100% scale with a ruler while contrasted against a white background, and was recorded (Figure 1C) [6].

#### 2.5. Lipid Peroxidation

The level of lipid peroxidation was assessed on fresh samples of roots and shoots from wheat by monitoring the thiobarbituric-acid-reacting substances (TBARS). For this, a concentration of malondialdehyde (MDA) was used as an indicative of cell damage. The absorbance was measured with a UV-vis spectrophotometer at 532, 600, and 440 nm to correct the interference generated by TBARS–sugar complexes [36]. The unit for LP was determined as equivalents of MDA contents (nmol g<sup>-1</sup> FW).

## 2.6. Superoxide Dismutase (SOD) Activity

The SOD activity was assayed by monitoring the photochemical inhibition of nitroblue tetrazolium (NBT) [37]. Briefly, 20  $\mu\text{L}$  of samples were exposed to a reaction mixture containing potassium phosphate buffer pH 7.0 (0.1 M), ethylenediaminetetraacetic acid (EDTA) (10 mM), methionine (260 mM), and NBT (4.2 mM). The reaction was started by adding riboflavin (0.13 mM). The mixtures containing samples were illuminated, while blanks were kept in dark for 15 min. The absorbance was measured at 560 nm by a multimode microplate reader (Synergy H1 Hybrid Multi-Mode Reader; BioTek Instruments, Inc., Winooski, VT, USA). One unit of SOD activity ( $\text{U g}^{-1}$ ) was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT.

## 2.7. Field Assay

### 2.7.1. Bacteria Inoculation under Field Conditions

Wheat seeds were surface sterilized (15% ethanol plus 1% sodium hypochlorite for 2 min); 500 g of seeds were used in total, which after disinfection were mixed with 100 g of magnecal, 75 mL of water, and 10 mL of diluted glue (1:9). The seeds were separated into two groups, and one was added 1 g of the lyophilized consortium at  $\sim 10^9$  CFU  $\text{mL}^{-1}$  (estimated by agar plate-counting after lyophilization), and the other group was not added treatment of bacteria (control). For lyophilization, bacterial strains were grown according to point 2.2 and then lyophilized (freeze-dried) in a FreeZone Freeze Dry System (Labconco, Kansas City, MO, USA) according to previous studies [38]. Freeze-drying was performed using 20% (*w/v*) of skim milk as a cryoprotective additive. The mixtures were placed in a pelletizer pond for 10 min and then allowed to dry for 3 h in open air.

### 2.7.2. *Gaeumannomyces graminis* Inoculum (Powder Inoculum)

Ggt inoculum was prepared as described by Durán et al., (2017). Briefly, oat kernels were soaked in water for 24 h and sterilized for three consecutive days at 121 °C for 15 min. Then, Ggt strain KY689233 were grown on PDA for 7 days, put on the sterile oat, and maintained at room temperature for 30 days. Colonized oat kernels were blended, sieved to a particle size of 0.5–1.0 mm, and stored at 4 °C until usage.

### 2.7.3. Treatments and Plant Samples Collection and Analysis

The best treatment to biocontrol take-all disease in the greenhouse experiment was used in the field assay at the Maquehue Experimental Station of La Frontera University (38°50' S, 72°41' W) during the spring of 2018 (November). In this respect, we considered one season, since two or more seasons could affect the suppression of the pathogen when it was applied in the same location in conducive soils [39,40]. Plants were sown in plots (2 × 1 m) with the following treatments: (1) wheat without Ggt and without consortium (control); (2) wheat with Ggt 1% and without consortium; (3) wheat with Ggt 1% and with consortium; and (4) wheat without Ggt and with consortium. Three plots were used for each treatment, and each one in triplicate. After 90 days of the assay, the plants were randomly gathered after proper homogenization and mixture in order to assure representative samples. Then, they were carefully removed, washed, and separated into shoots and roots, and the samples were stored at −80 °C for subsequent analysis. Ggt was detected and quantified according to Section 2.4. Lipid peroxidation and SOD activity were determined according to Sections 2.5 and 2.6, respectively.

