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Cucumber Rhizosphere Microbial Community Response to Biocontrol Agent *Bacillus subtilis* B068150

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Abstract: Gram-positive bacteria *Bacillus subtilis* B068150 has been used as a biocontrol agent against the pathogen Fusarium oxysporum cucumerinum. Cucumber was grown in three soils with strain B068150 inoculated in a greenhouse for 90 days, and the colonization ability of strain B068150 in cucumber rhizosphere and non-rhizosphere soils was determined. Changes in total bacteria and fungi community composition and structures using denaturing gradient gel electrophoresis (DGGE) and sequencing were determined. Colony counts showed that B068150 colonization in the rhizosphere was significantly higher ($p < 0.001$) than in non-rhizosphere soils. Based on our data, the introduction of *B. bacillus* B068150 did not change the diversity of microbial communities significantly in the rhizosphere of three soils. Our data showed that population density of B068150 in clay soil had a significant negative correlation on bacterial diversity in cucumber rhizosphere in comparison to loam and sandy soils, suggesting that the impact of B068150 might be soil specific.

Keywords: cucumber; *Bacillus subtilis*; Microbial community; colonization; diversity

1. Introduction

Biological control may be a potential alternative approach to controlling soil-borne plant diseases, since chemical methods may have some adverse effects on the environment and food safety, and at the same time, destroy both pathogenic and beneficial organisms in soils [1,2]. At present, the acceptability of biocontrol agents [3], and the predicted rate of growth on this method is about 10% yearly [4]. However, an introduced biocontrol agent will interact not only with the pathogen to be controlled but also with all the biotic components of the soil. For example, the biocontrol agent *Fusariumoxy sporum* Fo47 introduced to soils affected the structures of the bacterial and fungal communities in silt loamy soil and sandy soil [5]. Similar effects of biocontrol agents on soil microbial communities have also been reported in the rhizosphere and non-rhizosphere of plants [6,7]. Additionally, it was reported that the introduction of *Trichoderma harzianum* ThzID1-M3 in natural soils resulted in an increase in microbial populations during the experimental period, while potentially reducing its biocontrol efficacy due to competition [8]. These studies provided a basic environmental assessment for the application of biocontrol agents.

Soil is a medium for growth and survival of microorganisms. Different soils with distinct properties also affect bacterial populations and their growth differently [9]. Soil microbial community is thought to be necessary in maintaining a healthy soil and suppressing plant diseases [10–13]. It has been reported that a decrease in soil microbial diversity was responsible for the development of soil-borne plant diseases [14,15]. Some management practices like fertilizer, tillage and pesticide application, *etc*. have been reported as major factors affecting the bacterial composition of rhizosphere soil [16–18]. A potentially good biocontrol agent should be able to be established in different soil environments without affecting the investigated microbial structures, except inhibiting the growth of the pathogen. The soil microbial community played a major role in the retention and cycling of nutrients, in disease suppression, and in the maintenance of plant health [12,19,20]. Therefore, assessing the structure of soil microbial community is important for determining the biological processes that occur in soil after the application of bio-control agent. The use of molecular biological methods currently offers the opportunity to monitor the changes of microbial diversity in soil and rhizosphere samples without cultivation [21,22].

The ability of members of the Gram-positive genus *Bacillus* to form spores is advantageous for controlling a variety of soil-borne phytopathogenic fungi [23–27], and some are commercially marketed as biopesticides, biofertilizers and soil amendments because of their easy colonization, good competition and broad antimicrobial spectrum [28–32]. For example, these bacteria: *B. subtilis* B579 [33], *B. subtilis* N7 [34], *B. amyloliquefaciens* SQR-9 [35], *Pseudomonas fluorescens* [15,36] are effective biocontrol agents. According to these researchers, biocontrol agents have always shown variable results, because they must compete for nutrients with other native soil microbes to survive [37]. Rhizosphere competence, including colonization, is still considered a prerequisite of effective biological control for a biological agent.

In our previous study, we isolated *B. subtilis* strain B068150 from surface sterilized roots of cucumber plants that effectively control cucumber *Fusarium* wilt disease in a greenhouse experiment [38]. However, strain B068150 has not been studied in many soils. In this study, we assess the ecological fitness of *B. subtilis* strain B068150 by using DGGE to explore the influence of soil types on the rhizosphere competence and biocontrol activity of strain B068150 and its effects on the indigenous soil bacteria and fungi. We hypothesized that B068150 would establish in different soil environments without affecting the native microbial communities. Therefore, understanding how B068150 influences soil microbial communities in the greenhouse may be helpful in the development of practices to suppress *Fusarium oxysporum* f. sp. *Cucumerinum* associated with cucumber *Fusarium* wilt.

2. Materials and Methods

2.1. Strain

B. subtilis strain B068150 used in this study was originally isolated from surface sterilized roots of cucumber plants grown in greenhouse in Beijing, China. The strain was effective in controlling *Fusarium* wilt of cucumber during our previous laboratory study [38]. The strain was maintained in Luria-Bertani (LB) broth containing 15% glycerol and was stored at −80 °C before use.

2.2. Preparation of Antibiotic Resistant Mutant from Wild-Type Isolate

B. subtilis B068150 was tagged with nalidixic acid (50 $\mu g \cdot mL^{-1}$) and rifampicin (150 $\mu g \cdot mL^{-1}$), in LB plates as previously described [39]. Antibiotic resistance status and stability in strain B068150 was confirmed by growing on LB plates containing 50 $\mu g \cdot mL^{-1}$ nalidixic acid and 150 $\mu g \cdot mL^{-1}$ rifampicin. Strain B068150 extracted from soil samples was cultured in LB plates containing 25 $\mu g \cdot mL^{-1}$ nalidixic acid and 50 $\mu g \cdot mL^{-1}$ rifampicin.

Tests showed that the growth and survival of the rifampicin/nalidixic acid-resistant isolates were the same as the wild-type on LB plates. All strains of B068150 were separately stored on cryoprotect beads in MicroBank microbial storage tubes (Pro-Lab Diagnostics, Richmond Hill, ON, Canada) under −80 °C.

2.3. B. subtilis B068150 Inoculum Preparation

A single bead was removed from storage at −80 °C under aseptic conditions and plated onto LB broth containing rifampicin (150 μg· mL^{-1}) and nalidixic acid (50 μg· mL^{-1}). After 24 h at 37 °C, a loop of bacterial growth was streaked across a second LB plate with rifampicin/nalidixic acid-amended, and subsequent single colonies were used for inoculum production in a sterile LB broth (100 mL) in a 250 mL flask to obtain a batch culture. The flask was incubated overnight at 37 °C in a shaker at 180 rpm min^{-1}. An aliquot (1 mL) of the overnight culture was used to inoculate one fresh LB plate (rifampicin/nalidixic acid) the next day. A total of 80 LB plates (rifampicin/nalidixic acid) were incubated for approximately 2 days at 37 °C, washed by sterilized water, and filtered with sterile layer gauze to collect the suspension of spore of *B. subtilis* B068150.

