Book Chapter

Microbial Community Composition in Take-All Suppressive Soils

Paola Durán[1,2]*, Gonzalo Tortella[1], Sharon Viscardi[1,2], María de la Luz Mora[1] and María José Pozo[3]

[1]Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Chile
[2]Biocontrol Research Laboratory, Universidad de La Frontera, Chile
[3]Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín (CSIC), Spain

***Corresponding Author:** Paola Durán, Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Temuco, Chile, Tel: (56) 45 2596855; Fax: (56) 45 2592822

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Abstract

Gaeumannomyces graminis var. tritici (Ggt) is the main biotic factor that affects wheat production around the world. Recently we reported the occurrence of six suppressive soils in monoculture areas from indigenous "Mapuche" communities, and evidenced that the suppression relied on the microbial component of those soils. Here, we determine the occurrence of the microbial community (total bacteria, actinomycete, total fungi, and ascomycete) on the rhizosphere and endosphere of wheat plants grown in suppressive soils compared with a conducive soil. We found that Ggt suppression is mediated mostly by endophytic, rather than rhizosphere microorganisms, especially by bacteria since the community structure was similar in all suppressive soils. Interestingly, despite the lower incidence of take-all disease in suppressive soils, the Ggt concentration quantified by qPCR was not different among plants grown in suppressive soils compared to conducive soil. Therefore, the fungal activity is not a determinant factor for suppressiveness classification. Due to the importance of endophytic bacteria, we isolated *Serratia* sp and *Enterobacter* sp from suppressive soils, which only diminish around 20% of fungal growth. Thus, induced resistance or tolerance mechanisms in plants are

involved in suppression against take-all more than direct effect of microorganisms.

Introduction

Take-all disease is caused by *Gaeumannomyces graminis* (Sacc.) Arx et Olivier var. tritici (Walker) or Ggt. This fungus is an ascomycete belonging to the Magnaportheaceae family, and it also affects barley, rye, and other related grasses as triticale. However, it is best known for their notorious negative impact on wheat [1]. Southern Chile produces around 85% cereals, where 40% is wheat (ODEPA, 2016) , and Ggt is the main biotic factor causing crop loss [2].

G. graminis can survive as saprophytically on infected or dead root and crown debris from previous crops causing primary infection through parasitism, thus this pathogen infects the next wheat crop [3]. Roots come into contact with the ascospores and dark runner hyphae of Ggt, they colonize the surface, and then penetrate directly through hyaline hyphae beneath the hyphopodia into the roots cortex and across the endodermis into the stele, obtaining nutrients, carbon, and energy, hence triggering a secondary infection [4,5] [5, 6,7]. Consequently, crop rotation is determinant cultural practice to diminish disease incidence due to the control of the pathogen is inefficient.

Suppression is the ability of a natural soil to reduce or suppress the activity of plant pathogens, mostly due to the presence and activity of soil microorganisms. Their presence increases the ecosystem resilience by creating redundancy in ecosystem services, making soil less vulnerable to short-term changes in the environment [6]. Studies showed that conducive soils, where the incidence of take-all disease is elevated [7], become suppressive under certain characteristics: monoculture of susceptible host, Ggt presence, and take-all disease outbreak [8]. Thus, suppressive soils are defined as soils where disease development is minimal despite the presence of an infective pathogen and a susceptible plant host [9].

Despite suppressive soils have been known for over 100 years and have been studied for more than five decades, they remain poorly understood [10,11]. Considering the great potential that this type of soil offers for sustainable pest management and plant biocontrol, understanding the mechanisms underlying suppression and soil properties should be a priority line of research. Recently, we found six suppressive soils against take-all disease, managed under ancestral agronomic practices as monoculture for more than 10 years and we demonstrated the essential role of soil microbial communities in Ggt suppression [12]. Therefore, the main objective of our study was to determine the structure of the microbial community in these soils and the role it plays on soil suppression. We used denaturing gradient gel electrophoresis (DGGE) to profile total bacteria, actinomycete, total fungi, and ascomycete group community composition present in rhizosphere and endosphere samples of suppressive soils compared with a conducive control. Additionally, the presence of the Ggt was quantified by qPCR, and endophytic bacteria from wheat grown in suppressive soils were assayed in order to determinate their putative role in pathogen inhibition.