## 2.8. Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA). Mean comparisons were made by Tukey's test using SPSS software (IBM SPSS, Inc., Chicago, IL, USA). All experiments were performed in quadruplicate, and the values are shown as mean  $\pm$  standard error (SE). The differences were significant with a *p*-value less than or equal to 0.05.

### 3. Results

#### 3.1. Microbial Traits

The Ggt inhibition by *Acinetobacter* sp. was around 100%, by the consortium was around 80%, by *Bacillus* sp. was around 40%, and by *Serratia* sp. was around 20%. With respect to plant-growth-promoting traits, *Bacillus* sp. and the consortium showed the strongest ability to solubilize tricalcium phosphate on NBRIP media based on halo formation around colonies (Table 2). *Serratia* sp. also showed this ability, but to a lesser extent. *Acinetobacter* sp. did not show this ability. Similarly, all strains produced siderophore production except *Acinetobacter* sp.

**Table 2.** Some plant-growth-promoting traits of *Bacillus* sp., *Serratia* sp., *Acinetobacter* sp., and the consortium.

Strain	PS *	Siderophores	Ggt Inhibition (%)	Phenazine	Chitinase ** ( $\mu\text{mol mg protein}^{-1}$ )
<i>Bacillus</i> sp.	+++	+	40	+	2.1 <sup>bc</sup> $\pm$ 0.43
<i>Serratia</i> sp.	+	+	20	+	1.9 <sup>bc</sup> $\pm$ 0.35
<i>Acinetobacter</i> sp.	-	-	100	+	4.1 <sup>ab</sup> $\pm$ 0.52
Consortium	+++	+	80	+	9.9 <sup>a</sup> $\pm$ 2.1

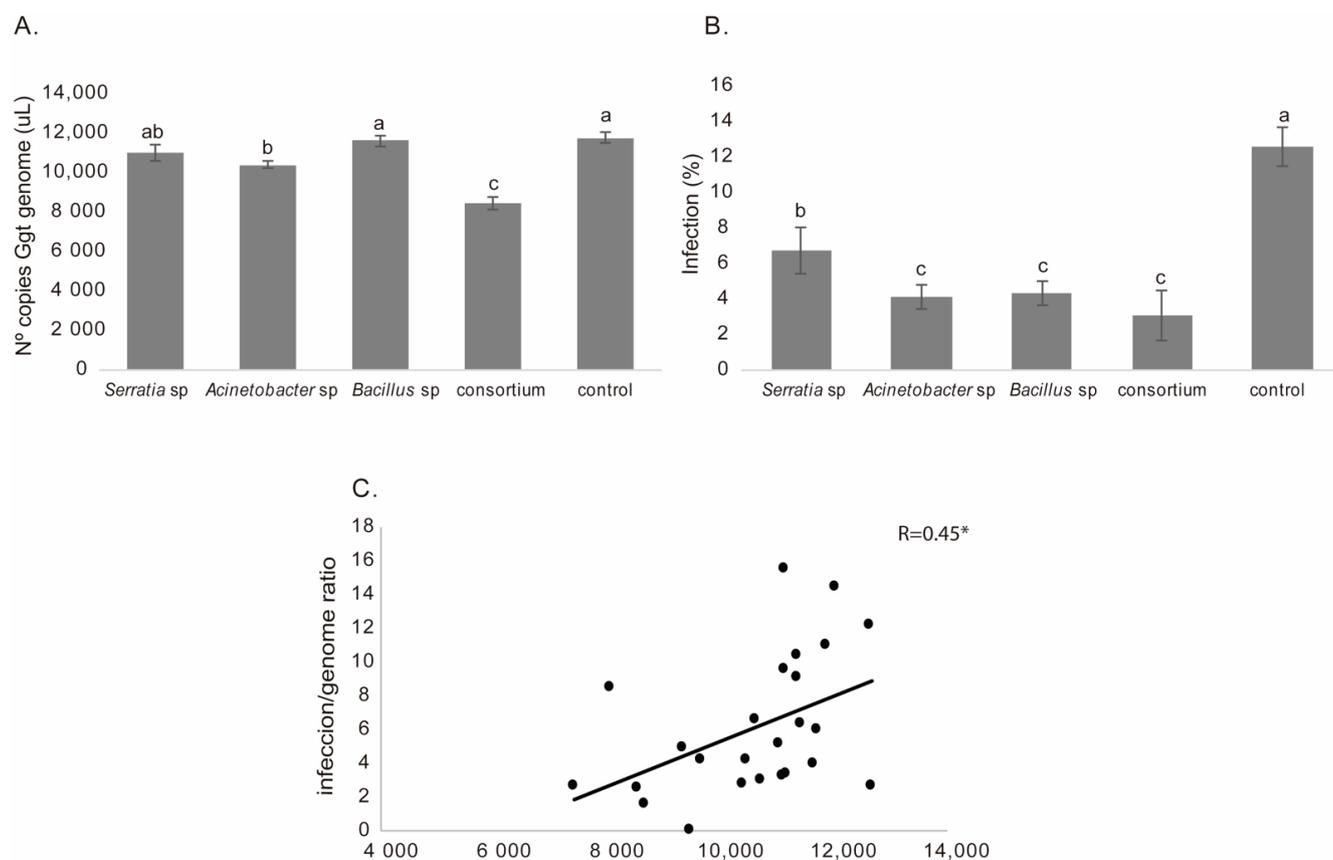
\* PS solubilization of phosphate on NBRIP. \*\* Values represent mean  $\pm$  standard error (average of three repeats). Different letters in a column denote a significant difference using Tukey's test ( $p \leq 0.05$ ).