The suspension was centrifuged at 4000 rev min^{-1} for 10 min at 4 °C, and the spores were suspended in sterilized water again, which contained approximately 2×10^8 colony forming units (CFU) per milliliters of sterilized water. This suspension was added into the soil with a target level of 1×10^6 CFU per gram of dry soil.

2.4. Soils Source

Three soils used for this study were Dello loamy sand (sand), Arlington sandy loam (loam) and Willow silty clay (clay), with organic carbon (OC) contents of 0.58, 5.4 and 20.4 g· kg^{-1}, respectively (Table 1). Soils were collected from the Santa Ana River bed (sand), fallow field at the University of California-Riverside (loam), and Mystic Lake dry bed (clay). Soil texture and chemistry were the same as previously described [39].

2.5. Soils Preparation

Before use, soils were sieved to pass through a 2-mm sieve and placed in plastic barrels (diameter, 16.5 cm; height, 25 cm). Moisture content of the soil sample was maintained constantly (50% of WHC) during the experiment by differences in soil weight after every 3 days. Based on water loss from each soil, the amount of irrigation water to be added automatically to each soil was determined.

2.6. Strain B068150 Introduction into Soils

The *B. subtilis* B068150 spore suspension was mixed into soils to a final concentration of 1.00×10^6 CFU per gram of dry soil. The CFU was counted according to the methods described previously [39]. Soil without strain B068150 was used as control for three soils. All plastic barrels were maintained in the greenhouse after introducing B068150 into soils and planting cucumber (Hybrid cucumber, Seminis vegetable seeds, Oxnard, CA, USA).

Cucumber seeds were surface-sterilized by soaking in 3% NaOCl and incubated at 30 °C for 24 h for germination [38]. Triplicate plastic barrels containing the soils inoculated with *B. subtilis* B068150 was set up for each soil type.

2.7. Pot Experimental Design

Three soils that had never been planted with cucumber were used in the seedling growth. The method and the amount of soils loaded per barrel were as previously described [38]. Germinating cucumber seeds were planted in the control soils and soils inoculated with B068150 in barrels (16.5 cm in diameter, 25 cm in height), under the simulated natural conditions with the average daytime temperature at 30 °C, and 24 °C in the night. Moisture content of the soil sample was maintained constantly (50% of WHC) during the experiment by dripping water automatically to make up for evaporation based on differences in soil weight. Based on evaporation, the amount of irrigation water applied to each soil was 27.33 mL, 38.67 mL and 18 mL for clay, loam, and sandy soil per day and per barrel, respectively.

Table 1. Soils texture and chemistry.

Soil Type	Sand (%)	Silt (%)	Clay (%)	Bulk Density (g/cm)	WHC (%)	pH	T-N (g/kg)	OC (g/kg)	WSOC (mg/kg)	MBC (mg/kg)	AOC (mg/kg)
Dwllo loamy sand	99.1	0.2	0.7	1.67	17	7.1	0.07	0.58	10	11	0.2
Arlington sandy loam	70.9	20.8	8.3	1.54	21	7.2	0.061	5.4	44	56	0.9
Willow silty clay	3.7	49.1	47.2	1.51	63	7.2	1.61	20.4	242	278	4.94

WHC, water holding capacity; T-N, Total nitrogen; OC, Total organic carbon; WSOC, Water soluble organic carbon; MBC, Microbial biomass organic carbon; AOC, Assimilable organic carbon. From Ma *et al.* [39].

Non-rhizosphere soil samples from the pot experiments were collected at days 0, 3, 5, 10, 15, 20, 25, 28 and 32, and continue for days 39, 53, 74 and 91, for the rhizosphere soils. The experimental design consist of 15 seeds per barrel, 12 barrels per treatment, and three barrels per sampled time. After 32 days, the barrels were filled with cucumber roots, and hence the collection of non-rhizosphere soils was stopped. The rhizosphere samples were collected after shaking loosely held soil on the roots by taking out whole root systems and non-rhizosphere samples were collected using a cylindrical soil corer with diameter of 1.5 cm at least 10 cm away from plants. All soil samples were collected in depth of 5–10 cm below the surface soil. Cells were extracted from cucumber rhizosphere and non-rhizosphere soil samples inoculated with B068150 and cultured on the LB plates (25 $\mu g \cdot mL^{-1}$ nalidixic acid, 50 $\mu g \cdot mL^{-1}$ rifampicin) for the assessment of the colonization patterns of strain B068150.

2.8. DNA Extraction, PCR Amplification, and DGGE Analysis

Total microbial community DNA was extracted from soil samples (0.5 g; at 0, 10, 32 days for all rhizosphere soils) with the Power Soil DNA Kit (MoBio Laboratories, Solana Beach, CA, USA) and stored at –20 °C.

A 236 bp DNA fragment in the V3 region of 16Sr RNA genes of bacteria were amplified. The primers used were 338F [40] and 518R [41]. The 200 bp DNA fragment in the ITS genes region of fungi were amplified with primers EF4F and EF3R for the first PCR step [42], and NS3F and Fungal5R for the second nested PCR [42,43]. The reverse primers used for PCR of bacteria and nested PCR of fungi included a GC clamp [41]. Ready-To-Go PCR beads (GE Healthcare Biotech, Piscataway, NJ, USA) and 5 pmol of primers in a total volume of 25 μL were used in the PCR reaction as previously described [16].

The PCR amplification conditions of fungi were 95 °C for 5 min; 35 cycles of 94 °C for 1 min, 48 °C for 1 min, 72 °C for 1 min and a final incubation at 72 °C for 10 min. Nest PCR was performed with 95 °C for 5 min; 35 cycles of 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min and a final incubation at 72 °C for 10 min.

DGGE was performed with 8% (wt/vol) acrylamide gels containing a linear chemical gradient ranging from 30%–70% denaturant with 100% defined as 7 M urea and 40% formamide. Gels were run for 3.5 h at 200 V with the Dcode™ Universal Mutation System (Bio-Rad Laboratories, Hercules, CA, USA). The gel was then stained with ethidium bromide, visualized and photographed using a gel imaging system (Bio-Rad Lab., Hercules, CA, USA). Cluster analysis was constructed using PCORD 5.0 [44]. Distance measure was based on Jaccard index, and group linkage method was Ward's method. Major bands were excised for identification of microbial species as previously described [45]. All bands were sent to Sunbiotech Co. (Beijing, China) for sequencing. The sequences recovered were aligned with bacterial and fungal gene fragments available from the National Center for Biotechnology Information (NCBI) databases.

2.9. Statistical Analysis

The data for colonization of strain B068150 in rhizosphere and non-rhizosphere soil were analyzed by analysis of variance (ANOVA) and the means were separated by Duncan's tests at $p < 0.05$. General linear model ANOVA (repeated measures) procedure in randomized complete block design was used with soil type and inoculation as the main plot and sampling time as the subplot. Both ANOVA and general linear model ANOVA were using SPSS (SPSS statistics 17.0, 2008, SPSS Inc., Chicago, IL, USA). The rhizosphere and non-rhizosphere colonization data were converted to log10 values before the statistical analysis. The richness, evenness, diversity indexes and colonization were compared by Repeated Measures ANOVA and Pearson Correlation analysis using SPSS (SPSS statistics 17.0, 2008), and the means were separated by Duncan's tests at $p < 0.05$. The richness, evenness, Shannon index of diversity and Simpson's diversity index were calculated by PCORD 5.0 [44]. Richness (S) refers to the number of bands detected in a given soil sample. The DGGE evenness (E), a measure of how evenly DGGE bands were distributed in a given soil sample, was calculated as $E = H'/\ln(S)$. Diversity was

calculated by using the Shannon index of diversity (H') to compare changes in diversity of microbial communities within all treatments at each time [46] by using the following function:

$$H' = -\Sigma P_i \, log \, P_i$$

where $P_i = n_i / N$, n_i is the height of peak, and N is the sum of all peak heights in the curve.

