UNIVERSIDAD AUTÓNOMA AGRARIA ANTONIO NARRO

SUBDIRECCIÓN DE POSTGRADO

BIOCONTROL DE *Fusarium oxysporum* Y *Rhizoctonia solani* CON BACTERIAS ENDÓFITAS EN PAPA Y SU EFECTO EN LA INDUCCIÓN DE RESISTENCIA Y PROMOCIÓN DE CRECIMIENTO

Tesis

Que presenta EPIFANIO CASTRO DEL ÁNGEL

como requisito parcial para obtener el Grado de
DOCTOR EN CIENCIAS EN PARASITOLOGÍA AGRÍCOLA

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Resumen

BIOCONTROL DE *Fusarium oxysporum* Y *Rhizoctonia solani* CON BACTERIAS ENDÓFITAS EN PAPA Y SU EFECTO EN LA INDUCCIÓN DE RESISTENCIA Y PROMOCIÓN DE CRECIMIENTO

POR

EPIFANIO CASTRO DEL ÁNGEL

DOCTOR EN CIENCIAS
EN PARASITOLOGÍA AGRÍCOLA

UNIVERSIDAD AUTÓNOMA AGRARIA ANTONIO NARRO

DR. FRANCSICO DANIEL HERNÁNDEZ CASTILLO - ASESOR –

Saltillo, Coahuila Junio 2017

Más de doscientas especies de plantas dicotiledóneas y monocotiledóneas pueden ser afectadas por hongos como *Fusarium oxysporum* y *Rhizoctonia solani*; estos patógenos son responsables de pérdidas económicas significativas en los cultivos antes, durante y después de la cosecha. El control biológico es una alternativa adecuada para disminuir el impacto de estas enfermedades en diversos cultivos. Bajo este criterio, las bacterias endofíticas pueden utilizarse para contrarrestar el uso indebido de productos químicos. En esta investigación se obtuvieron 26 aislamientos de bacterias endofíticas y se ensayaron en cuanto a su actividad antagonista por confrontación en cultivo dual contra *F. oxysporum* y *R. solani*. Se seleccionaron dos cepas bacterianas por su fuerte actividad antagónica, las cuales se identificaron como *Bacillus amyloliquefaciens* (cepa 21 y cepa 53), por tal motivo dichas cepas fueron empleadas para formar un consorcio microbiano. El consorcio se aplicó a plantas de papa en tres oportunidades. Las plantas inoculadas se utilizaron para la evaluación del biocontrol, desarrollo agronómico de la planta y para determinar en tejido foliar la actividad de proteína, de fenilalanina amonioliasa y peroxidasa, como factores de inducción de resistencia. Los resultados obtenidos bajo condiciones de invernadero, demostraron que los consorcios bacterianos favorecieron la sanidad de planta en 909% contra *R. solani* y 303% contra *F. oxysporum*, promovieron el crecimiento vegetal, aumentaron el contenido de clorofila, el peso de la biomasa, diámetro del tallo, peso de raíz, así como la altura de planta, también aumentaron el rendimiento en 135% y 275% sobre el testigo, en comparación de donde no fueron aplicados. Finalmente los microorganismos en consorcio mejoraron la activación de vías metabólicas como inducción de resistencia sistémica en plantas de papa, elevando los niveles de proteínas totales, peroxidasa y fenilalanina amonioliasa, bajo condiciones de invernadero.

Palabras clave:

Bacillus amyloliquefaciens, bioconsorcio, inducción de resistencia, promoción de crecimiento y sanidad de planta.

Abstract

Fusarium oxysporum AND *Rhizoctonia solani* BIOCONTROL WITH ENDOPHYIC BACTERIA IN POTATO AND ITS EFFECT ON RESISTANCE INDUCTION AND GROWTH PROMOTION

BY

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More than two hundred species of dicotyledonous and monocotyledonous plants can be affected by fungi such as *Fusarium oxysporum* and *Rhizoctonia solani*; these pathogens are responsible for significant economic losses to crops before, during and after harvest. Biological control is a suitable alternative to reduce the impact of these diseases on several crops. Under this judgment, endophytic bacteria can be used to counteract the misuse of chemicals compounds. In this investigation 26 isolates of endophytic bacteria were obtained and tested for their antagonistic activity by confrontation in dual culture test against *F. oxysporum* and *R. solani*. Two bacterial strains were selected for their strong antagonistic activity, which were identified as *Bacillus amyloliquefaciens* (strain 21 and strain 53), for which reason these strains were used to form a microbial consortium. The consortium was applied to potato plants three times. The inoculated plants were used for the evaluation of biocontrol, agronomic development of the plant and to determine in foliar tissue the activity of protein, phenylalanine ammonia and peroxidase, as resistance induction factors. The results obtained under greenhouse conditions, showed that the bacterial consortium favored plant health in 909% against *R. solani* and 303% against *F. oxysporum*, promoted plant growth, increased chlorophyll content, biomass weight, stem diameter, root weight, as well as plant height, also increased the yield by 135% and 275% over the control, compared to where they were not applied. Finally, the microorganisms in consortium improved the activation of metabolic pathways as induction of systemic resistance in potato plants, raising levels of total proteins, peroxidase and phenylalanine ammonium lyase, under greenhouse conditions.