Phenazine production evaluated by specific primers was evidenced in all strains and the consortium, denoting the presence of a gene involved in the biosynthesis of phenazines highly associated with biocontrol traits. Interestingly, a significant higher chitinase activity ( $p < 0.05$ ) was observed in the consortium ( $\sim 10 \mu\text{mol chitinase mg protein}^{-1}$ ) compared with each single bacterial strain (which ranged from 2 to 4  $\mu\text{mol chitinase mg protein}^{-1}$ ) (Table 2). The enzymatic activity measured in *Escherichia coli* (CCCT 15.08) that was used as a negative control showed  $< 2 \mu\text{mol chitinase mg protein}^{-1}$  (data not shown).

#### 3.2. Greenhouse Experiment

##### 3.2.1. *Gaeumannomyces graminis* var. *Tritici* Detection and Quantification in Wheat Roots

According to our results, plants inoculated with bacterial strains (both individual or the consortium) showed major root biomass. This response was more evident in plants inoculated with *Acinetobacter* sp., which showed significant differences compared to the control (Figure 1A). The percentage of root infection (determined by necrotic root evaluation) showed a significant ( $p < 0.01$ ) inverse correlation with the weight of plants infected with Ggt inoculum (Figure 1B), evidencing less weight in infected roots according to the infection scale (Figure 1C). With respect to the molecular quantification of pathogens, the number of Ggt copies was decreased when plants were inoculated with the consortium (by  $\sim 25\%$ ) and with the *Acinetobacter* sp. strain (by  $\sim 10\%$ ), which was accompanied by a significant decrease of necrotic roots (Figure 2A,B) by  $\sim 80\%$  and  $68\%$ , respectively. No fungicidal capacity was evidenced by *Bacillus* sp. or *Serratia* sp. In general, the necrotic roots were directly related to the number of copies of the Ggt genome (Figure 2C).