Simpson's diversity index (D) for infinite population was calculated by the following equation:

$$D = 1 - \text{sum}\,(Pi \times Pi)$$

where Pi = importance probability in element i (element i relativized by row total).

3. Results

3.1. Abundance of B. subtilis B068150 in Cucumber Rhizosphere and Non-Rhizosphere

The abundance of *B. subtilis* B068150 with the two antibiotics was detected in the clay, loam and sand rhizosphere and non-rhizosphere soils (Figure 1). A significant decrease was seen after 10 days in rhizosphere soil samples and after three days in the non-rhizosphere soil. Thereafter, the numbers stabilized and remained between 4.68 and 5.21 log CFU g^{-1} soil (0.68×10^4 and 0.21×10^5 CFU g^{-1} soil). The abundances of strain B068150 did not differ significantly in day three from any of the three soils from non-rhizosphere samples ($p = 0.068$). However, there was significant interaction between soil type and sampling time in days ($p < 0.001$). The abundance of strain B068150 was significantly different in three soils after day 10 of the study in both rhizosphere and non-rhizosphere soils ($p < 0.0001$). The order of colonization of strain B068150 in rhizosphere and non-rhizosphere in different soils was clay > loam > sand. Furthermore, B068150 colonization in the rhizosphere was significantly different from the non-rhizosphere soils ($p < 0.0001$). The abundance of strain B068150 in sand decreased significantly faster than the other two soils from day 25 (Figure 1).

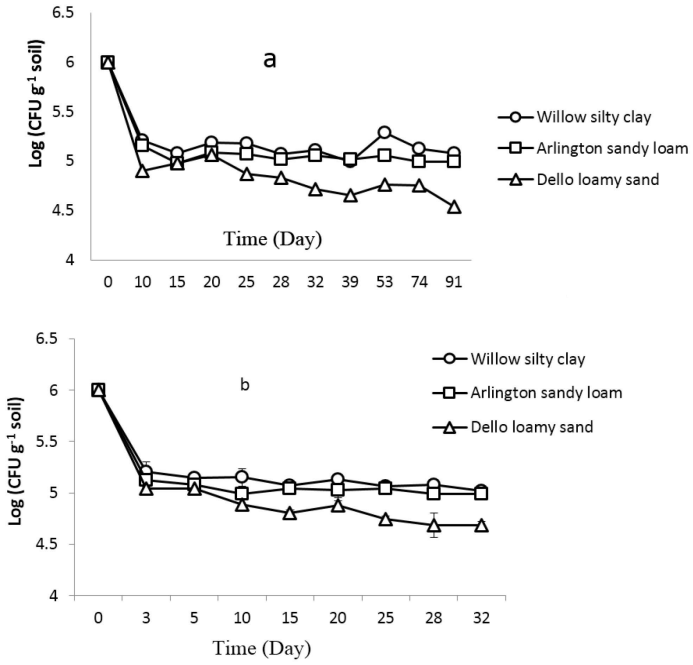

Figure 1. Abundance of *B. subtilis* B068150 in (**a**) rhizosphere; (**b**) non-rhizosphere clay, loam, and sandy soils.

3.2. Changes in Bacterial Community Structure in Cucumber Rhizosphere with *B. subtilis* B068150

Rhizosphere and non-rhizopshere soils inoculated with *B. subtilis* B068150 were analyzed over time to determine the changes in microbial community structure (Figure 2). To compare DGGE patterns, Pearson's indices were determined for comparisons of all profiles, and unweighted pair group method with mathematical averages (UPGMA) was used to create a dendrogram describing pattern similarities in the rhizosphere (Figure 3). The analysis clearly distinguished among bacterial communities in the three soils studied. Analysis of DGGE profiles (Figure 3) from 18 soil samples grouped them into three main clusters. The UPGMA result revealed that the cucumber rhizosphere bacterial community of both treated and control were similar in day 0, and thereafter some differences were observed in individual soil types. For example, the main cluster of clay divided into two branches, one branch contained two samples of treatment and control in day 0, and the other branch contained treatment samples in day 10 and 32. The same trend was also observed in the other two soils.

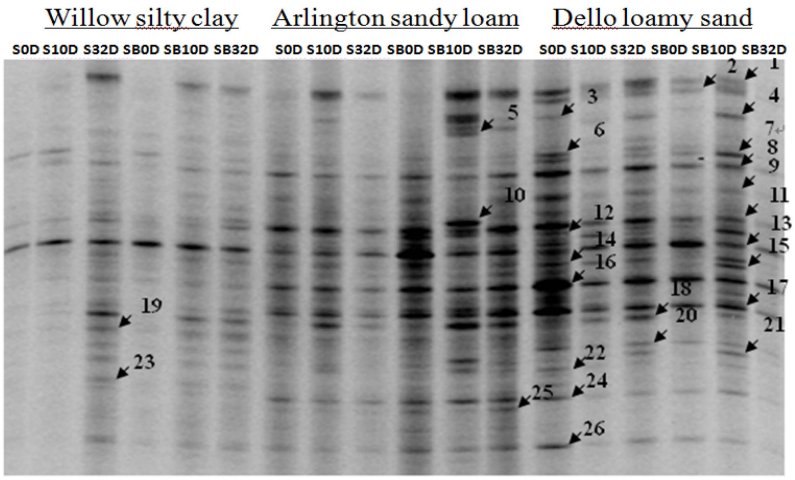

Figure 2. DGGE banding patterns of 16S rDNA fragments. Numbers indicate the excised and sequenced 16S rDNA bands. Lane labels denote samples from the treatments in this study (S0D, S10D, S32D: soils untreated and sampled in 0, 10 and 32 day. SB0D, SB10D, SB32D: *B subtilis* B068150-NR was introduced in to soils and sampled in 0, 10 and 32 day.)