Keywords: *Bacillus amyloliquefaciens*, consortium, resistance induction, growth promotion and plant health.

INTRODUCCIÓN

El cultivo de la papa tiene un gran número de problemas fitosanitarios como son las enfermedades causadas por hongos como: *Phytophthora infestans* (Claude *et al*., 2016), *Fusarium oxysporum* (Castro *et al*., 2016), *Alternaria solani* (Landschoot *et al.,* 2017), y *Rhizoctonia solani* (Yang *et al*., 2017), entre otras. A medida que la producción agrícola se intensificó durante las últimas décadas, los productores se volvieron cada vez más dependientes de los agroquímicos como un método relativamente fiable para la protección de los cultivos, sin embargo, el uso creciente de insumos químicos causa efectos negativos, como, el desarrollo de resistencia de patógenos a los agentes aplicados y el impacto ambiental que ocasionan (Gerhardson, 2002; Arredondo *et al*., 2017). Además, el costo creciente de los plaguicidas, particularmente en las regiones menos favorecidas del mundo, y la demanda de los consumidores de alimentos libres de plaguicidas ha llevado a la búsqueda de sustitutos de estos productos. El control biológico ofrece una alternativa a los productos químicos, contribuyendo a minimizar las consecuencias negativas para la salud humana y el medio ambiente (Kim *et al*., 2003; O'Brien, 2017). El control biológico puede definirse como una reducción de la cantidad de inóculo o enfermedad producida por la actividad de un patógeno, basada en el uso de enemigos naturales o el uso de compuestos derivados de su metabolismo (Soria *et al*. 2012). Las bacterias asociadas a las plantas residen en la rizósfera (Figueroa *et al*., 2016), la filósfera (Bringel and Couée, 2015) y dentro de los tejidos de las plantas sanas (Taghavi, *et al*., 2009; Martín *et al*., 2015). Se ha reportado que los endófitos contribuyen al crecimiento y sanidad de diferntes plantas a través de mecanismos como competencia, antibiosis (Castro *et al*., 2017) y/o resistencia inducida, son portadores de productos químicos específicos o genes y promotores del crecimiento vegetal, especialmente en la etapa de planta joven para sobrevivir al ataque de los hongos del suelo, basándose en la promoción del crecimiento hormonal o nutricional (Alström and Van Vuurde, 2001; Pageni *et al*., 2014). Dado a lo anterior la parte de investigación se llevó a cabo para probar múltiples bacterias endofíticas como agentes de control biológico contra *Rhizoctonia solani* y *Fusarium oxysporum*.

Objetivo general

Estudiar el efecto de un consorcio de bacterias endófitas, para el control de *Fusarium oxysporum* y *Rhizoctonia solani* así como su respuesta en la activación de mecanismos de defensa y la promoción de crecimiento en plantas de papa, bajo condiciones de invernadero.

Objetivos específicos

Estudiar el antagonismo *in vitro* de bacterias endófitas sobre *Fusarium oxysporum* y *Rhizoctonia solani*.

Determinar la compatibilidad *in vitro* de bacterias endófitas y formar consorcios.

Evaluar el efecto de consorcio para el control de *Fusarium oxysporum* y *Rhizoctonia solani* bajo condiciones de invernadero.

Estudiar el efecto del consorcio en la promoción de crecimiento y desarrollo.

Determinar el efecto del consorcio en la inducción de resistencia.

Hipótesis

La aplicación de bacterias endófitas tendrá efecto en el control de *Fusarium oxysporum* y *Rhizoctonia solani*, además promoverán el crecimiento, activarán los mecanismos de defensa y aumentarán el rendimiento en plantas de papa, bajo condiciones de invernadero.

REVISIÓN DE LITERATURA

1. El Cultivo de la Papa

1.1. Origen y distribución

La papa (*Solanum tuberosum* L.) es originaria de la cordillera de los Andes, en el altiplano andino. Se considera que *S*. *tuberosum* ssp. *andigenum* se originó en el sur de Perú, en los límites de Bolivia a partir del complejo *Solanum brevicaule* y la sección *Etuberosum*, en las tierras bajas de la parte central de Chile (Spooner *et al*., 2005).

En América existen alrededor de 190 especies de papa silvestre, en Sudamérica se localizan a lo largo de los Andes desde Venezuela hasta el noroeste de Argentina y en las tierras bajas de Chile, Argentina, Uruguay, Paraguay y el sureste de Brasil (Spooner *et al*., 2004; Spooner *et al*., 2005).