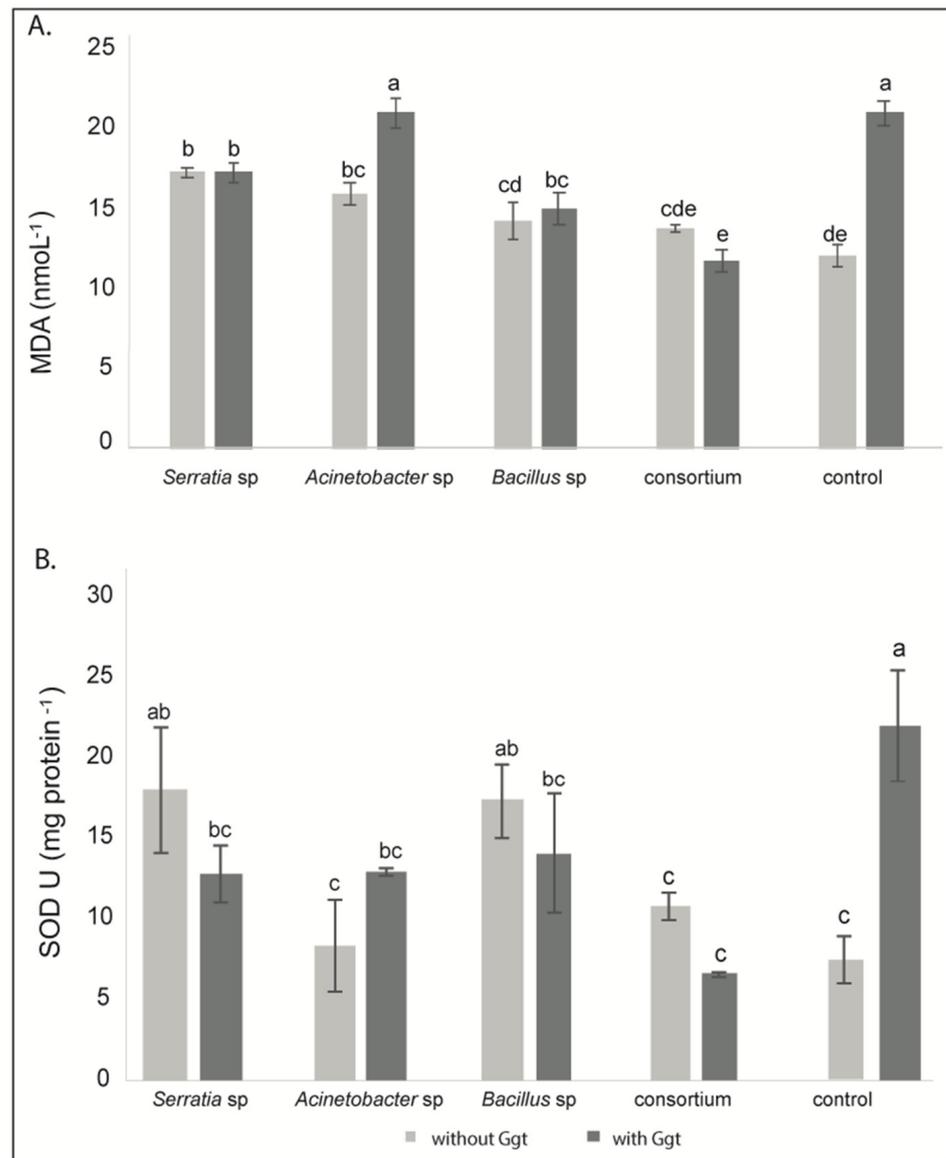
**Figure 2.** (A) Detection and quantification of the number of Ggt genome copies in infected roots 40 days after inoculation with 1% of Ggt; (B) infection measured by blackening of roots 40 days after inoculation with 1% of Ggt; and (C) relationship between Ggt genome copies (quantified in real time using the specific primer set GGT2F/GGT168R) and blackening of roots 40 days after inoculation with 1% of Ggt. Tukey's test was used to compare treatment means; values followed by the same letter did not differ at  $p < 0.05$  ( $n = 5$ ). \* Denotes significant differences at  $p < 0.05$ .

### 3.2.2. Lipid Peroxidation

The cellular effects of bacterial strains and fungal inoculation were determined by measuring changes in the thiobarbituric-acid-reactive substances (TBARS) content. According to the results, no effects were evidenced in shoots (data not shown). The roots of plants inoculated with Ggt (c + Ggt) and *Acinetobacter* sp. showed the highest production of TBARS compared with the rest of treatments, whereas plants inoculated with the consortium showed the lowest TBARS content. In treatments without Ggt inocula, only *Serratia* sp. and *Acinetobacter* sp. showed significantly higher production of TBARS compared with the negative control (c) (Figure 3A).