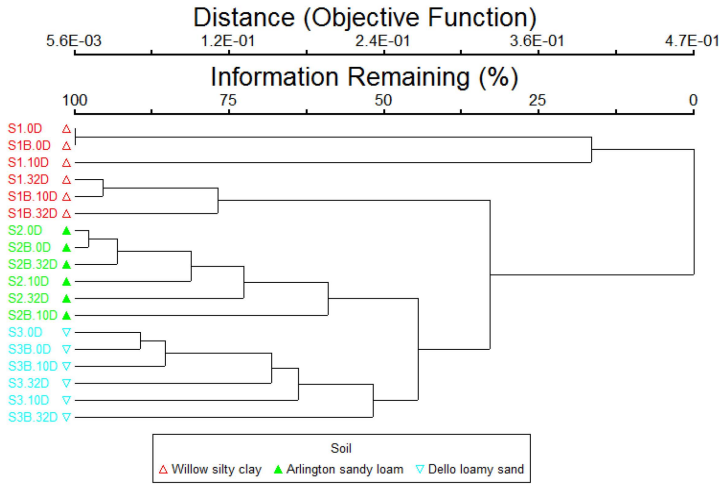

Figure 3. Cluster dendrogram of bacterial community in cucumber rhizosphere by PCORD. Figures next to the branches are cophenetic correlations value that is a parameter to express the consistence of a cluster. (S0D, S10D and S32D indicate soils untreated and sampled in 0, 10 and 32 day. SB0D, SB10D and SB32D indicate *B subtilis* B068150-NR was introduced in to soils and sampled in 0, 10 and 32 day).

The diversity of bacterial communities were examined in detail using analysis of variance with repeated measures to determine differences in species richness, evenness, Shannon, and the Simpson diversity indices among the treatments with strain B068150 and non-inoculated controls at day 0, 10 and 32 (Table 2). Pearson correlation coefficients showed that there were no significant correlation between bacteria diversity and colonization of *B subtilis* B068150 in cucumber rhizosphere in loam and sand. However, in clay, the introduction of B068150 had a significant negative correlation on bacterial diversity in cucumber rhizosphere (Table 2), suggesting that the impact of B068150 might be soil specific.

Table 2. Pearson correlation coefficients and significance levels between B068150 colonization and bacteria diversity indices in cucumber rhizosphere samples.

	Colonization in Clay	Colonization in Loam	Colonization in Sand
Richness	−1.000 * (0.017)	−0.286 (0.815)	−0.109 (0.930)
Evenness	+0.685 (0.520)	+0.584 (0.603)	+0. 611 (0.582)
Shannon diversity	−1.000 ** (0.007)	−0.253 (0.837)	+0. 018 (0.988)
Simpson diversity	−0.999 * (0.034)	+0.411 (0.730)	−0.057 (0.964)

"**" indicate significant correlation at $p < 0.01$; "*" indicate significant correlation at $p < 0.05$; "−" indicates a negative correlation; "+" indicates a positive correlation.

Bands indicated by numbers were excised and sequenced (Table 3) to gain insight into the identities of major bacterial populations. Bands excised were correctly identified with 97%–100% identity to closely related database sequences. Most of the sequences were uncultured bacteria, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroides*. *B. subtilis* B068150 belongs to *Firmicutes* phylum.

Table 3. Sequence similarity of the main bacterial in different cucumber rhizosphere soil samples in DGGE.

Band	The Most Similar Species	Similarity (%)	Phylum	Accesion #
2	*Bacillus asahii*	98	*Firmicutes*	KF387676.1
8	*Bacillus* sp.	100	*Firmicutes*	AB748970.1
10	*Bacillus* sp.	100	*Firmicutes*	KJ935909.2
11	*Bacillus megaterium*	99	*Firmicutes*	KC485314.1
13	*Bacillus* sp.	99	*Firmicutes*	KF747082.1
14	*Bacillus licheniformis*	100	*Firmicutes*	HQ009796.1
15	*Bacillus* sp.	100	*Firmicutes*	GQ487543.1
16	*Bacillus jeotgali*	99	*Firmicutes*	HM854268.1
17	*Bacillus jeotgali*	100	*Firmicutes*	HM854268.1
18	*Bacillus* sp.	100	*Firmicutes*	HQ141380.1
3	*Pedobacter* sp.	99	*Bacteroidetes*	KF817798.1
4	*Chryseobacterium* sp.	99	*Bacteroidetes*	GU451187.1
12	*Chryseobacterium* sp.	100	*Bacteroidetes*	AB581570.1
7	*Herbaspirillum* sp.	97	*Proteobacteria*	EU549851.1
20	Uncultured *Sphingomonas* sp.	100	*Proteobacteria*	LN568861.1
25	*Polyangium* sp.	100	*Proteobacteria*	KJ611227.1
26	*Arthrobacter nicotianae*	100	*Actinobacteria*	HM209738.1
1	Uncultured bacterium	100	unclassified	LN571102.1
5	Uncultured bacterium	99	unclassified	KJ956651.1
6	Uncultured bacterium	100	unclassified	GQ866159.1
9	Uncultured bacterium	99	unclassified	HM273912.1
19	Uncultured bacterium	100	unclassified	KM207257.1
21	Uncultured bacterium	99	unclassified	AM935657.1
22	Uncultured bacterium	99	unclassified	KC605411.1
23	Uncultured bacterium	99	unclassified	FN567976.1
24	Uncultured bacterium	99	unclassified	FN567976.1

3.3. Fungal Community in Cucumber Rhizosphere

The fungal communities in cucumber rhizosphere were analyzed after inoculating *B. subtilis* B068150 into clay, loam and sand (Figure 4). The bands indicated by numbers were excised and sequenced (Table 4). *Ascomycota* was the largest phylum in cucumber rhizosphere among the three types of soil (Table 4). The pathogen of cucumber wilt disease *F. oxysporum* belongs to the *Ascomycota* phylum. PCR-DGGE gels (Figure 4) revealed that the rhizosphere fungal communities were different among the treatments with strain B068150 and non-inoculated controls at each time point examined (0, 10 and 32 day).

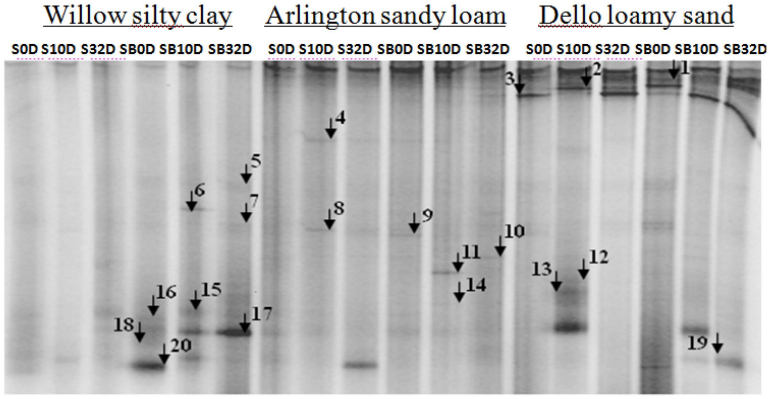

Figure 4. DGGE banding patterns of ITS fragments. Numbers indicate the excised and sequenced ITS bands. Lane labels denote samples from the treatments in this study (S0D, S10D, S32D: soils untreated and sampled in 0, 10 and 32 day. SB0D, SB10D, SB32D: *B subtilis* B068150-NR was introduced in to soils and sampled in 0, 10 and 32 day).

Table 4. Sequence similarity of bands of the main fungi in different cucumber rhizosphere soil samples in DGGE.