Los centros de diversificación de las papas silvestres son México, Bolivia y el norte de Argentina. La mayoría de especies crecen en los Andes, y 28 de ellas en México (Spooner *et al*., 2004). La papa se cultiva en alrededor de 20 estados de la república Mexicana durante todo el año, tanto en el ciclo de primavera-verano como otoño-invierno, bajo condiciones de riego o de temporal (Villa y Rodríguez, 2010). Los estados productores en 2016 fueron: Baja California, Baja California Sur, Coahuila, Chiapas, Chihuahua, Distrito Federal, Durango, Guanajuato, Hidalgo, Jalisco, Estado de México, Michoacán, Morelos, Nuevo León, Oaxaca, Puebla, Sinaloa, Sonora, Tamaulipas, Tlaxcala, Veracruz y Zacatecas (SIAP, 2016).

1.2. Importancia económica

La papa forma parte de los primeros cuatro cultivos de mayor importancia en el mundo, después del arroz (*Oriza sativa* L.), el trigo (*Triticum aestivum* L.) y el maíz (*Zea mays* L.) con una producción mundial, de 320 711 961 t, Asia, Oceanía, África y América Latina producen 169 477 301 t. En 2007 la superficie

cultivada con esta especie en 149 países fue de 19 264 021 ha, y los principales productores fueron China (72 000 000 t), la Federación Rusa (35 718 000 t), India (26 280 000 t) y los Estados Unidos (20 000 000 t). El consumo per cápita es mucho mayor en Rusia (142 kg) que en los otros países (34 kg). El rendimiento es fluctuante de una región a otra y puede ser de 11 (países africanos) a 37 (Estados Unidos) t/ha, y un promedio mundial de 16.6 t/ha (FAOSTAT, 2008).

En México en 2016 se sembraron 64,879 ha; más del 60 % se cultivaron bajo condiciones de riego con rendimiento promedio de 31.2 t/ha, y alrededor del 30% corresponde a condiciones de temporal, cuyo rendimiento promedio fue de 16.5 t/ha (SIAP 2016). De la producción total, el 17, 25 y 58 % son destinados para semilla, la industria y consumo en fresco, respectivamente, con 16.2 kg de consumo per cápita (Devaux *et al*., 2006).

1.3. Generalidades de la planta

La papa pertenece a la familia *Solanaceae*. Es una planta suculenta, herbácea y anual. Posee un **tallo** aéreo; que puede ser ramificado hueco y triangular en su sección transversal. Se considera principal, él que crece directamente del tubérculo y a las ramas laterales de éste, se les denomina tallos secundarios. Las **hojas** son alternas al igual que los estolones; consisten en un pecíolo con folíolo terminal; folíolos laterales secundarios y a veces terciarios intersticiales. Las **flores** son pentámeras, de colores diversos, tienen estilo y estigma simples y ovario bilocular, la inflorescencia de la papa es una cima terminal que puede ser simple o compuesta, no todas las variedades provenientes de papa tubérculo y de semilla sexual florecen y forman bayas, en las variedades provenientes de semilla sexual la floración se retarda una o dos semanas más, las flores se autopolinizan en un 98 % y un 2 % es por polinización cruzada. El **fruto** es una baya de color verde, donde se encuentra la verdadera semilla sexual, es de forma redonda y llega a medir hasta 2.5 cm, en el interior del fruto crecen las semillas, unas 200 por baya, el tiempo de maduración de las bayas es de 45 a 60 días después de la floración. Los **tubérculos** comienzan a

formarse a partir de los estolones, que son tallos laterales que crecen dentro del suelo y son emitidos por los tallos principales, cuando la planta comienza la floración (en variedades que florecen), esto ocurre entre los 35 a 45 días después de la siembra, los tubérculos están formados a los 60 días, desarrollándose hasta cuando la planta alcanza su madurez fisiológica: 90 días para variedades precoces; 110 a 120 días para variedades de ciclo intermedio y más de 120 para variedades tardías, (Rubio *et al*., 2000; Molina *et al*., 2014).

2. Enfermedades de la papa

Las enfermedades de la papa son causadas por hongos, bacterias, virus, fitoplasmas, viroides, nematodos y por condiciones abióticas o no infecciosas. Todos los problemas de enfermedades se derivan de la interacción de muchos factores. Entre estos factores se encuentran: (1) El vigor de la planta, o su falta; (2) las condiciones ambientales durante el crecimiento de la planta; (3) las condiciones en la época de cosecha; (4) almacenamiento adecuado de los tubérculos; y (5) la presencia de organismos patógenos, insectos o la aparición de alteraciones fisiológicas (Muriel *et al.*, 1979).

2.1. Generalidades de *Rhizoctonia solani* (teleomorfo: *Thanatephorus cucumeris*).

R. solani es uno de los patógenos fúngicos más importantes del suelo, se desarrolla tanto en suelos cultivados como no cultivados, causando enfermedades en diferentes cultivos como papa, frijol, tomate, entre otros (Montealegre *et al*., 2003; Meza *et al*., 2007; DOF, 2017). Las enfermedades por *Rhizoctonia* inician por semilla o inóculo en el suelo. El hongo sobrevive en forma de esclerocio o micelio en tubérculos infectados, en residuos de plantas o suelo infestado. El mayor daño puede ocurrir a temperatura entre los 10°C y alrededor de los 24°C. Después de la emergencia de los brotes infectados estos pueden morir y como consecuencia, el cultivo tendrá un rendimiento

pobre. La infección de estolones jóvenes puede causar que sus puntas mueran y como consecuencia el número de tubérculos puede decrecer. Este patógeno también puede producirse ramificación múltiple de estolones, lo que conduce a la formación de tubérculos cerca del tallo, dando como resultado tubérculos aéreos. También los tubérculos en el suelo pueden mostrar una gran cantidad de deformación y grietas. La presencia de esclerocios en los tubérculos degrada la calidad de estos, especialmente en la producción de semilla para siembra (Tsror, 2010).