### 3.2.3. Superoxide Dismutase (SOD) Activity

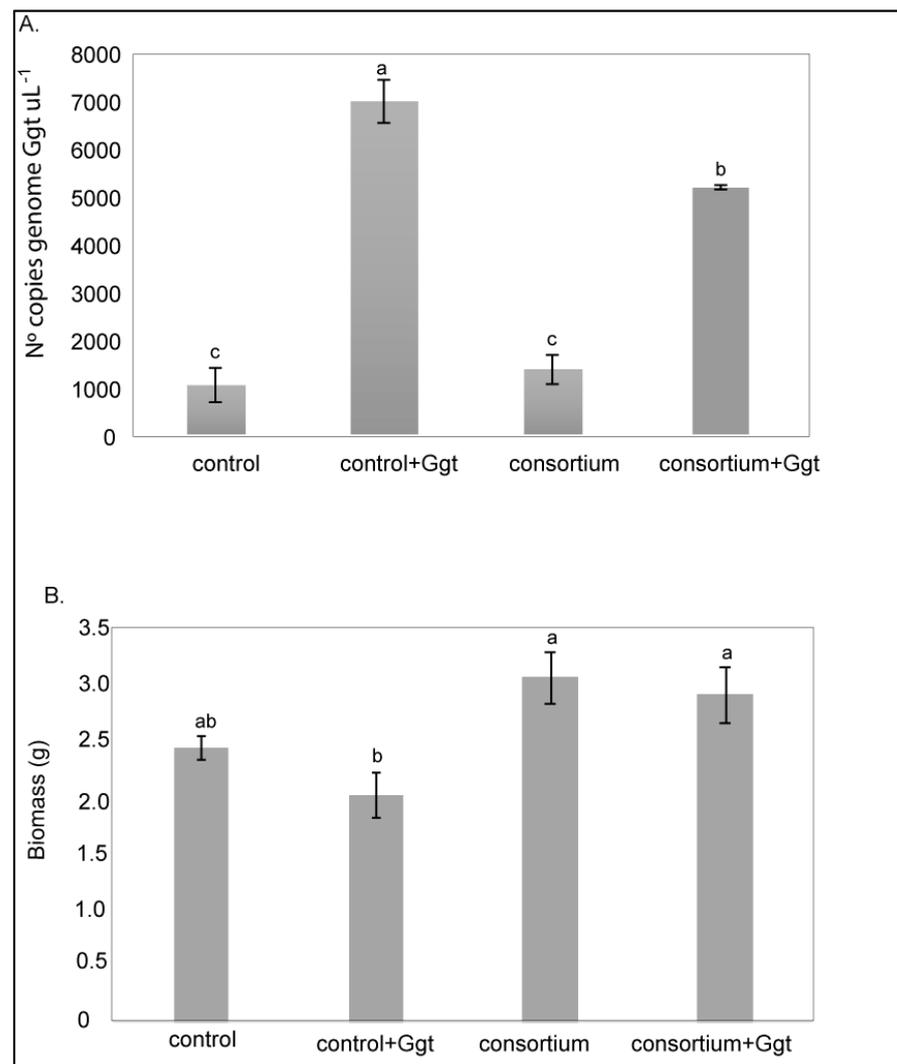
The enzymatic SOD activity showed a significant increase when plants were inoculated with Ggt, evidencing the oxidative stress due to disease incidence. This trend was even more evident in the control, in which infected wheat showed 3-fold more SOD activity than noninfected plants. However, when plants were inoculated with bacteria, the production of SOD was significantly decreased ( $p < 0.05$ ), mainly when plants were inoculated with the bacterial consortium (Figure 3B).



**Figure 3.** (A) Lipid peroxidation measured as TBARS (thiobarbituric-acid-reactive substance) accumulation in roots of wheat plants inoculated with 1% of Ggt and without Ggt inoculation; and (B) superoxide dismutase activity in roots of wheat plants inoculated with 1% of Ggt. Tukey's test was used to compare treatment means; values followed by the same letter did not differ at  $p < 0.05$  ( $n = 6$ ).

