

**ENDOPHYTIC BACTERIA CONTROLLING *FUSARIUM OXYSPORUM*
AND *RHIZOCTONIA SOLANI* IN *SOLANUM TUBEROSUM***

Epifanio Castro del Ángel
Universidad Autónoma Agraria
Antonio Narro, Department of
Agricultural Parasitology **Mexico**
epifaniocastrodelangel@hotmail.com

**Francisco Daniel Hernández
Castillo**
Universidad Autónoma
Agraria Antonio Narro,
Department of Agricultural
Parasitology **Mexico**
fdanielhc@hotmail.com
(Corresponding author)

Yisa María Ochoa Fuentes
Universidad Autónoma
Agraria Antonio Narro,
Department of Agricultural
Parasitology **Mexico**
yisa8a@yahoo.com

Gabriel Gallegos Morales
Universidad Autónoma Agraria
Antonio Narro, Department of
Agricultural Parasitology **Mexico**
ggalmor@uaaan.mx

Francisco Castillo Reyes
Instituto Nacional de
Investigaciones Forestales,
Agrícolas y Pecuarias **Mexico**
reyes.francisco@inifap.gob.mx

**Fulgencio Martín Tucuch
Cauch**
GreenCorp Biorganiks de
México, S.A. DE C.V. **Mexico**
fm.tucuch@greencorp.mx

ABSTRACT

This study was conducted to test multiple endophytic bacteria as biological control agents against *Rhizoctonia solani* and *Fusarium oxysporum*. A total of 26 endophytic bacteria were isolated from potato plants. Two strains of *Bacillus amyloliquefaciens* (strain21 and strain53) were found to be potential biological control agents based on their radial growth inhibition percentage (RGIP) in dual culture test. The biocontrol potential of the two most effective antagonist strains was evaluated in potato plants under greenhouse conditions against *R. solani* and *F. oxysporum*. As a result, both bacteria promoted growth and development of the crop by increasing chlorophyll content, biomass fresh weight, root weight, stem diameter, plant height and crop yield. Both bacteria favored the health of potato plants in 909.09% against *R. solani* and 303.03% against *F. oxysporum*. This study suggests the use of endophytic bacteria to minimize losses caused by wilt diseases and root rot in greenhouses.

Keywords: Antagonism, endophytes, incidence, severity, *Bacillus amyloliquefaciens*.

INTRODUCTION

It has been estimated that potato crop in Mexico requires most of the fungicide supply to prevent and control several diseases; around 21.3% are applied only on this crop, from the total available fungicides (Hernández *et al.*, 2008). *Rhizoctonia solani* causes the disease known as black scurf of the potato, which causes damage to underground stems, roots, stolons and tubers, which is reflected in yield losses, another of the limiting pathogens in potato production is *Fusarium* spp. Both pathogens cause losses ranging from 7 to 64% (Hernández *et al.*, 2001). Endophytic bacteria have been used to control these kind of diseases, these bacteria live in plant tissues for at least part of their life cycle without causing any damage to the host, they promote plant growth and health among other beneficial effects, in many cases caused by metabolic interactions, and the phytoremediation process of toxic compounds efficiency improvement in the rhizosphere (Pérez *et al.*, 2013). They use mechanisms such as: antibiosis, competition for nutrients, ecological niches and induced systemic resistance (ISR) to displace the phytopathogen (Aliye *et al.*, 2008). The efficacy of

endophytes depends on factors such as: host specificity, population dynamics, colonization pattern, ability to move within host tissues and induce systemic resistance (Barka et al., 2002). Endophytic bacteria suppress pathogens that cause diseases of economic importance in several crops (Sharma et al., 2009; Maksimov et al., 2011), they have the ability to produce antibiotics and enzymes such as chitinases, glucanases, proteases and lipases, which cause cellular lysis (Neeraja et al., 2010). The objective of this study was to evaluate the biocontrol potential of several endophytic bacteria strains against *R. solani* and *F. oxysporum*.

MATERIALS AND METHODS

Endophytic bacteria strains

To isolate endophytic bacteria, potato plant stems were collected from potato fields of the Galeana region, Nuevo Leon, Mexico in 2014, the bacteria were cultured by plate dilution in nutrient agar (NA) and King's B (KB) media, previously surface sterilized. Stems were cut into 4 cm pieces and then surface sterilized by sequential immersion in ethanol 70% for 1 minute, 2% of NaOCl for 3 min, and 70% of ethanol for 30 sec, followed by three washes in distilled water and blotted dry on sterile filter paper. Both ends of each stem were burnt into a flame and fragmented to about 1 cm segments. The success of surface sterilization was checked by rolling the stem pieces on the surface of nutrient agar medium. Succeeded sterilization was indicated by no bacterial growth on the medium after three days of incubation. Each piece of stem was macerated in a sterile mortar and resuspended in 5mL of phosphate buffer. Aliquots of 50 μ L from a serial dilution up to 10⁻⁶ were plated on NA medium in triplicate. Petri dishes were incubated at 27 \pm 2 °C for 24 to 72h. Bacterial colonies were purified on NA medium as described by Perez et al. (2010).