2.2. Síntomas de la enfermedad

2.2.1. Costra negra en tubérculo

La costra negra es el mejor síntoma conocido de *Rhizoctonia* en papa. Se denotan esclerocios oscuros sobre los tuberculos como estructuras costrosas (Johnson and Leach, 2003).

2.2.2. Daño en brotes jóvenes

La penetración subterranea de los brotes en desarrollo causa daños considerables al cultivo, pudiéndose observar lesiones hundidas de color rojizo a gris en las plantas jóvenes. Con la ayuda de una lupa, se pueden ver muchas hifas de color marrón oscuro en las lesiones. Las lesiones pueden ceñir el brote joven completamente, causando que la parte sobre la lesión muera. Como consecuencia, los brotes dejarán de emerger o se marchitarán después de la emergencia (Mulder and Turkensteen, 2005).

2.2.3. Lesiones en tallos y estolones

En la base de los tallos y en los estolones se ven lesiones de color marrón rojizo a marrón. A medida que estas lesiones maduran, se convierten en cancros que son ásperos y marrones y pueden tener cráteres, grietas o ambos. En la base del tallo y en las partes de la planta que están en contacto con el suelo puede formarse una lámina de hongo blanco (Van den Brink and Wustman, 2014).

2.2.4. Tubérculos aereos

La interferencia del movimiento de carbohidratos también puede causar la formación de tubérculos aéreos en las axilas de las hojas, con unas cuantas hojas pequeñas en la parte superior. Los tubérculos aéreos no sólo son causados por *Rhizoctonia*, sino que también pueden ser el resultado de daños en el tallo causados por plagas, maquinaria, viento, tizón tardío, pudrición rosada y fitoplasmas.

2.3. Descripción de *Rhizoctonia solani*

Rhizoctonia forma un micelio estéril y desarrolla pequeños esclerocios que no muestran la diferenciación del tejido interno. Las células miceliales de las especies más importantes de *R. solani*, contienen varios núcleos (*Rhizoctonia* multinucleada), mientras que las células miceliales de otras especies contienen dos núcleos (*Rhizoctonia* binucleada). El micelio, es incoloro cuando joven, pero se vuelve amarillento o de color marrón claro con la edad, se compone de células alargadas y produce ramificaciones que crecen en ángulos aproximadamente rectos a la hifa principal, están ligeramente constreñidos en la unión, y tienen una pared transversal cerca de la unión. Esta característica es considerada básica para la identificación de *Rhizoctonia*. Bajo ciertas condiciones, el hongo produce esclerocios, de células cortas y anchas que funcionan como propágulos, son de color marrón a negro y son comunes en algunos huéspedes como la papa (Agrios, 2005).

2.3.1. Grupos de anastomosis

Los aislamientos de *Rhizoctonia* se clasifican en base a la reacción de anastomosis hifal, la cual es una respuesta de incompatibilidad vegetativa entre

las cepas confrontadas (Carling and Leiner 1990; Carling *et al*., 2002a; Carling *et al*., 2002b). Actualmente se consideran cuatro categorías: C0 indica que los aislamientos confrontados pertenecen a diferentes grupos de anastomosis (GA) y carecen de una relación vegetativa; C1, representa una relación distante, que puede ser encontrada dentro de GA altamente heterogéneos; la categoría C2 comprende aislamientos relacionados que corresponden al mismo grupo anastomósico, pero a diferentes poblaciones vegetativamente compatibles; y la categoría C3 incluye una relación muy cercana, las cepas pertenecen al mismo GA y a la misma población vegetativamente compatible, siendo una posibilidad que se trate del mismo individuo (Carling 1996). Estos estudios han permitido diferenciar grupos de anastomosis (GA) definidos sobre la base de reacción de anastomosis hifal. Actualmente se han descrito 14 grupos que son: GA-1, GA-2, GA-3, GA-4, GA-5, GA-6, GA-7, GA-8, GA-9, GA-10, GA-11, GA-12, GA-13, y GA-B1 (Hernández *et al*., 2001; Carling *et al*., 2002b; Hernández *et al*., 2005; Chávez *et al*., 2011).