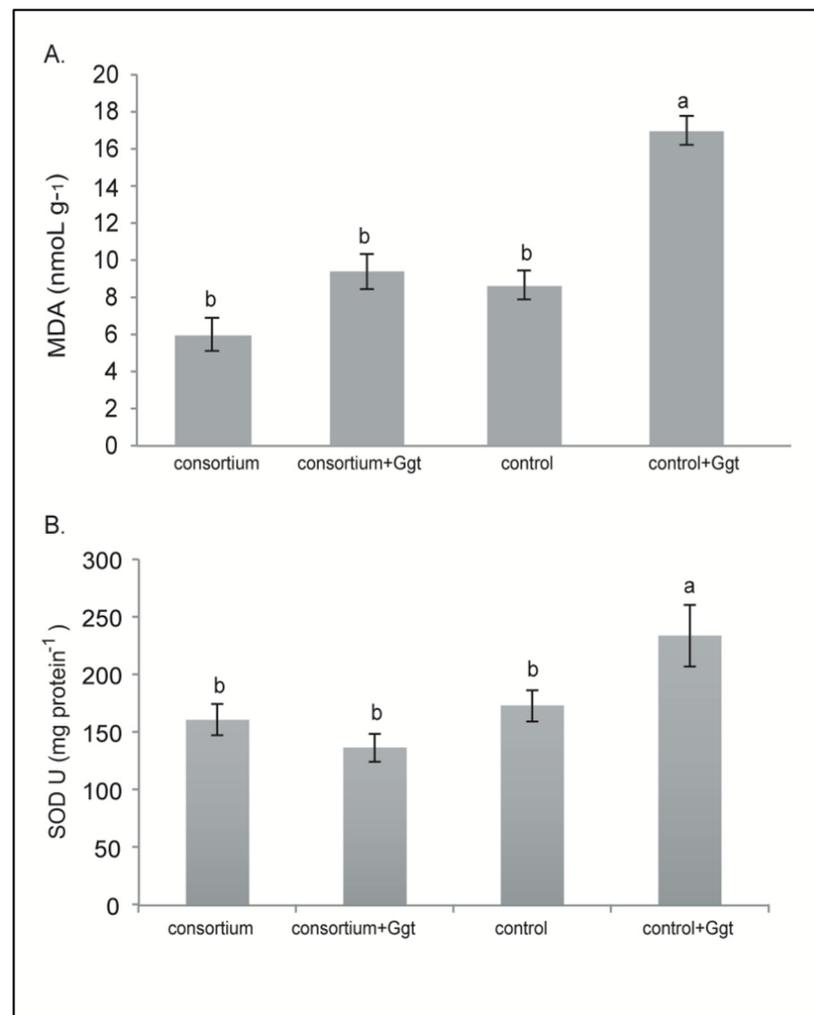
### 3.3. Field Assay

After 90 days of field assay, the number of Ggt copies in wheat roots was evaluated (Figure 4A). A significantly ( $p < 0.05$ ) lower number of copies was observed when plants were inoculated with the bacterial consortium compared with infected plants (c + Ggt). Plants grown in soils without fungal infection evidenced at least 1000 copies genome Ggt  $\mu\text{L}^{-1}$  that were intrinsic from soil. We also found that in field conditions, the number of Ggt copies was significantly lower than in the greenhouse conditions ( $\sim 8000$  and 10,000 copies genome Ggt  $\mu\text{L}^{-1}$ , respectively, in the case of infected control). Similar to the greenhouse assay, a higher root biomass was observed in plants inoculated with the consortium.



**Figure 4.** (A) Detection and quantification of the number of Ggt genome copies in infected roots 90 days after inoculation with Ggt; and (B) root biomass (g). Tukey's test was used to compare treatment means; values followed by the same letter did not differ at  $p < 0.05$  ( $n = 5$ ).