Isolation and morphological identification of phytopathogens

The strains of *Rhizoctonia solani* and *Fusarium oxysporum* were isolated from potato plants with necrosis and wilt symptoms, both strains were cultured in PDA medium. The morphological identification of *F. oxysporum* was made using the keys of Leslie and Summerell (2006) and *R. solani* by the Sneh et al. (1991).

Identification Using 16S rRNA Gene and ITS1-ITS4 regions

The isolation of genomic DNA from the bacteria and fungi by PCR amplification of the 16S rRNA Gene and ITS1-ITS4 internal transcribed regions were performed using the previously described methods (Ríos et al. 2016). Polymerase chain reaction (PCR) amplification of the 16S–23S rDNA gene and ITS1-ITS4 internal transcribed regions between ribosomal genes (rDNA) 18S-5.8S and 5.8S-28S from strains was performed as described by Ríos et al. (2016). Pure colonies of the bacteria were inoculated in LB broth, and incubated during 48h at 26°C on a rotary shaker. Fungal cultures were grown on PDA at 25 \pm 2 °C for 14 days. Each bacteria reaction mixture (20 μ L) contained 0.2 μ L of Taq DNA polymerase (1U/ μ L), 2 μ L of 10x PCR Buffer + MgCl₂, 0.32 of MgCl₂ (25mmol/L), 0.5 μ L of DMSO, 0.4 μ L dNTPs (10 mmol/L), 0.5 μ L (10 μ mol/L) of each primer, primers ITS1 (KIO Fw 3'-TAGAGGAAGTAAAAGTCGTAA-5') and ITS4 (KIO Rv 5'-TCCTCCGCTTWTGWTGTC-3'), 13.58 μ L of Milli-Q water and 2 μ L of template DNA at 40ng/ μ L. After denaturation of the template at 95°C for 3min, 35 rounds of temperature cycling (95°C for 15 seconds, 48°C for 15 seconds, and 72°C for 45 seconds) were followed by final extension at 72°C for 7 min. Genomic DNA of bacteria was amplified through F1624 (3'-CCTTTGTACACACCGCCCGTCG-5') and R1494 (5'-CTACGGRTACCTTGTACGAC-3') primers. Each reaction mixture (20 μ L) contained 0.2 μ L of Taq DNA polymerase (1U/ μ L), 2 μ L of 10x PCR Buffer QIA, 0.5 μ L of DMSO,

0.4 μ L dNTPs (10 mmol/L), 0.8 μ L (5 μ mol/L) of each primer, 14.3 μ L of Milli-Q water and 1 μ L of template DNA at 20ng/ μ L. After denaturation of the template at 95°C for 2 min, 35 rounds of temperature cycling (95°C for 40 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds) were followed by a final extension at 72°C for 7 min. The amplification was observed in agarose gel at 1% through electrophoresis at 60 V. The PCR products were increased and purified by using a GeneAll®Expin™ SV PCR purification kit. The sequencing of the partial 16S rRNA Gene and ITS1-ITS4 regions was carried out using the service of Macrogen (Rockville Maryland, USA), and the obtained sequences were identified using the NCBI GenBank database and were stripped from initial and final part to increase the sensitivity of the analysis.

Antagonistic activity *in vitro*

Twenty-six strains of endophytic bacteria were screened for their antagonistic activity against *F. oxysporum* and *R. solani* by the dual culture test in PDA medium as described Hernández et al. (2014), An explant of each of the phytopathogens with active mycelia of seven days old was placed at the center of a Petri dish, then a sample of each endophytic bacteria was placed in direction of the cardinal points. The antagonistic effect was determined by the equation of Jomduang and Sariah (1995). The experiment was arranged in a completely randomized design with five replicates per bacterial isolation and one control without antagonist for each phytopathogen, dual cultures were kept in incubation at 26 \pm 2 °C.

Consortia formation

Compatibility between strains that showed the highest antagonism levels and maintained their continuous action on phytopathogens was determined. Compatibility was performed in Petri dishes considering the single and combined antagonism of the endophytic bacteria strains using the methodology of Sueke et al. (2010).