Materials and Methods

Three soils previously identified as suppressive (soils 2, 4, and 13) and one as conductive control soil (soil 1) were used in this study [12] (Table 1). All suppressive soils have a large history of wheat monoculture for more than 10 years.

Microbial Communities of Suppressive Soils

The microbial community composition in most representative suppressive soils (soil 2, soil 4, soil 13) regarding the conductive sample (soil 1) was evaluated with DGGE by using universal primer sets for total bacteria, actinomycete, total fungi, and ascomycete.

DNA was extracted from endosphere and rhizosphere soils in three replicates with the Power Soil DNA Isolation Kit (QIAGEN, US), according to the manufacturer's instructions. Primers used for each microbial group are showed in Table 2.

The quality and quantity of the resulting amplicons for all DDGE were assessed by electrophoresis in a 1.5% agarose gel and compared to 100 bp DNA mass ladder bands (Invitrogen). The primer set generates amplicons between 300-500bp. The DGGE analysis was performed using a DCode system (Bio-Rad Laboratories, Inc.). Twenty-five µL PCR product were loaded onto 6% (w/v) polyacrilamide gel with 40-70% gradient (urea and formamide). The electrophoresis was run for 16h at 75V. The gel was then stained with SYBR Gold (Molecular Probes, Invitrogen Co.) for 30 min and photographed on an UV transilluminator.

The DGGE banding profiles Clustering as dendrogram was carried out by using Phoretix 1D analysis software [13] (TotalLab Ltd., UK). The correlation between bacterial communities (biological parameters) and chemical soil properties (ecological parameters) was visualized by non-metric multidimensional scaling (MDS) analysis by using Primer 7+ Permanova software (Primer-E Ltd., Ivybridge, UK) [13]. The *in silico* analysis was also used to estimate bacterial diversity by richness (S), Shannon–Wiener, and dominance by Simpson Index (D) represented by 1- D or 1-λ [14].

Geumannomyces graminis var. tritici DNA Concentration in Endosphere and Rhizosphere

Real Time Quantitative PCR (*q-PCR*) was used to study *Ggt* DNA concentration in wheat roots and rhizosphere soils of wheat being grown in suppressive soils. Briefly, 0.25g samples belonging to rhizosphere and endosphere of conductive soil 1, and suppressive soils 2, 4 and 13 were subjected to DNA extraction by using Power Soil DNA Isolation Kit (QIAGEN, USA) according to the manufacturer's instructions. DNA from pure Ggt was used as positive control and *Aspergillus niger* CCCT-15.62 (CCT-UFRO) and plants without inoculated Ggt were used as negative control.

Abundance of Ggt was performed in an Applied Biosystems Step One™ Real-Time PCR System in 12 µl reaction mixtures, containing Brillant®II SYBR®, Green QPCR master mix

(Stratagene, Agilent Technologies Company, USA), 1 µl 1:10 Ggt DNA dilution (to determine standard curve) and 1 µL sample DNA and 600 nM of each primer. Specific primers GGT2F /GGT168R (Duran *et al.*, in preparation) were used. PCR was performed in triplicate under the following conditions: an initial denaturing step at 95°C for 10min, and 40 cycles at 95°C for 30 s, 52°C for 30 s, and 60°C for 1 min. The PCR efficiency (E) was calculated from the slope of the standard curve as target by the equation: $E=10^{[-1/slope]}$. The specificity of amplified products was checked immediately after the amplification process, by analysing the dissociation curves generated from 60 to 95°C at 0.3 °C intervals. Cycle threshold values (Ct) (the number of cycles required for the fluorescent signal to cross the threshold value, i.e. exceeds back-ground level) were converted to picograms of DNA per gram of sample (soil or wheat root) using a reference standard curve made of 10-fold dilutions for DNA at 0.8 ng to 8×10^{-7} ng DNA from Ggt isolate [12].