Aunque los diversos grupos de anastomosis no son del todo específicos del hospedero, muestran ciertas tendencias bastante bien definidas, por ejemplo; los aislados de GA-1 causan pudrición de semilla y de hipocotilo y de tizones de muchas especies de plantas; los aislados de GA-2 causan cancro en raíces de los cultivos, y la enfermedad de dólar en céspedes; los aislamientos de GA-3 afectan principalmente a papa, causando cancros al tallo y lesiones a los estolones y produce esclerocios negros en los tubérculos; los aislados de GA-4 infectan a una amplia variedad de especies de plantas, causando pudrición de la semilla y del hipocotilo en casi todas las angiospermas y lesiones de tallo cerca de la línea del suelo en la mayoría de las leguminosas, algodón, remolacha azucarera y papa. Otros seis grupos de anastomosis son conocidos dentro de *R. solani* y hay muchos más en otros en *Rhizoctonia* (Agrios, 2005).

2.4. **Generalidades de *Fusarium oxysporum***

El género *Fusarium* fue introducido por Link en 1809, y ahora se acerca a su tercer siglo como un género que una gran cantidad de plantas. Los miembros de este género pueden incitar directamente enfermedades en plantas, humanos y animales domesticados (Goldschmied *et al*., 1993; Krcmery *et al*., 1993; Rabodonirina *et al*., 1994; Boonpasart *et al*., 2002). *F. oxysporum* es el más disperso de las especies de *Fusarium* y puede ser recuperado de la mayoría de los suelos árticos (Kommedahl *et al*., 1988), tropicales o desérticos (Simpson *et al*., 2004) y cultivados o no cultivados (Hu *et al*., 2015). También puede ser dispersado por insectos (SENASICA, 2016) y recuperado de algas marinas (Granchinho *et al*., 2002). *F. oxysporum* también es sin duda la especie económicamente más importante en el género *Fusarium* dado sus numerosos huéspedes y el nivel de pérdida que puede producirse cuando infecta una planta. Incluye muchas especies que son patógenos para las plantas que a menudo causan síntomas de marchitez vascular, (García *et al*., 2011), problemas de secadera, y las pudriciones de corona y raíz. Puede ser dispersado por muchos medios diferentes, incluyendo el viento y en el suelo, las semillas o el material de siembra infectado (Garibaldi *et al*., 2004).

2.5. Descripción de *F. oxysporum*

Los macroconidios se forman en esporodoquios naranja pálido, generalmente abundantes, son de corta a mediana longitud, de forma falcada a casi recta, de paredes finas y usualmente triseptados. La célula apical es corta y ligeramente ensanchada en algunos aislamientos. La célula basal tiene muescas o forma de pie. Los macroconidios se forman a partir de monofialides en conidióforos ramificados en esporodoquios y en menor grado en monofialides de hifas. Los microconidios usualmente son sin septos, pueden ser ovales, elípticos o reniformes (en forma de riñón), y se forman abundantemente en cabezas falsas de monofialides cortos. Las clamidosporas se forman abundantemente en hifas en la superficie del agar por la mayoría de los aislamientos, especialmente los clones saprofíticos del suelo, pero pueden ser lentas (4-6 semanas) en algunos

aislamientos (Leslie and Summerell, 2006).

2.6. Síntomas de la enfermedad

2.6.1 Marchitez

La marchitez de la papa es causada por el crecimiento de organismos en los tejidos del tallo y las raíces de la planta. Esto da como resultado un taponamiento de los vasos conductores de agua, la producción de sustancias toxicas o la destrucción de los tejidos hasta el punto de que no se pueden realizar funciones normales. Bajo tales circunstancias las hojas pierden su color, se marchitan, y la planta entera finalmente muere. Se han encontrado varios organismos diferentes que producen el marchitamiento de la papa. Los principales organismos productores de marchitez son *Verticillium albo-atrum*, *F. oxysporum y F. eumartii* (Eljounaidi *et al*., 2016). Los síntomas de marchitez de *Fusarium* son similares en todas las plantas y dependen de varios factores, incluyendo la cantidad de inóculo en el suelo, las condiciones ambientales, los nutrientes (particularmente el nitrógeno) y la susceptibilidad del huésped. El síntoma se caracteriza por la pérdida de presión de turgencia de las nervaduras. Los hases pueden recuperarse durante la noche, pero finalmente se marchitan permanentemente. Los síntomas iniciales a menudo incluyen una apariencia verde grisácea de las hojas que precede a una pérdida de presión de turgencia y el marchitamiento. El marchitamiento es seguido por un amarillamiento de las hojas y finalmente la necrosis. El marchitamiento comienza generalmente con las hojas más viejas y progresa al follaje más joven. Bajo condiciones de densidad de inóculo suficientemente alta o un huésped muy susceptible, toda la planta puede marchitarse y morir en un corto tiempo. Bajo alta presión de inóculo, las plántulas pueden secarse cuando emergen del suelo (Egel and Martyn, 2007). Los tubérculos afectados por el marchitamiento a menudo, aunque con menos frecuencia, muestran un pardeamiento de los vasos de xilema. Estos tejidos descoloridos muestran como líneas amarillas a pardas o negras, o un anillo que se extiende por distancias variables en el tubérculo en el extremo del tallo desde el punto de

fijación del estolón. La decoloración se limita a los tejidos vasculares, que cuando son normales aparecen como una capa débil situada alrededor de 0.6mM debajo de la epidermis.