Band	The Most Similar Species	Similarity (%)	Phylum	Accesion #
3	*Rhizophydium* sp.	95	*Chytridiomycota*	AY635821.1
12	Uncultured *Ceratobasidium*	99	*Basidiomycotina*	EF154347.1
5	Uncultured *ascomycete*	100	*Ascomycota*	FN390517.1
8	*Stilbella fimetaria*	99	*Ascomycota*	FJ939395.1
10	Uncultured *ascomycete*	100	*Ascomycota*	FN390419.1
11	*Microascus cirrosus*	100	*Ascomycota*	KM222204.1
13	*Chaetomium* sp.	99	*Ascomycota*	AB521039.1
14	*Stilbella fimetaria*	98	*Ascomycota*	FJ939395.1
15	Uncultured *ascomycete*	99	*Ascomycota*	FN390729.1
17	*Aspergillus ustus*	99	*Ascomycota*	GQ856237.1
18	*Stilbella fimetaria*	98	*Ascomycota*	FJ939395.1
19	*Aspergillus* sp.	100	*Ascomycota*	EU795695.1
1	Uncultured fungus	100	unclassified	KF192297.1
2	Uncultured fungus	99	unclassified	AJ635526.1
4	Uncultured fungus	99	unclassified	AJ635526.1
6	Uncultured fungus	99	unclassified	EU657332.1 |
7	Uncultured fungus	100	unclassified	AB534361.1
9	Uncultured fungus	100	unclassified	EU657047.1
16	Uncultured fungus	100	unclassified	AB534361.1
20	Uncultured fungus	100	unclassified	EU795695.1

When fungal diversity indices were compared in different cucumber rhizosphere soils, the Shannon diversity and Simpson diversity indices showed significant difference in different cucumber

rhizosphere soils (data not shown). The order of both Shannon diversity and Simpson diversity indices of rhizosphere fungi was sand > clay > loam ($p < 0.05$).

Cluster and NMDS analysis of DGGE bands of the 18 soil samples grouped the soils into three main clusters based on soil types (Figures 5 and 6). The analysis did not distinguish between fungal communities in the three types of soil, and between the controls compared to the samples inoculated with strain B068150 as observed with bacterial community structures above.

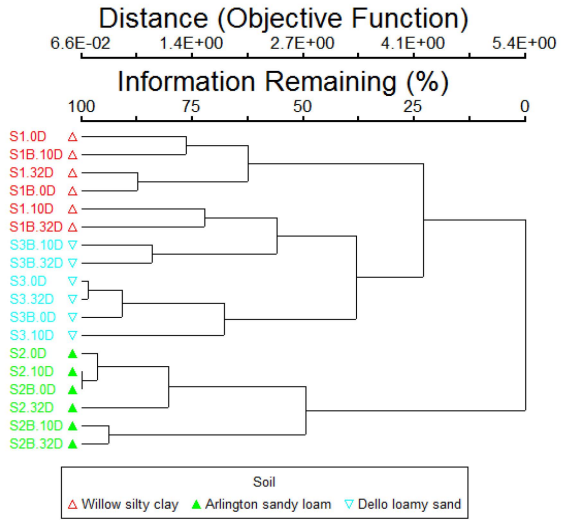

Figure 5. A comparison of the fungal community structure of cluster dendrogram by using PCORD. Figures next to the branches are the cophenetic correlations value that is a parameter to express the consistency of a cluster. (S0D, S10D and S32D indicate soils untreated and sampled in 0, 10 and 32 day. SB0D, SB10D and SB32D indicate *B subtilis* B068150 was introduced in to soils and sampled in 0, 10 and 32 days).

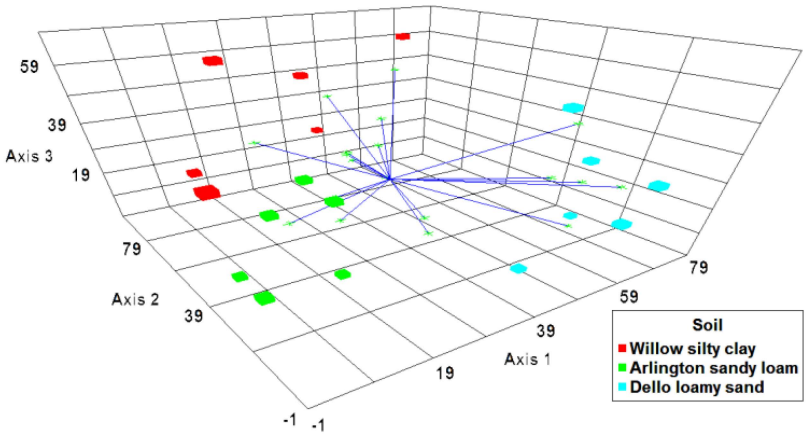

Figure 6. Analysis the similarity of fungi communities in different cucumber rhizosphere soils based on NMDS analysis of DGGE bands of the 18 soil samples.

4. Discussion

Colonization of roots and soils through adding bio-control agent is considered a prerequisite for successful biological control [47,48]. *B. subtilis* B068150 strain, originally isolated from surface sterilized roots of cucumber plants, inhibited the cucumber *Fusarium* wilt [38]. In the first part of this study, the ability of strain B068150 to colonize cucumber rhizosphere and non-rhizosphere under controlled conditions in greenhouse was assessed. To determine B068150 colonization, three soils

with contrasting properties were used (Table 1). The population of *B. subtilis* B068150 significantly decreased in non-rhizosphere and rhizosphere soil samples, and then stabilized after three and 10 days, respectively. This pattern was observed in other studies [49,50]. The stabilization in the number of viable cells likely corresponded to spore formation, which is the ability of the Gram-positive genus *Bacillus* to adapt to the soil environment [51,52]. The abundance of *B. subtilis* B068150 in rhizosphere was significantly higher than in non-rhizosphere in the three soils (clay, $p = 0.021$; loam, $p = 0.04$; sand, $p = 0.002$). The colonization in the cucumber rhizosphere in the three soils may be related to root exudates as previously reported [53,54]. Our data suggest that the survival of strain B068150 in cucumber rhizosphere and non-rhizosphere was successful, indicating positive impacts on biocontrol of *Fusarium* wilt as previously reported [55].

The use of microorganisms to control plant diseases has been a common practice during the last few decades. Application of biocontrol agent directly into soils to control plant diseases was shown to have no negative impact on other organisms or on biogeochemical cycles [7,48,56]. One of the objectives described in this study was to elucidate the influence of *B. bacillus* B068150 on the native microbial communities in the cucumber rhizosphere. Based on our data, the introduction of *B. bacillus* B068150 did not change the diversity of microbial communities significantly in the rhizosphere of three soils, although the abundance of *B. subtilis* B068150 in the rhizosphere was significantly higher than in the non-rhizosphere. Pearson indices also indicated that there were no significant correlation between microbial diversity and abundance of *B subtilis* B068150 in the rhizosphere except in the clay rhizosphere soil (Table 3). The significant negative correlation in clay soil may be related to the higher natural microbiota or competition for nutrients as previously reported [7]. However, there were no significant differences in microbial diversity between the control and cucumber rhizosphere inoculated with *B subtilis* B068150. Therefore, the effects of B068150 on native microbial communities were temporary as previously reported [57]. For example, application of a commercially available *B. amyloliquefaciens* FZB42 had a temporary negative impact on the indigenous lettuce rhizosphere microbial community [58]. However, the majority of the reported non-target studies of BCAs pointed to no substantial effects on bacterial abundance [7].