Endophytic Bacteria Isolation from Wheat Plants Grown in Suppressive Soils

Given the importance of endophytic bacteria strains denoted in this study, roots and shoots from wheat plants growing in the different soils were separated, surface sterilized by repeatedly immersing the samples in 80 %v/v ethanol for 5min and 4 %v/v NaOCl for 20 min, and then rinsed three times with sterile distilled water. Tissue samples were macerated and homogenized in 1mL sterile saline solution (0.85 %v/v NaCl). One hundred microliters of homogenized tissue dilutions were spread onto the general media Luria-Bertani (LB) and incubated at 30 °C for 2 days. Additionally, the efficacy of tissue surface sterilization was confirmed by spreading last-run rinsing water onto LB and PD media as described above [15]. Endophytic bacteria isolated from suppressive soils as *G. graminis* biocontrol in vitro were assayed by a dual-culture technique for their biocontrol against *G. graminis* var. *tritici* (Ggt) pathogen. The Ggt was grown on PDA plates at 25 °C for 1 week. Agar disks (4-mm diameter) containing Ggt were aseptically incised and transferred to the center of agar plates containing fresh LB/PD (1:1) media. Then,

two drops (5 µL) of each selected endophytic bacteria suspension were taken from overnight LB cultures, washed threefold with NaCl (0.85 %) and placed on two diametric positions at 2 cm from the agar disk containing the Ggt inoculum. Fungal mycelia growth was measured after 3, 5, and 7 days of incubation at 25 °C in the darkness [15].

Identification of Endophytic Selected Strains

Genetic characterization of selected bacteria was based on partial sequencing of 16S rRNA gene. The 16S rRNA gene fragments were amplified by PCR with universal bacterial primers set 27f and 1492r [16]. After starting at 94°C for 5 min, PCR amplification was carried out for 35 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2min. The PCR products were purified and sequenced by Macrogen Inc., (Korea). Sequences were deposited under accession nos. MF623050, MF623051 and MF623052, and then compared with those in the GenBank database. Similarly, strains were submitted to the Chilean Culture Collection from La Frontera University (CCCT- UFRO).

Statistical Analyses

Data normality was analysed according to Kolmogorov's test. Data obtained in 2.5 (*in vitro* biocontrol plate assay) were analysed by a one-way analysis of variance (ANOVA) and compared by Tukey test, using SPSS software (SPSS, Inc.). The similarity between bacterial communities was visualized in non-metric multidimensional scaling analysis (MDS), by using Primer 7 software (Primer-E Ltd., Ivybridge, UK), which showed a Bray–Curtis similarity index higher than 60% and 0.14 stress values [13]. Values were given as means ± standard errors. Differences were considered significant when the *P* value was lower than or equal to 0.01.

Results

Microbial Community Composition of different Microbiological Groups (Total Bacteria, Actinomycetes, Total Fungi and Ascomycetes)

Non metric multidimensional scaling (NMDS) analysis of the soils based on the DGGE profiles of the different microorganism groups is shown in Figure 1. At 60% similarity, all endophytic bacteria from suppressive soils are separated from those in control conductive soil. In contrast, this separation is not evidenced in rhizosphere soils, where all samples are grouped together and were mostly influenced by chemical parameters mainly P, Al sat, P, pH, and K (Figure 1). In relation to the biodiversity index, we found that despite an elevated diversity expressed in richness (N from 325 to 540 in endosphere, and from 30,000 to 35,000 in rhizosphere) and lower dominance in the case of endophytic bacteria (Simpson 0.74 to 0.85) respect to rhizosphere soil (Simpson >0.95), no significant differences were found between suppressive and conductive soils in both endosphere and rhizosphere microorganisms (Table 3).

In relation to actinomycetes group, all samples from plants endosphere are separated independently in different clusters, but regarding to rhizosphere soil, those from soils 2 and 4 are grouped together. Similar to total bacteria, rhizosphere actinomycetes are most influenced by soil chemical parameters. Regarding the biodiversity index in endosphere, no significant differences were found in suppressive soils as compared to conductive control. Whereas in rhizosphere soils there is higher number of species and richness (d), and less dominance (1- λ); hence there is higher biodiversity when compared with conductive control (soil 1).