Microorganisms and culture conditions

The bacterial inoculum was prepared with the strains 21 and 53, both of them were grown in potato dextrose liquid medium (PD) and incubated on a rotary shaker at 120 rpm at 26 \pm 2 °C for seven days. After incubation, the spores were recovered by centrifugation at 3500 rpm and resuspended in sterile distilled water; the solution was adjusted to 1x10⁶ spores/mL and 1x10⁸ spores/mL.

The mycelia of *F. oxysporum* was recovered by scraping the Petri dish and suspending it in sterile distilled water, and finally adjusted to 1x10⁶ conidia/mL. The inoculum of *R. solani* was obtained using the methodology of Schneider et al. (1997) modified; 300 g of wheat grain were placed in 1000 mL flasks with 100 mL of PD and autoclaved sterilized for 30 min for three consecutive days. The flasks were inoculated with three 5 mm diameter disks with active mycelia of the seven day old pathogen, the flasks were incubated at 24 \pm 2 °C for 21 days.

Biocontrol activity *in plant*

The two isolates that yielded the greatest inhibition zones of phytopathogens *in vitro* growth were selected to demonstrate their biocontrol activity in plants against *F. oxysporum* and *R. solani* development. Minutubers of potato cv. Fianna were planted into pots of 5 kg with sterilized soil. The pathogens were inoculated at sowing time; application of *F. oxysporum* was at 1x10⁶ conidia/mL in 20 mL of sterile distilled water, the inoculation of *R. solani* was made with ten infected wheat seeds with mycelia and sclerotia. The consortia were applied on three occasions: the first one at sowing time, the second when the plants reached about 15 cm

of length and the third was at an interval of 15 days after the second. The treatments of this research were: (FoC1, RsC1) Pathogen + consortium 1 1×10^6 spores/mL, (FoC2, RsC2) Pathogen + consortium 2 1×10^8 spores/mL, (BC1) consortium 1 1×10^6 spores/mL, (BC2) consortium 2 1×10^8 spores/mL, (Fo, Rs) pathogen and control. The experiment was in a randomized block design with 6 replicates per treatment and was kept under greenhouse conditions at the Universidad Autonoma Agraria Antonio Narro, Saltillo, Coahuila, Mexico. The biocontrol effect was checked 130 days after inoculation, the disease incidence was determined, and it was expressed as a diseased plants percentage. Severity was assessed with a six-class scale; where: 0 - Plants with healthy stems and roots, 1 - Plants with minimal damage in stems and roots (less than 10%), 2 - Plants with slight damage in stems and root (25%), 3 - Plants with medium damage in stems and root (50%), 4 - Plants with severe damage in stems and root (75%) and 5 - Plants with dead stems (100%). The effect on growth promotion and development was measured, as plant height (cm), stem diameter (mm), chlorophyll content (SPAD units), fresh root weight (g), fresh biomass weight (g) and tuber weight (g).

Statistical analysis

Data were subjected to analysis of variance using the software SAS 9.0 for Windows and the means were separated by the least significant difference (LSD) tested at $P \leq 0.05$ to detect statistical differences.

RESULTS

Morphological and molecular phytopathogens identification

Fungi phytopathogens isolated from diseased potato plants were identified by morphological characteristics as *F. oxysporum* (Leslie y Summerell 2006) and *R. solani* (Sneh et al.1991). Morphological identification confirmation of the species was obtained by sequencing the internal space transcribed ITS1 and ITS4, the sequences obtained in BLAST showed 99% homology with *Fusarium oxysporum* isolate 20160115-F and *Rhizoctonia solani* isolate JZB-34, with access key in GenBank: KU533843.1 and JX050236.

Antagonistic activity *in vitro*

After screening their antagonistic activity, all the endophytic bacteria tested showed different degrees of inhibition towards the mycelial growth of *F. oxysporum* and *R. solani*. Both isolates of endophytic bacteria identified as *Bacillus amyloliquefaciens* based on the sequence analysis of the 16S rRNA gene, showed 99% homology with access key in Geenbank: KU570451.1 and KX665550.1, respectively. Strain 21 and 53 produced significantly ($P < 0.0001$) higher PIRG values based on dual culture (Table 1 and Fig. 1). It is relevant to mention that although some strains exhibited considerable antagonistic activity they were not able to maintain their continuous activity and the phytopathogen grew on them.