2.6.2 Pudrición seca

La enfermedad, es causada por varias especies de *Fusarium* como *F. solani* var. *coeruleum*, *F. sambucinum*, *F. oxysporum*, *F. avenaceum* y *F. culmorum*, dando como resultado pérdidas significativas de rendimiento. Los síntomas iniciales de pudrición seca aparecen en el tubérculo en los sitios de la herida como pequeñas lesiones marrones poco profundas después de aproximadamente un mes de almacenamiento. Las lesiones se agrandan en todas las direcciones y el peridermo eventualmente se hunde y puede arrugarse en anillos concéntricos a medida que el tejido muerto subyacente se seca (Bojanowski *et al*., 2013; Mejdoub *et al*. 2015).

2.7. Formas especiales

Los aislamientos parecen ser de un hospedero específico, el cual ha resultado en la subdivisión de las especies dentro de formas especiales y razas que representan la especialización fitopatogénica. Alrededor de 150 formas especiales y razas de *F. oxysporum* han sido descritas (Booth, 1971; Baayen *et al*., 2000; O'Donnell *et al*., 2009).

3. Control biológico de enfermedades

El control biológico puede definirse como una reducción de la cantidad de inóculo o enfermedad producida por la actividad de un patógeno, basada en el uso de enemigos naturales o el uso de compuestos derivados de su metabolismo (Soria *et al*., 2012; Harding and Raizada, 2015; Sundin *et al*., 2016). Ofrece una alternativa a los productos químicos, contribuyendo a

minimizar las consecuencias negativas para la salud humana y el medio ambiente (O'Brien, 2017). Los agentes del control biológico son frecuentemente probados, desarrollados y usados como un esfuerzo para el control de varios fitopatógenos del suelo (Yobo *et al*., 2010). Una de la más seria desventaja es que en muchos casos el control ofrecido no es equivalente al control químico (Alamri *et al*., 2012).

3.2 Bacterias endófitas

Las bacterias endofíticas pueden definirse como aquellas que se pueden aislar de tejidos vegetales sanos y superficialmente desinfectados sin causar ningún daño a la planta huésped (Companant *et al*., 2005); son capaces de penetrar y diseminarse sistémicamente en la planta huésped, colonizando activamente los vasos conductores, y ocasionalmente los espacios intracelulares. Esta colonización presenta un nicho ecológico, similar al ocupado por los patógenos vegetales, y estas bacterias endofíticas pueden actuar como agentes de control biológico contra patógenos (Dai *et al*., 2016).

3.2.1. Beneficios de las bacterias endófitas

No se ha resuelto si las plantas se benefician más de un endófito que de una bacteria de la rizósfera o si es más ventajoso para que las bacterias se conviertan en endofíticas en comparación con las rizosféricas. Aún no está claro qué población de microorganismos (endófitos o bacterias rizosféricas) promueve el crecimiento de las plantas; Sin embargo, los beneficios conferidos por los endófitos son bien reconocidos (Rosenblueth and Martínez, 2006). La diversidad de endófitos bacterianos garantiza que hay endófitos capaces de formar una asociación compatible con todas las plantas importantes desde el punto de vista agronómico, incluidas las monocotiledóneas y las dicotiledóneas (Bacon and Hiton, 2007).

3.2.2. Promoción de crecimiento y desarrollo

El efecto de los endófitos sobre el crecimiento y desarrollo vegetal se debe a mecanismos de acción directos o indirectos. Entre los mecanismos directos se encuentra la producción de hormonas como auxinas, giberelinas, citoquininas y etileno. La producción de ácidos orgánicos, la fijación de nitrógeno, la solubilización de fosfato y otros nutrientes y la movilización de los mismos (Wall, 2001; Sevilla *et al.,* 2001; Hurek *et al*., 2002, Iniguez *et al*., 2004; Khalifa *et al*., 2015). La elucidación de los mecanismos que promueven el crecimiento de las plantas ayudará a favorecer las especies y las condiciones que conducen a mayores beneficios para las plantas. Las sustancias volátiles como el 2-3 butanodiol y la aceotina producidas por bacterias parecen ser un mecanismo responsable de la promoción del crecimiento de las plantas (Ryu *et al*., 2003). Los endófitos producen ribosidos de adenina que estimulan el crecimiento y mitigan el pardeamiento de los tejidos de pino (Pirttilä *et al*., 2004). La producción de fitohormonas es considerada uno de los mecanismos más importantes para la promoción del crecimiento vegetal, estos compuestos orgánicos regulan el crecimiento y desarrollo en plantas, y en bajas concentraciones influencian procesos bioquímicos, fisiológicos y morfológicos. El AIA (ácido 3-indol acético) es la fitohormona más común, mejor caracterizada y la auxina fisiológicamente más activa en las plantas (Soler *et al*., 2012), el AIA es el responsable de la división, expansión y diferenciación de las células y tejidos de las plantas y estimula la elongación de las raíces (Martínez *et al*., 2010; Rojas *et al*., 2016). Liaqat and Eltem (2016) reportan endófitos con potencial de promoción de crecimiento de las plantas de durazno y peral de acuerdo con la producción de ácido indolacético (IAA), fijación de nitrógeno, solubilización de fosfatos y producción de sideróforos. Los aislamientos endofíticos mostraron resultados positivos para las pruebas anteriores. Sobre la base de estas competencias promotoras del crecimiento, se puede presumir que los endófitos aislados tienen una influencia significativa en el crecimiento de las plantas huésped.