Regarding total fungi, according to NMDS analyzes (Figure 1), we found that there is no separation between suppressive and conducive control soil in endosphere. In the case of the rhizosphere samples, 2 are grouped independently and are influenced mainly by K differences of samples 13 and 4 as compared with their replicates, which could be attributed to slight variations of the community structures or during the band

recognition by the phoretic software. No differences were found concerning the biodiversity index in the endosphere, but in the rhizosphere there is a fewer number of individuals in suppressive soils (N from 5,300 to 14,600) in relation to conductive control (N= 22, 200).

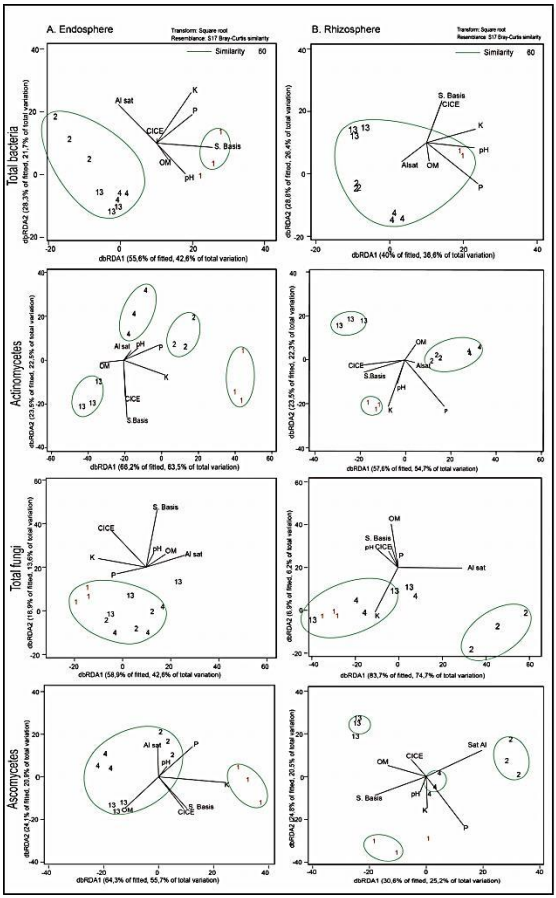

Figure 1: Non-metric Multidimensional Scaling (NMDS) analysis of soils was used in this study, based in DGGE profiles of total bacterial, actinomycetes, total fungi, and ascomycetes communities, in relation with soil parameters (P, K, OM, Al sat, CICE, and Σ basis). The length and position of the black lines (soil parameters) indicate correlation strength and direction of significant variables ($P<0.05$) with the microbial community of endosphere (A) and rhizosphere soils (B). Red letters represent control conductive soil.

In contrast, in the case of the ascomycetes group, we found that the control conductive soil grouped separately respect to suppressive soils at 60% of similarly. Regarding rhizosphere, all groups were separated independently. In relation with the biodiversity index in suppressive soil endosphere, there is lower richness expressed in S, N, d, and mayor dominance (1- λ) in comparison with conductive control (soil 1), except in soil 2.

Biodiversity Index and Chemical Parameters of Suppressive and Conducive Soils of different Microbial Groups

The correlation between biodiversity index and chemical parameters was evaluated (see supplementary Table 1). In general, we found that rhizosphere microorganisms are more influenced by chemical parameters than endosphere microorganisms. Considering the main soil factors that influence the chemical parameters of andisol soils, as pH, OM and Al Sat, we found that all biodiversity indexes, such as S, d, H' and 1- λ, are positively correlated with pH and OM, and negatively correlated with Al sat in rhizosphere soil (Figure 2). Thus, the higher the pH and OM the higher the biodiversity, whereas it decreases in relation to Al Sat. Interestingly, ascomycete group in the endosphere is related positively with K content in soil.

Correlation between Endophytic Ascomycete and other Microbial Groups

In order to determine the influence of the endophytic ascomycete group (belonging to the pathogen) on the rest of microbial groups, we analyzed the Pearson correlation between different microbial groups and their respective Shannon index (see supplementary Table 2). Hence we found that the ascomycete, both rhizosphere and endosphere in suppressive soils, are correlated with endophytic bacteria and conductive control, whereas endophytic ascomycetes are not related with any other microbial groups. In this context, and considering all biodiversity indexes, we found that endophytic bacteria are related with both ascomycete group (rhizosphere and endosphere), except in the

Simpson index (1- λ) where it is only related with endosphere ascomycete (Figure 3).