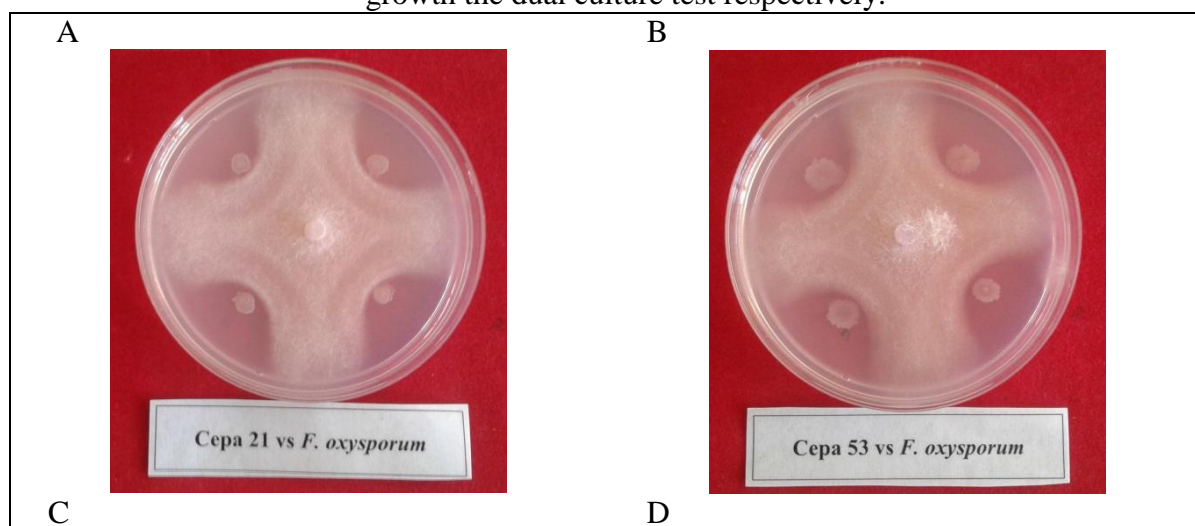
Table 1. Antagonistic activity of endophytic bacteria in dual culture test against *F. oxysporum* and *R. solani* *in vitro*.

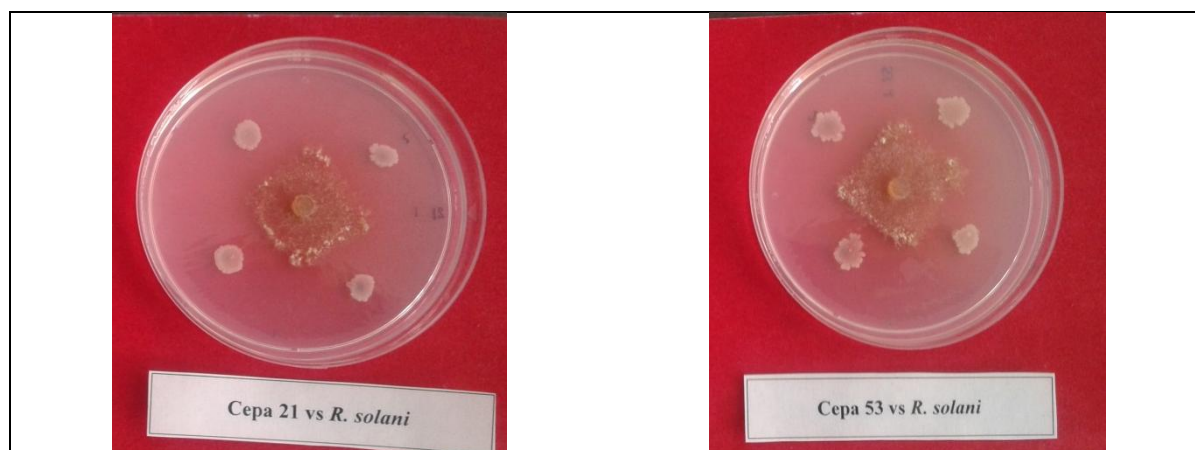
Strain	Radial growth inhibition percentage (%)	
	<i>Rhizoctonia solani</i>	<i>Fusarium oxysporum</i>
Strain 9	5.00 r	0.00 h
Strain 11	10.00 p	0.00 h
Strain 14	34.38 e	31.13 c
Strain 21	73.33 a**	55.00 b**
Strain 23	30.00 g	0.00 h
Strain 27	35.00 d	0.00 h

Strain 30	26.88 l	0.00 h
Strain 33	31.88 f	0.00 h
Strain 34	24.38 o	0.00 h
Strain 37	30.00 g	0.00 h
Strain 38	29.36 i	0.00 h
Strain 41	28.75 j	0.00 h
Strain 45	8.13 q	0.00 h
Strain 47	2.50 s	0.00 h
Strain 50	26.25 m	0.00 h
Strain 52	35.00 d	28.75 d
Strain 53	72.71 b**	57.30 a**
Strain 54	36.25 c	0.00 h
Strain 55	25.00 n	31.13 c
Strain 56	26.88 l	0.00 h
Strain 57	25.00 n	18.75 f
Strain 59	26.88 l	0.00 h
Strain 61	29.38 h	13.75 g
Strain 68	28.75 j	26.25 d
Strain 72	8.13 q	0.00 h
Strain 80	28.13 k	0.00 h

Note: Means with the same letter in the same column are not significantly different according to the least significant difference (LSD) tested at $P=0.05$. ** Strains with better antagonistic capacity.