Although exogenous microbes like biocontrol agents are often correlated with the observed indigenous microbial community structure and diversity changes, the structure and diversity of soil bacterial communities have been found to be closely related to soil environmental characteristics [59,60]. The abundance of strain B068150 was significantly different in three soils after day 10 of the study ($p < 0.01$) in both rhizosphere and non-rhizosphere soils, and the observed order in our study was clay > loam > sand. It is known that the soil physico-chemical properties may influence the biological balance considerably. Therefore, B068150 survival at low population densities in sand may be related to a lower level of organic matter and lower water potential (Table 1) [61,62].

B. bacillus B068150 is known for its ability to suppress cucumber *Fusarium* wilt, which showed no obvious antagonistic activity to *F. oxysporum* f. sp. *cucumerinum* on potato dextrose agar plate. The mechanisms of action of reported biocontrol agents had been shown to include biofilm formation, competition for root niches and nutrients, antibiosis and induced systemic resistance in host plants [55,63–65]. Studies have shown that biofilm formation by *B. subtilis* may likely act as a biobarrier on the roots to protect plants from pathogens being infected [63]. *Ascomycota* was the largest community in our study, and the pathogen of cucumber wilt disease *F. oxysporum* belongs to the *Ascomycota* phylum. During our study, there were no symptoms of cucumber *Fusarium* Wilt in cucumber. The disease generally manifests itself in young and mature plants throughout cucumber-growing stages [66]. Our study showed that *Ascomycota* was the dominant fungal community in the plant rhizosphere, which is contrary to previous reports [67,68]. One possible explanation for lack of disease symptoms by cucumber may be the ability of *B. bacillus* B068150 to out-compete the pathogen on the rhizosphere for nutrients, and establish itself as one of the dominant bacteria in the soil.

5. Conclusions

B. bacillus B068150 is known for its ability to suppress cucumber *Fusarium* wilt, which showed no obvious antagonistic activity to *F. oxysporum* f. sp. *cucumerinum* on potato dextrose agar plate. During our study, no significant differences were found between native microbial communities with strain B068150 except in bacterial community in clay. There were also no significant differences in microbial diversity between the control and cucumber rhizosphere inoculated with *B subtilis* B068150. Therefore, the ability of *B. bacillus* B068150 to easily colonize different types of soils could make it an environmentally compatible plant protective agent in soils.

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References

1. Becker, J.O.; Schwinn, F.J. Control of soil-borne pathogens with living bacteria and fungi: Status and outlook. *Pest. Sci.* **1993**, *37*, 355–363. [CrossRef]
2. Minuto, A.; Spadaro, D.; Garibaldi, A.; Gullino, M.L. Control of soilborne pathogens of tomato using a commercial formulation of *Streptomyces griseoviridis* and solarization. *Crop Protect.* **2006**, *25*, 468–475. [CrossRef]
3. Bailey, K.L.; Boyetchko, S.M.; Längle, T. Social and economic drivers shaping the future of biological control: A Canadian perspective on the factors affecting the development and use of microbial biopesticides. *Biol. Control* **2010**, *52*, 221–219. [CrossRef]
4. Bailey, K.L.; Mupondwa, E.K. Developing microbial weed control products: Commercialization, biological, and technological considerations. In *Handbook of Sustainable Weed Management*; Singh, H.P., Batish, D.R., Kohli, R.K., Eds.; The Haworth Press Inc.: Binghamton, NY, USA, 2006; pp. 431–473.
5. Véronique, E.H.; Sylvie, B.; Nadine, G.; Sébastien, A.; Claude, A.; Christian, S. Ecological fitness of the biocontrol agent *Fusarium oxysporum* Fo47 in soil and its impact on the soil microbial communities. *FEMS Microbiol. Ecol.* **2009**, *68*, 37–45.
6. Timms-Wilson, T.; Kilshaw, K.; Bailey, M.J. Risk assessment for engineered bacteria used in biocontrol of fungal disease in agricultural crops. *Plant Soil* **2004**, *266*, 57–67. [CrossRef]
7. Winding, A.; Binnerup, S.J.; Pritchard, H. Non-target effects of bacterial biological control agents suppressing root pathogenic fungi. *FEMS Microbiol. Ecol.* **2004**, *47*, 129–141. [CrossRef]
8. Bae, Y.S.; Knudsen, G.R. Soil microbial biomass influence on growth and biocontrol efficacy of *Trichoderma harzianum*. *Biol. Control* **2005**, *32*, 236–242. [CrossRef]
9. Pratibha, P.; Neera, K.; Sarita, S. Rhizosphere: Its structure, bacterial diversity and significance. *Rev. Environ. Sci. Biotech.* **2014**, *13*, 63–77.
10. Van Bruggen, A.H.C.; Semenov, A.M. A new approach to the search for indicators of root disease suppression. *Aust. Plant Pathol.* **1999**, *28*, 4–10. [CrossRef]
11. Van Bruggen, A.H.C.; Semenov, A.M. In search of biological indicators for soil health and disease suppression. *Appl. Soil Ecol.* **2000**, *15*, 13–24. [CrossRef]
12. Garbeva, P.; van Veen, J.A.; van Elsas, J.D. Mocrobial diversity in soil: Selection of microbial populations by plant and soil type and implications for disease suppressiveness. *Annu. Rev. Phytopathol.* **2004**, *42*, 243–270. [CrossRef] [PubMed]