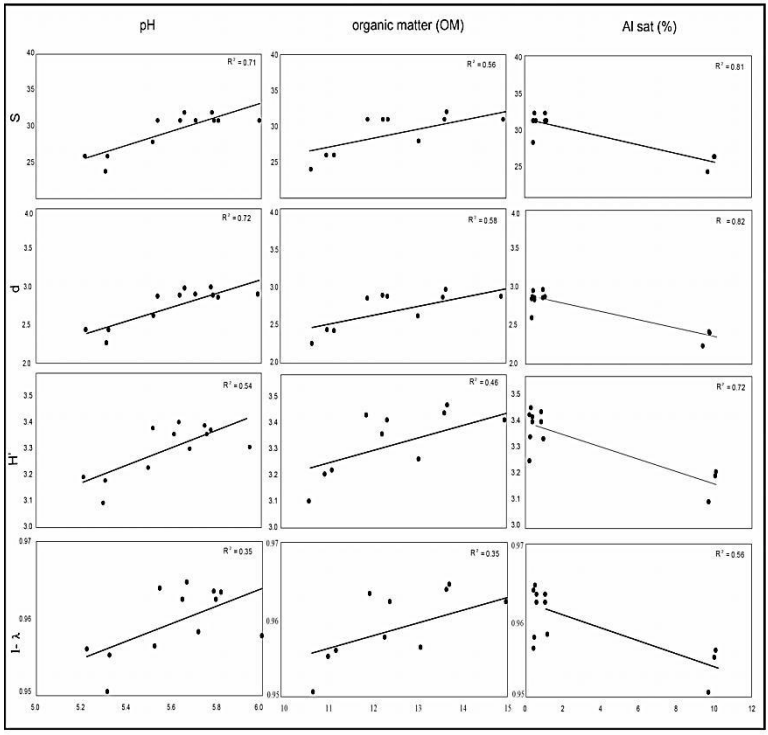

Figure 2: Correlation between biodiversity index S (species), d (individual), H' (Shanon), and Simpson (expresed as 1- λ) of total bacteria rhizosphere, pH, OM, and Sat Al .

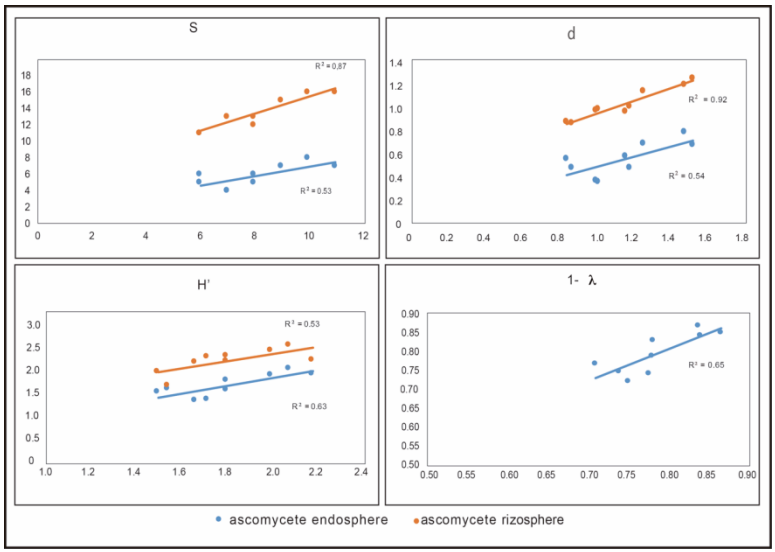

Figure 3: Correlation between biodiversity index between biodiversity index H' (Shanon) of endophytic bacteria and rhizosphere and endosphere ascomycete.