3.2.3. Biocontrol

Para considerar un microorganismo endófito como potencial agente para control biológico se requieren ciertas características. En primer lugar, que no sea patógeno para los vegetales, el hombre o animales. Debe tener una elevada capacidad de colonización y reproducción en los tejidos internos después de su inoculación en las plantas, ya que una población que declina rápidamente tiene una baja capacidad competitiva con la microflora presente en los cultivos. También es muy importante que tenga la capacidad de reproducirse abundantemente en condiciones *in vitro* para asegurar su reproducción y conservación (Hernández y Escalona, 2003). El control biológico de fitopatógenos en la zona de raíces es el resultado de la producción de antifúngicos o agentes antibacterianos, sideróforos, la competencia de nutrientes (Sturz *et al*., 2000) y la inducción de la resistencia sistemática adquirida del huésped (González *et al*., 2015), o inmunidad, aumentando la disponibilidad de minerales (Sessitsch *et al*., 2002). En este sentido, se ha demostrado la supresión de enfermedades de las plantas debido a la acción de microorganismos endofíticos en varios patosistemas. El microbioma bacteriano endofítico promueve el crecimiento de las plantas y la sanidad; los efectos benéficos son en muchos casos mediados y caracterizados por interacciones metabólicas. Se ha estudiado la producción de metabolitos por microbiontes de plantas, lo que demuestra que pueden producir una gama de diferentes tipos de metabolitos que desempeñan un papel en la defensa y la competencia, pero también pueden ser necesarias para la interacción específica y la comunicación con el huésped de la planta (Brader *et al*., 2014). Varios mecanismos pueden controlar esta supresión, ya sea directamente sobre el patógeno dentro de la planta por antibiosis y la competencia por los nutrientes (Franco *et al*., 2006), o indirectamente por la inducción de la resistencia a las plantas (Hao, *et al*., 2017). Las bacterias en las raíces y en la rizósfera se benefician de exudados radiculares, pero algunas bacterias y hongos son capaces de entrar en la planta

como endófitos sin causar daño y podrían establecer una asociación mutualista (Azevedo *et al*., 2000). Las plantas constituyen nichos vastos y diversos para los organismos endofíticos. Las bacterias endofíticas han sido aisladas de una gran diversidad de plantas según lo revisado por Sturz *et al*. (2000). En general, las bacterias endofíticas se producen a densidades de población más bajas que las bacterias rizosféricas o patógenos bacterianos (Hallmann *et al*., 1997; Rosenblueth and Martínez, 2004).

3.1.2 Antagonismo

El nicho endofítico ofrece una oportunidad única para el control de patógenos. El endófito está protegido dentro de la planta y se multiplica en los espacios intercelulares cuando la planta crece. Las cepas de bacterias endófitas aisladas de plantas de vainilla inhibieron el crecimiento de *F. oxysporum* f. sp*. vanillae* mediante antibiosis y por competencia por espacio y de nutrientes (Jiménez *et al*., 2015). Por otra parte Berg *et al*. (2005) encontraron que a pesar de un número amplio de aislamientos correspondientes de la rizósfera, la filósfera, la endosfera y la endorhiza de papa, sólo un aislamiento de la endorhiza identificado como *Serratia plymuthica*, fue antagonista fúngico eficaz contra *Verticillium dahliae* y *Rhizoctonia solani*.

Del mismo modo Coomb *et al*. (2004) aislaron actinobacterias endofíticas que resultaron ser biocontroladoras efectivas contra *Gaeumannomyces graminis*, *R. solani* y *Pythium* spp en trigo, tanto *in vitro* como *in vivo*. Los aislamientos fueron ubicados en los géneros *Streptomyces*, *Microbispora*, *Micromonospora* y *Nocardioidies*. También Abla *et al*. (2015) ensayaron aislamientos de bacterias endofíticas de *Mentha rotundifolia* L. contra *F*. *oxysporum*, *Aspergillus niger* y *Botrytis cinerea*, de las cepas endofíticas ocho fueron ubicadas en la familia Bacillaceae, dos correspondieron a la familia Pseudomonadaceae. Por otra parte Liu *et al.* (2016) a través de ensayos antagónicos de *Paenibacillus* sp. aislada de semillas de maíz demostró buena actividad antagonista contra los patógenos *F*. *graminearum*, *Bipolaris maydis*, *B*. *sorokiniana*, *Cochliobolus*