13. Van Bruggen, A.H.C.; Semenov, A.M.; van Diepeningen, A.D.; de Vos, O.J.; Blok, W.J. Relation between soil health, wave-like fluctuations in microbial populations, and soil-borne plant disease management. *Eur. J. Plant Pathol.* **2006**, *115*, 105–122. [CrossRef]
14. Mazzola, M. Assessment and management of soil microbial community structure for disease suppression. *Annu. Rev. Phytopathol.* **2004**, *42*, 35–59. [CrossRef] [PubMed]
15. Hiddink, G.A.; van Bruggen, A.H.C.; Termorshuizen, A.J.; Raaijmakers, J.M.; Semenov, A.V. Effect of organic management of soils on suppressiveness to *Gaeumannomyces graminis* var. *tritici* and its antagonist, *Pseudomonas fluorescens*. *Eur. J. Plant Pathol.* **2005**, *113*, 417–435. [CrossRef]
16. Ibekwe, A.M.; Poss, J.A.; Grattan, S.R.; Grieve, C.M.; Suarez, D. Bacterial diversity in cucumber (*Cucumis sativus*) rhizosphere in response to salinity, soil pH, and boron. *Soil Biol. Biochem.* **2010**, *42*, 567–575. [CrossRef]
17. Fang, M.; Kremer, R.J.; Motavalli, P.P.; Davis, G. Bacterial diversity of rhizospheres of non-transgenic and transgenic corn. *Appl. Environ. Microbiol.* **2005**, *71*, 4132–4136. [CrossRef] [PubMed]
18. Grayston, S.J.; Wang, S.Q.; Campbell, C.D.; Edwards, A.C. Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biol. Biochem.* **1998**, *30*, 369–378. [CrossRef]
19. Larkin, R.; Tavantzis, S.; Bernard, E.; Alyokhin, A.; Erich, S.; Gross, S. Compost and biological amendment effects on soil borne disease and soil microbial communities. *Phytopathology* **2008**, *98*, S86.
20. Manici, L.M.; Caputo, F. Soil fungal communities as indicators for replanting new peach orchards in intensively cultivated areas. *Eur. J. Agron.* **2010**, *33*, 188–196. [CrossRef]
21. Bai, Y.H.; Liang, J.S.; Liu, R.P.; Hu, C.Z.; Qu, J.H. Metagenomic analysis reveals microbial diversity and function in the rhizosphere soil of a constructed wetland. *Environ. Tech.* **2014**, *35*, 2521–2527. [CrossRef] [PubMed]
22. Van Diepeningen, A.D.; Vos, O.J.; Zelenev, V.V.; Semenov, A.M.; van Bruggen, A.H.C. DGGE fragments oscillate with or counter to fluctuations of cultivable bacteria along wheat roots. *Microb. Ecol.* **2005**, *50*, 506–517. [CrossRef] [PubMed]
23. Grosch, R.; Junge, H.; Krebs, B.; Bochow, H. Use of *Bacillus subtilis* as a biocontrol agent. III. Influence of *Bacillus subtilis* on fungal root diseases and on yield in soil less culture. *J. Plant Dis. Protect.* **1999**, *106*, 568–580.
24. Idriss, E.E.; Bochow, H.; Ross, H.; Borriss, R. Use of *Bacillus subtilis* as biocontrol agent. VI. Phytohormone-like action of culture filtrates prepared from plant growth-promoting *Bacillus amyloliquefaciens* FZB24, FZB42, FZB45 and *Bacillus subtilis* FZB37. *J. Plant Dis. Protect.* **2004**, *111*, 583–397.
25. Koumoutsi, A.; Chen, X.H.; Vater, J.; Borriss, R. DegU and YczE positively regulate the synthesis of bacillomycin D by *Bacillusamy-loliquefaciens* strain FZB42. *Appl. Environ. Microbiol.* **2007**, *73*, 6953–6964. [CrossRef] [PubMed]
26. Arguelles-Arias, A.; Ongena, M.; Halimi, B.; Lara, Y.; Brans, A.; Joris, B.; Fickers, P. *Bacillusamy loliquefaciens* GA1 asasource of potentanti biotics and other secondary metabolites for biocontrol of plant pathogens. *Microbial Cell Fact.* **2009**, *8*, 63–68. [CrossRef] [PubMed]
27. Chowdhury, S.P.; Dietel, K.; Rändler, M.; Schmid, M.; Junge, H.; Boriss, R.; Hartmann, A.; Grosch, R. Effects of *Bacillusamy loliquefaciens* FZB42 on lettuce growth and health under pathogen pressure and its impact on the rhizosphere bacterial community. *PLoS ONE* **2013**, *8*, e68818. [CrossRef] [PubMed]
28. Baysala, Ö.; Çalışkanc, M.; Yeşilovaa, Ö. An inhibitory effect of a new *Bacillus subtilis* strain (EU07) against *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Physiol. Mol. Plant Path.* **2008**, *73*, 25–32. [CrossRef]
29. Cazorla, F.M.; Romero, D.; Pérez-García, A.; Lugtenberg, B.J.J.; de Vicente, A.; Bloemberg, G. Isolation and characterization of antagonistic *Bacillus subtilis* strains from the avocado rhizoplane displaying biocontrol activity. *J. Appl. Microbiol.* **2007**, *103*, 1950–1959. [CrossRef] [PubMed]
30. Choudhary, D.K.; Johri, B.N. Interactions of *Bacillus* spp. and plants with special reference to induced systemic resistance (ISR). *Microbiol. Res.* **2009**, *164*, 493–513. [CrossRef] [PubMed]
31. Chung, S.; Kong, H.; Buyer, J.S.; Lakshman, D.K.; Lydon, J.; Kim, S.; Roberts, D.P. Isolation and partial characterization of *Bacillus subtilis* ME488 for suppression of soilborne pathogens of cucumber and pepper. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 115–123. [CrossRef] [PubMed]
32. Hervás, A.; Landa, B.; Datnof, L.E.; Jiménez-Díaz, R.M. Effects of commercial and indigenous microorganisms on *Fusarium* wilt development in chickpea. *Biol. Control* **1998**, *13*, 166–176. [CrossRef]