Ggt DNA Concentration in Endosphere and Rhizosphere

Quantitative real-time PCR was used to determine Ggt DNA concentration in endosphere and rhizosphere belonging to suppressive soil 2, 4 and 13, using as positive control conductive soil (sample 1). Negative control (*Aspergillus niger* and wheat plants without Ggt) no ampliefied (data no showed). The standard curve constructed with Ggt purified DNA showed an amplification efficiency of 98% (Figure 4A.). Dissociation curves indicated the presence of a single amplicon and the specificity of primer set was confirmed when purified Ggt DNA was used in PCR reactions (Figure 4B.). Results illustrated in Figure 4C showed that only in soil 13 exist significant differences respect to conducive control, in the rest of soils no differences were evidenced. In fact, samples 2 and 4 show similar Ggt concentration to positive control (around 30 μg g

sample^{-1}), but Ggt was not detected in the rhizosphere of these samples (2 and 4).

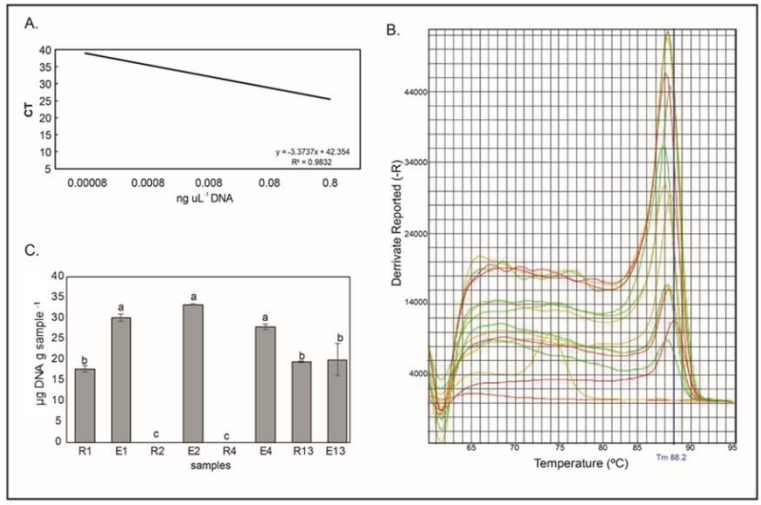

Figure 4: Ggt DNA quantification by quantitative PCR: A) Standard curve, B) Dissociation curve, and C) Ggt DNA concentration (µg g sample^{-1}).

Endophytic Bacteria Isolated from Wheat Grown in Suppressive Soils Capacity to biocontrol *G. graminis in vitro*

Due the importance of endophytic bacteria denoted in this study, we isolated 9 endophytic bacteria form suppressive soils: SS2-1, SS2-2 and SS2-3 from soil 2; SS4-1, SS4-2 and SS4-3 from soil 4 and SS13-1, SS13-2 and SS13-3 from suppressive soil 13. Strains SS2-2, SS2-3, and SS13-3 inhibited mycelia growth 7 days after inoculation (Table 4). The fungal inhibition was around 15% for SS2-2, 20% for SS2-3 and 20% for SS13-3. The rest of the strains did not show fungal inhibition compared to control. The identification and phylogenetic affiliation of these isolates based on 16S rRNA genes partial sequencing are revealed that the endophytic bacteria with antagonistic activity were species from the genera *Serratia* sp. (SS2-2, SS13-3) and *Enterobacter* sp. (SS2-3) (table 5).

Discussion

The important role of microbial communities on take-all suppression has been reported by several studies [9,11,12,17]. A recent study showed that the suppressive activity of soils isolated from mapuche communities, who have sown wheat for more than ten years (monoculture), was related to the microbial community composition, highlighting the essential role of microorganisms in take-all suppression in soils [12]. These soils are characterized by a low content of available P, moderate acidity and high Al saturation (>10%), which are main characteristics of agricultural soils in southern Chile [18]. Remarkably, in this study we found interesting that the total endophytic bacteria communities are similar in all suppressive soils (Figure 1) and are clearly different from conductive control soil. This is why we hypothesize that endophytic microorganisms have an important role in take-all suppression. Additionally, we found that the ascomycete group (group that belong Ggt) in suppressive soils are associated with endophytic bacteria in comparison with the rest of the microbial group analyzed (Figure 3), thus confirming the importance of these microorganisms in specific Ggt suppression as showed in other study where indicate that bacteria could be linked to a specific form of suppression [19]. A recent study established that *Rhizoctonia solani* suppression in potato plants was mediated by *Bacillus* sp and *Pseudomonas* sp. [20]. Similarly, actinomycetes group showed higher biodiversity (higher richness and lower dominance) in rhizosphere of suppressive soils, which could indicate that actinomycetes can also act as Ggt biocontrol agents in the rhizosphere. In this context, studies showed the importance of some strains of actinobacteria in reducing Ggt disease symptoms up to 70%, under steamed soil and field conditions [21]. A similar role of actinomycetes groups was found in suppression of other soil borne pathogens as *Pythium*, *Phytophthora, Ustilago crameri* and *Ralstonia solani* [22-24]. In contrast in fungal structure community no differences were found. Early studies reported that fungal microorganisms have a general role in suppression, but no role in specific suppression [19]. However, we found that the ascomycete group, where Ggt belongs, has major dominance in suppressive soils endosphere.