Fig. 1. Effect of strain 21(A, C) and 53 (B, D) on of *F. oxysporum* and *R. solani* radial growth the dual culture test respectively.





Biocontrol activity in plant

Results showed that the bacterial consortia reduce significantly the incidence and severity of the disease ($P \leq 0.0001$). Consortium 2 reduced the incidence of *R. solani* by 66.67% compared to the infested control, while *F. oxysporum* was reduced by 66.67% in the two tested concentrations (Table 2). Consortium 1 increased health of potato plants by 909.09% against *R. solani* and 303.03% in *F. oxysporum*. Disease severity was reduced by 9.91% on *R. solani* and 24.81% on *F. oxysporum* as compared with the infested control. In general, the disease development was least in plants treated with endophytic bacteria than in the untreated ones.

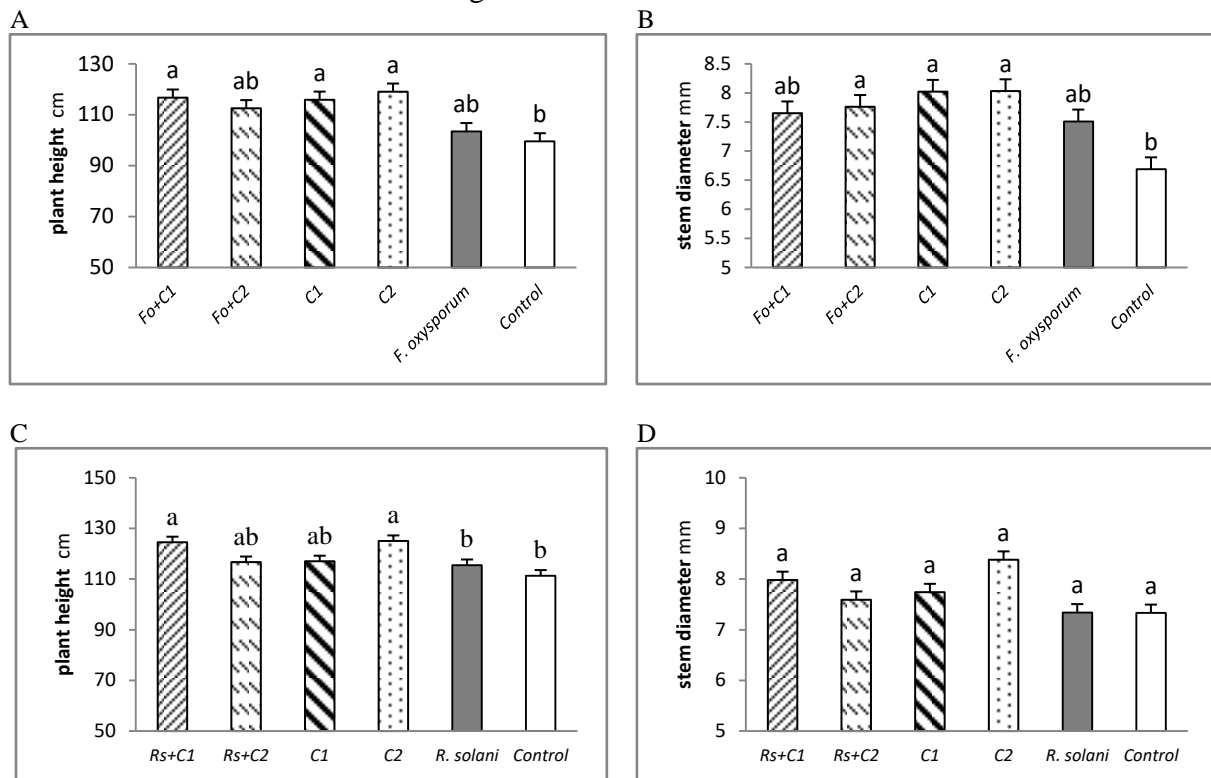
Table 2. Effect of antagonistic bacteria on disease incidence and severity of *F. oxysporum* and *R. solani*.