With respect to lipid peroxidation, a similar tendency to that found in greenhouse conditions was observed. Therefore, control plants inoculated with Ggt and without bacterial strains displayed significantly more TBARS content than plants inoculated with the bacterial consortium (Figure 5). In contrast, TBARS production in plants inoculated with consortium was similar to plants without infection (control). Similarly, SOD activity was significantly higher in plants infected with Ggt, evidencing the oxidative stress due to disease incidence (Figure 5A). However, when plants were inoculated with the bacterial consortium, the SOD activity was significantly decreased ( $p < 0.05$ ), reaching levels similar to those of noninfected plants (Figure 5B).



**Figure 5.** (A) Lipid peroxidation measured as TBARS (thiobarbituric-acid-reactive substance) accumulation; and (B) superoxide dismutase activity (SOD) in roots of wheat in the field assay. Tukey's test was used to compare the means of the treatments; values followed by the same letter indicate a nonsignificant difference at  $p < 0.05$  ( $n = 5$ ).

#### 4. Discussion

Over the last decade, several studies have evidenced and highlighted the important role of microbial communities residing in suppressive soils against soil-borne diseases [6,7,41]. Although not fully elucidated, the mechanisms beyond the suppressiveness of a soil have been attributed to specific bacterial taxa or particular strains, generally known as plant-growth-promoting bacteria (PGPB), which play important roles in the growth and development of their host plants [42,43]. In this regard, Firmicutes and Proteobacteria are reported as the most representative phyla, containing species recognized as PGPB [44]. Owing to the ability of the bacterial community from suppressive soils to suppress take-all disease, we evaluated the biocontrol ability of three selected bacterial strains: *Bacillus* sp. (Firmicutes), *Serratia* sp. (Proteobacteria), and *Acinetobacter* sp. (Proteobacteria) isolated from suppressive soils located in La Araucania [6,7,20].

Considering the pathogen response to bacteria inoculation, bacteria cultivated under in vitro conditions showed the ability to reduce the fungal growth by 100%, 40%, and 20% in the cases of *Acinetobacter* sp., *Bacillus* sp., and *Serratia* sp., respectively. Interestingly, the consortium increased the biocontrol activity by 80% by taking advantage of all microbial traits (*Acinetobacter* sp., *Bacillus* sp., and *Serratia* sp.). Similar results were reported recently, in which three indigenous isolates (*Bacillus subtilis*, *B. velezensis*, and *Penicillium* sp.) in

consortium were able to biocontrol around 60–63% of *Fusarium oxysporum* and *Alternaria* sp. in banana, whereas single strains no showed significant differences [45]. The authors attributed this result to the different action mode of each microorganism during invasion of phytopathogens.

The efficiency of the consortium also was evidenced in greenhouse and field conditions by reducing Ggt abundance in infected roots, verified by the copies of the Ggt genome. This also was reported for another soil-borne facultative parasite that causes economically important losses of watermelon, *Fusarium oxysporum* f. sp. Niveum [24], where the antagonistic *Paenibacillus polymyxa* SQR-21 significantly suppressed Fusarium wilt in watermelon plants by protecting the roots from infection. Thus, the decrease in the copy number of the pathogen had a concomitant effect in the blackening of roots, the main symptom attributed to take-all disease [6,20,46], thereby confirming the relationship between the suppression of the disease and the abundance of Ggt previously described [6,7,47].

Plant response to take-all disease was assessed through the lipid peroxidation and SOD activity. As expected, a decrease in TBARS content was observed in plants inoculated with the bacterial consortium under both greenhouse and field conditions, being similar to results obtained for noninfected plants (negative control). In contrast, plants infected with the pathogenic fungus, but not inoculated with the consortium, showed a significant membrane damage, reflecting a higher accumulation of oxidizing agents, which caused damage to proteins, membranes, and other cellular components [48]. Our findings were in agreement with the increase in SOD activity exhibited in plant roots in response to the infection with Ggt, since plants protected themselves against oxidative damage by using their antioxidant system, including antioxidative enzymes [49]. Despite the increased SOD activity in the infected control, plant roots showed less activity when treated with the bacterial consortium in both the greenhouse and field assays. Wheat plants under these conditions accumulated ROS, and their toxicity could affect biomolecules such as nucleic acids, proteins, and lipids, occasionally resulting in cell death, and consequently limiting biomass accumulation and yield [50]. These results therefore demonstrated a decline or suppression in take-all disease.