33. Rodgers, P.B. Potential of biological control organisms as a source of antifungal compounds for agrochemical and pharmaceutical product development. *Pest. Sci.* **1989**, *27*, 155–164. [CrossRef]
34. Luo, Y.; Sun, L.F.; Zhu, Z.; Shen, Q. Identification and characterization of an anti-fungi *Fusarium oxysporum* f. sp *cucumerium* protease from the *Bacillus subtilis* strain N7. *J. Microbiol.* **2013**, *51*, 359–366. [CrossRef] [PubMed]
35. Xu, Z.H.; Zhang, R.F.; Wang, D.D.; Qiu, M.H.; Feng, H.C.; Zhang, N.; Shen, Q.R. Enhanced Control of Cucumber Wilt Disease by *Bacillus amyloliquefaciens* SQR9 by Altering the Regulation of Its DegU Phosphorylation. *App. Environ. Microbiol.* **2014**, *80*, 2941–2950. [CrossRef] [PubMed]
36. Van Bruggen, A.H.C.; Semenov, A.M.; Zelenev, V.V.; Semenov, A.V.; Raaijmakers, J.M.; Sayler, R.; de Vos, O. Wave-like distribution patterns of gfp-marked *Pseudomonas* fluorescens along roots of wheat plants grown in two soils. *Microb. Ecol.* **2008**, *55*, 466–475. [CrossRef] [PubMed]
37. Cao, Y.; Zhang, Z.; Ling, N.; Yuan, Y.; Zhang, X.; Sun, B.; Shen, Q. *Bacillus subtilis* SQR9 can control *Fusarium* wilt in cucumber by colonizing plant roots. *Biol. Fertil. Soils* **2011**, *47*, 495–506. [CrossRef]
38. Li, L.; Ma, J.; Yan, L.; Wang, Z.; Gao, T.; Wang, Q. Screening and partial characterization of *Bacillus* with potential applications in biocontrol of cucumber *Fusarium* wilt. *Crop Protect.* **2012**, *35*, 29–35. [CrossRef]
39. Ma, J.; Ibekwe, A.M.; Yi, X.; Wang, H.Z.; Yamazaki, A.; Crowley, D.E.; Yang, C.H. Persistence of *Escherichia coli* O157:H7 and Its Mutants in Soils. *PLoS ONE* **2011**, *6*, e23191. [CrossRef] [PubMed]
40. Lane, D.J. 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematic*; Stackebrandt, E., Goodfellow, M.D., Eds.; John Wiley and Sons: New York, NY, USA, 1991; pp. 115–175.
41. Muyzer, G.; de Waal, E.C.; Uitterlinden, A.G. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **1993**, *59*, 695–700. [PubMed]
42. Smit, E.; Leeflang, P.; Glandorf, B.; van Elsas, J.D.; Wernars, K. Analysis of Fungal Diversity in the Wheat Rhizosphere by Sequencing of Cloned PCR-Amplified Genes Encoding 18S rRNA and Temperature Gradient Gel Electrophoresis. *Appl. Environ. Microbiol.* **1999**, *65*, 2614–2621. [PubMed]
43. White, T.J.; Burns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phyologenetics. In *PCR Protocols*; Innes, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: San Diego, CA, USA, 1990; pp. 315–322.
44. McCune, B.; Mefford, M.J. *PC-ORD for Windows. Multivariate Analysis of Ecological Data*; Version 4.01; MjM Sofware: Gleneden Beach, OR, USA, 1999.
45. Ibekwe, A.M.; Ma, J. Effects of fumigants on microbial diversity and persistence of *E. coli* O15:H7 in contrasting soil microcosms. *Sci. Total Environ.* **2011**, *409*, 3740–3748. [CrossRef] [PubMed]
46. Shannon, A.E.; Weaver, W. *The Mathematical Theory of Communication*; University Illinois Press: Urbana, IL, USA, 1963.
47. Duffy, B.K.; Weller, D.M. Biological control of take-all of wheat in the Pacific North-west of the USA using hypovirulent *Gaeumannomyces graminis* var. *tritici*, fluorescent *Pseudomonads*. *J. Phytopathol.* **1996**, *144*, 11–12. [CrossRef]
48. Cavaglieri, L.R.; Andres, L.; Ibáñez, M.; Etcheverry, M.G. Rhizobacteria and their potential to control *Fusarium verticillioides*: Effect of maize bacterisation and inoculum density. *Antonie van Leeuwenhoek* **2005**, *87*, 179–187. [CrossRef] [PubMed]
49. Akiba, Y. Microbial ecology of *Bacillus thuringiensis* VI. Germination of *Bacillus thuringiensis* spores in the soil. *Appl. Entomol. Zool.* **1986**, *21*, 76–80.
50. Pedersen, J.C.; Damgaard, P.H.; Eilenberg, J.; Hansen, B.M. Dispersal of *Bacillus thuringiensis var. kurstaki* in an experimental cabbage field. *Can. J. Microbiol.* **1995**, *41*, 118–125. [CrossRef]
51. Van Elsas, J.D.; Dijkstra, A.F.; Govaert, J.M.; van Veen, J.A. Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. *FEMS Microbiol. Ecol.* **1986**, *38*, 151–160. [CrossRef]
52. Backman, P.A.; Brannen, P.M.; Mahaffee, W.F. Plant response and disease control following seed inoculation with *Bacillus subtilis*. In *Improving Plant Productivity with Rhizosphere Bacteria*; Ryder, M.H., Stephens, P.M., Bowen, G.D., Eds.; CSIRO Division of Soils: Adclaidc, Australia, 1994; pp. 3–8.
53. Gamalero, E.; Lingua, G.; Capr, F.G.; Fusconi, A.; Berta, G.; Lemanceau, P. Colonization pattern of primary tomato roots by *Pseudomonas fluorescens* A6RI characterized by dilution plating, flow cytometry, fluorescence, confocal and scanning electron microscopy. *FEMS Microbiol. Ecol.* **2004**, *48*, 79–87. [CrossRef] [PubMed]

54. Liu, X.; Zhao, H.; Chen, S. Colonization of maize and rice plants by strain *Bacillus megaterium* C4. *Curr. Microbiol.* **2006**, *52*, 186–190. [CrossRef] [PubMed]
55. Compant, S.; Duffy, B.; Nowak, J.; Clément, C.; Barka, E.A. Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects. *Appl. Environ. Microbiol.* **2005**, *71*, 4951–4959. [CrossRef] [PubMed]
56. Príncipe, A.; Alvarez, F.; Castro, M.G.; Zachi, L.; Fischer, S.E.; Mori, G.B.; Jofré, E. Biocontrol and PGPR features in native strains isolated from saline soils of Argentina. *Curr. Microbiol.* **2007**, *55*, 314–322.
57. Domsch, K.H.; Jagnow, G.; Anderson, T.H. An ecological concept for the assessment of side effects of agrochemicals on soil microorganisms. *Residue Rev.* **1983**, *86*, 65–105.
58. Kröber, M.; Wibberg, D.; Grosch, R.; Eikmeyer, F.; Verwaaijen, B.; Chowdhury, S.P.; Hartmann, A.; Pühler, A.; Schlüter, A. Effect of the strain *Bacillus amyloliquefaciens* FZB42 on the microbial community in the rhizosphere of lettuce under field conditions analyzed by whole metagenome sequencing. *Front. Microbiol.* **2014**, *5*, 252. [CrossRef] [PubMed]
59. Bääth, E.; Anderson, T.H. Comparison of soil fungal/bacterial ratios in a pH gradient using physiological and PLFA-based techniques. *Soil Biol. Biochem.* **2003**, *35*, 955–963. [CrossRef]
60. Borneman, J.; Triplett, E.W. Molecular microbial diversity in soils from eastern Amazonia: Evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl. Environ. Microbiol.* **1997**, *63*, 2647–2653. [PubMed]
61. Acea, M.J.; Moore, C.R.; Alexander, M. Survival and growth of bacteria introduced into soil. *Soil Biol. Biochem.* **1998**, *20*, 509–515. [CrossRef]
62. Broeckling, C.D.; Broz, A.K.; Bergelson, J.; Manter, D.K.; Vivanco, J.M. Root exudates regulate soil fungal community composition and diversity. *Appl. Environ. Microbiol.* **2008**, *74*, 738–744. [CrossRef] [PubMed]
63. Bais, H.P.; Fall, R.; Vivanco, J.M. Biocontrol of *Bacillus subtilis* against infection of arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. *Plant Physiol.* **2004**, *134*, 307–319. [CrossRef] [PubMed]
64. Emmert, E.A.B.; Handelsman, J. Biocontrol of plant disease: A gram positive perspective. *FEMS Microbiol. Lett.* **1999**, *171*, 1–9. [CrossRef] [PubMed]
65. Haas, D.; Défago, G. Biological control of soil-borne pathogens by fluorescent *Pseudomonads*. *Nat. Rev. Microbiol.* **2005**, *3*, 307–319. [CrossRef] [PubMed]
66. Ahn, I.P.; Chung, H.S.; Lee, Y.H. Vegetative compatibility groups and pathogenicity among isolates of *Fusarium oxysporum* f. sp. *cucumerinum*. *Plant Dis.* **1997**, *82*, 244–246. [CrossRef]
67. O'Brien, H.E.; Parrent, J.L.; Jackson, J.A.; Moncalvo, J.M.; Vilgalys, R. Fungal community analysis by large-scale sequencing of environmental samples. *Appl. Environ. Microbiol.* **2005**, *71*, 5544–5550. [CrossRef] [PubMed]
68. Lim, Y.W.; Kim, B.K.; Kim, C.; Jung, H.S.; Kim, B.S.; Lee, J.H.; Chun, J. Assessment of soil fungal communities using pyrosequencing. *J. Microbiol.* **2010**, *48*, 284–289. [CrossRef] [PubMed]