Treatment	Incidence (%)	Reduction	Disease severity	Reduction
Rs+C1	50.00±4.6 ab	50.0	0.33±0.2 c	90.1
Rs+C2	33.33±18.3 b	66.7	0.50±0.0 c	85.0
Fo+C1	33.33±18.3 b	66.7	0.33±0.2 c	75.2
Fo+C2	33.33±18.3 b	66.7	0.33±0.2 c	75.2
C1	0.00±0.00 b	100.0	0.00±0.0 c	100.0
C2	0.00±0.00 b	100.0	0.00±0.0 c	100.0
<i>Rhizoctonia solani</i>	100.00±0.0 a	----	3.33±0.5 a	----
<i>Fusarium oxysporum</i>	50.00±4.8 ab	----	1.33±0.3 b	----
Control	0.00±0.00 b	100.0	0.00±0.0 c	100.0

Note: Means with the same letter in the same column are not significantly different according to the least significant difference (LSD) tested at $P=0.05$. \pm are mean standard deviation. ---- Used to compare. Disease reduction (DR) was calculated using the following equation: $DR = [1 - DT/DC] \times 100$, where DC and DT are the disease percentages in control and test treatments, respectively (Omar et al. 2006).

Plant height and stem diameter

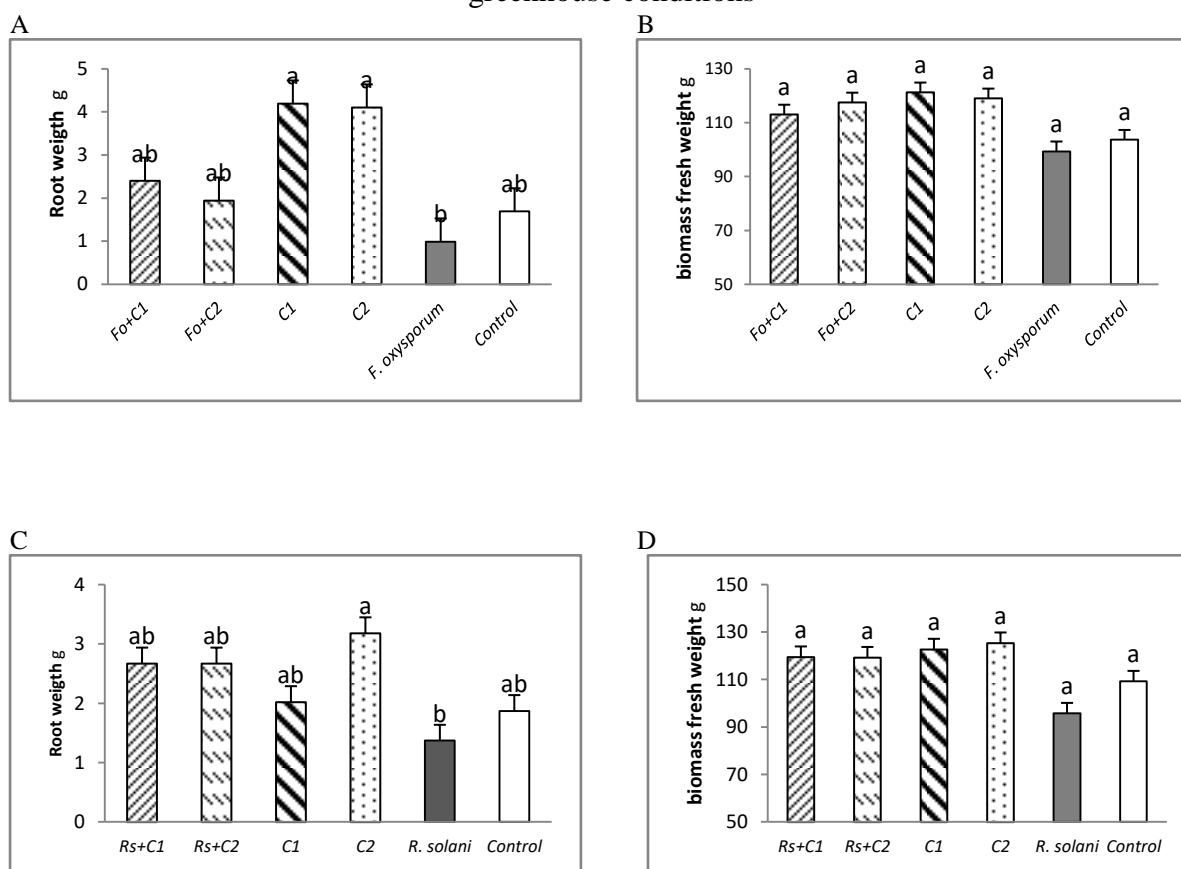
The increase of plant height was significantly different by the effect of bacterial consortia ($P < 0.05$) (Fig. 2), plant height on consortium 2 was increased by 8.22% and 12.28% on the control inoculated with *R. solani* and without inoculating it (Fig. 2A). Also, plants exposed to consortia in presence of pathogens, significantly increased plant height compared to untreated plants (Fig. 2A and Fig. 2C). The non-phytopathogenic consortium 2 showed a larger diameter compared to the infested and noninfested control plants with *R. solani* or *F. oxysporum* (Fig. 2B and Fig. 2D).