In contrast with the relevance of endophytic microorganisms in soil suppression found in this study, rhizosphere microorganism composition did not show significant differences in relation to microbial composition in suppressive soils as compared to the conductive control soil. On the other hand, microbial biodiversity was directly related to essential parameters of volcanic-ash-derived soils as pH, OM and Al Sat (Figure 2). Thus, biodiversity of the rhizosphere suppressive soils was higher when pH and SOM were high, and lower in relation to Al Sat, as we reported previously [12]. Other authors through DGGE analyses of the microbial communities showed similar banding patterns in suppressive and conducive soils supporting that rhizosphere communities have no direct influence over soil suppressive effects [7].

In order to determine Ggt concentration in wheat plants grown in suppressive soils compared with a conducive control, we quantified the pathogen by qPCR, and our results did not show differences in fungal concentration between suppressive and conducive soils. Thus, we confirm that suppressive soils had low disease incidence despite Ggt DNA concentration. For example, soils 2 and 4 showed around 30μg Ggt DNA g endosphere sample, and less than 3% blackening roots compared with the control with more than 30% blackening roots [12]. Similarly, other study on suppressive where low disease severity coupled with high Ggt DNA concentrations in roots [7]. On the other hand, we found Ggt presence in wheat plant endosphere with no associated symptoms, confirming our hypothesis that endophytic bacteria located inside the root control the infectivity of the pathogen.

Thus, we isolated endophytic bacteria from roots of wheat grown in suppressive soils and three Enterobacteriaceae strains identified as belonging to the genera *Serratia* and *Enterobacter* were able to inhibit Ggt mycelia growth around 20% and 15%, respectively, under *in vitro* conditions. However, previous studies have shown that endophytic bacteria *Acinetobacter* sp., *Bacillus* sp. and *Klebsiella* sp. isolated from conducive soils inhibited *G. graminis* mycelia growth *in vitro* from 30 to 100%.

Considering this, we hypothesize that the suppressivenes could be related with the capacity of endophytic bacteria to induce resistance to plants due to *Serratia* sp. and other Enterobacteriacea as *Enterobacter* sp. are involved in the N-acyl-L-homoserine lactone (AHL) and salicilic acid production, hence increasing systemic resistance [25-27]. Therefore, inducing hormone analysis and defense genes *in planta* and mechanisms related with plant tolerance are requested in order to determine the role of endophytic bacteria in take-all suppression.

Conclusions

Bacteria from wheat plants endosphere, grown in suppressive soils, have a direct effect on take-all suppression, whereas rhizosphere microorganisms are influenced by soil chemical parameters that are more relevant in volcanic soils such as pH, MO and Al sat. Notwithstanding, the lower incidence of take-all disease in suppressive soils and the Ggt concentration in wheat roots are not key factors to determine supressiveness, possibly due to the fact that the fungus could remain infective in endosphere as a result of the action of microorganisms, mainly endophytic bacteria. However, *Serratia* sp and *Enterobacter* sp isolated from suppressive soils endosphere were only able to inhibit mycelia growth around 20% under *in vitro* conditions. Regarding this, we consider that the suppressing effect of bacteria is related with factors involved in induced resistance in plants. Therefore, *in planta* analyses with endophytic bacteria isolated from wheat grown in suppressive soils are needed.

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