The positive response to bacterial consortium inoculation can be attributed to the fact that some plant-associated bacteria have several mechanisms, including 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, antibiotics production, enzymes that degrade the cell wall, and siderophores, amongst others, which directly help plants to deal with pathogens, as well as improve the response to the stress, usually associated with pathogen infection [44,51]. In this way, *B. subtilis* (YB-57) isolated from soil has been described by presenting a significant inhibitory effect against Ggt, which was attributed to antimicrobial compounds present in bacterial extracts [41]. In our study, we provided evidence for the presence of genes that are involved in the biosynthesis of phenazines, associated with biocontrol traits [31,52,53]. On the other hand, it has been described that plant-associated bacteria produce enzymes such as  $\beta$ -1,3-glucanase and chitinase, which can break down the cell wall components of pathogenic fungi [32,53,54]. In this context, we found a significant increase in enzymatic chitinase activity in the consortium over the individual strains. Recently, the production of a variety of chitinases has been evidenced by the *Trichoderma* species, strains known as biological control agents against phytopathogenic fungi [55], and rhizobacteria for biocontrol activities against *Fusarium* wilt [56]. With respect to PGPB traits, we noted that plants inoculated with the consortium showed major biomass, which could be attributed to the fact that different strains can act synergistically in consortium. For example, *Bacillus* sp. showed a high ability to solubilize P, and *Bacillus* sp. and *Serratia* sp. were able to produce siderophores; whereas *Acinetobacter* sp. showed major Ggt biocontrol ability. We demonstrated previously that the ability of P solubilization was very important in conditions of P-scarcity [57], and bacteria producing siderophores can alleviate Al stress (typical from acidic soil) by forming  $Al^{3+}$ -siderophore complexes [38], both indispensable traits to maintain suitable agricultural production in Andisol soils from La Araucania.

The efficiency of the consortium or multistrain biological control agents (MSBCAs) over single strains was recently reviewed [58]. The authors emphasized that members of the MSBCAs apply interspecies communication as a strategy to improve the control of soil-borne diseases, improving rhizosphere colonization and microbial–plant–soil interaction. Thus, in this study, we confirmed the existence of suppressive soil (specific suppression) management by long-term extensive wheat cropping, established by small farmers, that could be used as effective microorganism sources for take-all disease biocontrol. Future studies should explore plant defense mechanisms in conducive and suppressive soils, and take advantage of the microbiome of wheat grown in suppressive soil; e.g., based on the principle of host-mediated microbiome selection.

## 5. Conclusions

The bacterial consortium isolated from suppressive soils showed the ability to suppress take-all disease in greenhouse and field conditions, when inoculated in wheat plants. It was demonstrated that the biological control of Ggt diminished the severity of the disease, reducing the percentage of infection, oxidative damage, blackening of the roots, and number of copies of the Ggt genome. The capacity of the consortium, more so than the single strains, benefited immensely from the bacterial interaction, thereby greatly enhancing the levels of biocontrol. Finally, the study of microorganisms inhabiting suppressive soils is an urgent necessity to bioinoculant design, due to the unique niche of harboring specialized microbial strains, to provide soil immunity against a specific soil-borne disease. Special emphasis should be placed on the principle of host-mediated microbiome selection when developing to the new generation of bioinoculants.

## 6. Patents

Resulting from the work reported in this manuscript, we presented a patent titled: Consorcio microbiano de origen bacteriano para el control de *Gaeumaonyces graminis* var. Tritici (Requession N°:PCT/CL2020/050152).

**Author Contributions:** Writing—first draft preparation, I.M., A.F., P.D., P.J.B., I.S.; writing—review and editing, M.d.l.L.M., A.J.V., P.D.; Funding acquisition, P.D., M.d.l.L.M., G.T.; Figures and Tables, P.D. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the ANID-FONDECYT (regular project number 1201196, 1181050), ANID -FONDECYT (Initiation project number 11200377), INACH (number RT06-17) and Dirección de Investigación-Universidad de La Frontera DI21-1004. ANID Scholarship 21211649.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data sharing not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

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