Fig. 2. Effect of endophytic bacteria on plant height and stem diameter in potato plants under greenhouse conditions.

Means with the same letter are not significantly different according to the least significant difference (LSD) tested at $P=0.05$. Error bars are standard error of the mean.

Fresh root and biomass weight

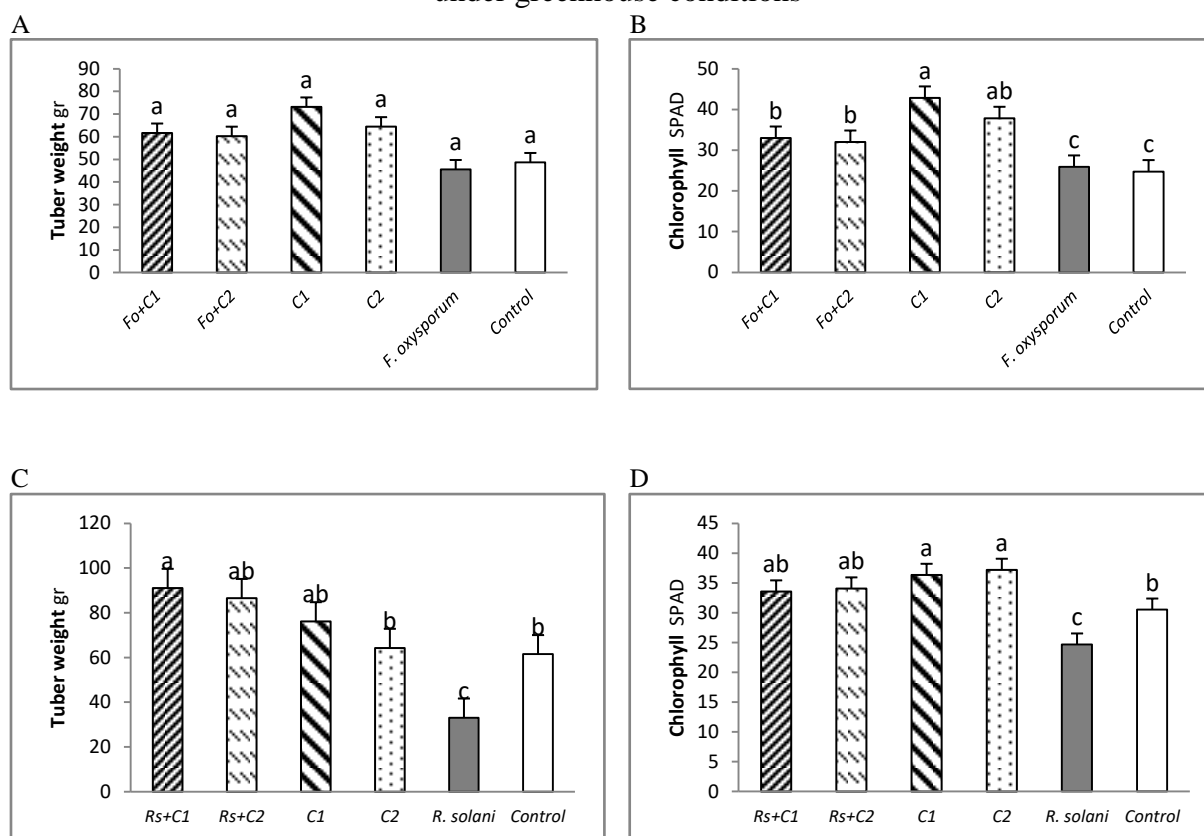
The consortia of endophytic bacteria promoted the highest fresh root and fresh biomass weight (Fig. 3). Plants exposed to consortium 2 without presence of *R. solani* showed maximum increases in fresh root weight by 132.92% over the control inoculated with the pathogen and 70.53% on uninoculated control plants (Fig. 3C). Meanwhile over *F. oxysporum*, the consortia 1 and 2 increased by 148.55% and 143.08% more than the free of any treatment control and from 310.82% to 320.14% compared to the inoculated control plants with *F. oxysporum* (Fig. 3A). No significant differences were found between treatments in biomass fresh weight, however the increase with the consortium 2 was 14.81% more than the plants inoculated with *R. solani* and 30.98% more than the free of any treatment plants (Fig. 3D), while over *F. oxysporum* the consortium 1 stimulated 22.00% and 16.96% more biomass fresh weight, compared to inoculated control plants and uninoculated with *F. oxysporum* (Fig. 3B).

Fig. 3. Effect of endophytic bacteria on root and biomass fresh weight in potato plants, under greenhouse conditions

Means with the same letter are not significantly different according to the least significant difference (LSD) tested at $P=0.05$. Error bars are standard error of the mean.

Tuber weight and chlorophyll content

Bacterial consortia increased the tuber weight in potato plants, in presence or absence of *R. solani* ($P < 0.001$), the values varied from 33.10 g to 91.10 g, the highest average weight was obtained in plants exposed to consortium 1 and in presence of the pathogen compared to the inoculated control with *R. solani*, this shows an increase in yield of 175.23% respectively (Fig. 4C). No significant difference ($P > 0.05$) was observed between the plants exposed to the consortium and *F. oxysporum*; however, inoculation with both consortia showed maximum increases in yield from 32.55% to 50.47% compared to inoculated control plants, while inoculation with consortia 1 and 2 compared to uninoculated control plants was obtained 41.58% and 60.73% more yield (Fig. 4A). Figure 4B and 4D illustrate SPAD units at 110 days after sowing potato plants, grown under greenhouse conditions, in plants inoculated with consortia, SPAD units were increased compared to untreated plants with endophytic bacteria. Specifically, the inoculation with consortium 1 and 2 in absence of pathogens showed the highest chlorophyll content. The inoculated and uninoculated control plants with *F. oxysporum* or *R. solani* showed the lowest chlorophyll contents in potato plants.

Fig. 4. Effect of endophytic bacteria application on tuber weight and chlorophyll content, under greenhouse conditions

Means with the same letter are not significantly different according to the least significant difference (LSD) tested at $P=0.05$. Error bars are standard error of the mean.

DISCUSSION

Development of disease management strategies using antagonistic bacteria is one of the most attractive alternatives to chemical fungicides. Endophytic bacteria are internal colonizers of root systems; therefore, they are able to compete within the vascular systems, inhibiting pathogens for both nutrient and space for their proliferation (Dalal et al. 2014). Species of the genus *Bacillus* are reported to be effective in controlling a wide range of diseases caused by fungi and bacteria; *Bacillus* spp produces secondary metabolites such as antibiotics, volatile and nonvolatile compounds and lytic enzymes (Tolba and Soliman 2013). Endophytic bacteria strains against *F. oxysporum* and *R. solani* had an antagonistic positive effect on the mycelial growth of *F. oxysporum*, but null for *R. solani* (Ji et al. 2014). Our results show strains with null antagonism for both *F. oxysporum* and *R. solani*. Proof of this is that some of the strains of the endophytic bacteria presented antagonism against pathogens at first, but lost their antagonistic activity and pathogens grew on them, only strains 21 and 53 showed favorable antagonistic capacity and were selected for *in plant* evaluation. Among the screened isolates, two antagonistic strains with strong inhibitory activity against *F. oxysporum* and *R. solani* were selected and subsequently identified in the genus *Bacillus*. Strain 21 and 53 showed antagonistic activity against *F. oxysporum* and *R. solani* were non-inhibitory to each other on agar dishes, and this compatibility among the two isolates of endophytic bacteria suggests their potential to be used as a mixture or consortium of isolates for disease management. Strains 21 and 53 in consortium reduced the incidence and severity of the disease, improving plant health. In general, the disease development was least in plants

treated with endophytic bacteria than in the untreated ones. Disease suppression could be due to the induction of the host defense mechanisms, such as the formation of structural barriers like lignified cell walls and production of antifungal metabolites to slow down the infection progress (Aliye et al., 2008). Overall, the selected antagonistic isolates proved to be efficient *in vitro* and significantly reduced the incidence and severity of the disease. In addition, the inoculation with endophytic bacteria yielded significant positive effects on plant growth parameters, including plant height, stem diameter, tuber weight, SPAD levels, biomass and root fresh weight. One of the mechanisms of stimulation of plant growth by bacteria involves the production of phytohormones, such as auxins, gibberellins and cytokinins. Auxins are known to be essential for plant physiology directly affecting the root and shoot architecture (Malfanova et al., 2011). In the inoculated potato plants with *R. solani* and *F. oxysporum* without the application of endophytic bacteria, a smaller tuber weight was obtained in comparison to the others, it is evident that when using these microorganisms we can obtain greater yield in comparison to where they are not applied. On the other hand Bautista et al. (2007) reported that *Pseudomonas fluorescens* increases significantly the number and weight of tubers of *Solanum phureja* in the presence and absence of *R. solani* compared to the control that was not treated.

CONCLUSION

The use of biocontrol agents such as endophytic bacteria as an alternative way to control *Fusarium oxysporum* and *Rhizoctonia solani* is an ideal option, apart from chemical and cultural control methods.

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