heterostrophus, *Aspergillus aculeatus*, *Phomopsis chimonanthi* y *V*. *dahliae*. En otro estudio Achari and Ramesh (2014) aislaron 167 bacterias del xilema de berenjenas sanas, de chile y de *Solanum torvum* Sw, del total de los aislamientos 28 cepas inhibieron el crecimiento de *Ralstonia solanacearum*, mediante producción de compuestos antagónicos volátiles y difusibles y sustancias promotoras del crecimiento de las plantas *in vitro*, estos aislamientos fueron identificados como *Staphylococcus* sp., *Bacillus* sp., *Streptomyces* sp., *Enterobacte*r sp., y *Agrobacterium* sp., además registraron una eficacia de biocontrol superior al 85% frente a la marchitez bacteriana. Vale la pena considerar que la mayoría de los ensayos para probar el antagonismo son *in vitro* y queda por establecer si esto se correlaciona con los efectos en la naturaleza. Por otra parte, estudios realizados por Müller *et al*. (2015) sobre endosfera de hoja de olivo encontraron que se albergan en su mayoría cepas de Proteobacteria, Firmicutes, Actinobacteria y Bacteroidetes, también determinaron el potencial antagonista de los endófitos cultivables; pertenecientes a *Bacillus amyloliquefaciens* con actividad antagonista frente a *Verticillium dahliae* Kleb causante de la marchitez en olivo

3.1.3. Inducción de resistencia

Las plantas establecen diferentes mecanismos de respuesta de defensa, incluyendo barreras físicas como la cutícula y la pared celular, entre otros, así como defensas químicas como la secreción de compuestos antimicrobianos (Hurek and Hurek 2011). Estudios sobre inducción de resistencia sistémica por su siglas en ingles (ISR), se han aplicado para suprimir enfermedades de las plantas en invernadero y en campo contra varios patógenos de plantas, incluyendo virus, hongos, bacterias y nematodos (Murphy, 2006; Kang *et al*., 2007). Sin embargo *Bacillus pumilus* INR7 aislado de tallo de pepino redujo significativamente la severidad de la mancha angular, la marchitez y la infestación de escarabajos en pepino mediante tratamiento de semilla o de plántula, también fue eficaz contra el virus del mosaico del pepino (CMV),

Sclerotium rolfsii, *Ralstonia solanacearum*, *Colletotrichum gloeosporoides* y *R*. *solani* en pimiento y tomate, de otra forma también redujo la incidencia de roya fusiforme causada por *Cronartium quercuum* f. sp. *fusiforme*, sobre pino (Enebak and Carey, 2000; Zehnder *et al*., 2001; Murphy *et al*., 2003). Otro estudio llevado a cabo bajo condiciones de invernadero en Tailandia, con especies endofíticas de *Bacillus* spp. se demostró que el ISR se obtuvo en varios cultivos, incluyendo una variedad local de pimiento (Jetiyanon *et al*., 2003). La mezcla de especies múltiples o los tratamientos de una sola especie de bacterias endofíticas formadoras de esporas provocaron ISR en el patosistema *Colletotrichum gloeosporoides* - pimiento (*Capsicum annuum* var. a*cuminatum*) (Jetiyanon and Kloepper, 2002). Por su parte Su *et al*. (2013) investigaron la capacidad promotora de ISR con *B. pumilus* INR7 de naturaleza endofítica, contra patógenos foliares y de suelo, incluyendo *Ralstonia solanacearum* y *Xanthomonas axonopodis* pv. *vesicatoria*, respectivamente, encontrando resultados favorables. Singh *et al*. (2013) demostraron que la deposición alta y uniforme de lignina en las células cambiales de garbanzo durante la infección de *Sclerotium rolfsii* se enfatiza cuando las plantas fueron tratadas con un consorcio microbiano triple (*Pseudomonas aeruginosa* PHU094, *Trichoderma harzianum* THU0816, *Mesorhizobium* RL091) en comparación con aplicaciones individuales microbianas. Además, las células del floema también representaron mejoras y más amplias lignificaciones en la capa de esclerénquima en las plantas tratadas con el consorcio de los tres microorganismos. El consorcio microbiano también aumentó la expresión de la primera enzima de la vía fenilpropanoide, la fenilalanina amonioliasa (PAL) que finalmente llevó a una mayor acumulación de compuestos fenólicos, siendo este paso importante para la lignificación. Del mismo modo, estos microorganismos normalmente aumentan las actividades antioxidantes relacionadas con las respuestas ISR y protegen las plantas de varios patógenos. Jetiyanon, (2007) determinó otros compuestos fenólicos como el ácido t-clorogénico, el ácido shikímico, la miricetina, el ácido ferúlico, el ácido jerárquico y la quercetina, los que se acumularon en mayor cantidad en las hojas de plantas de garbanzo

tratadas con el consorcio en comparación a las no tratadas. Se observó que las respuestas ISR estaban directamente relacionadas con la reprogramación y la movilización de las enzimas involucradas en la defensa del huésped, tales como Proteínas (PR), finilalanina amoniolisa (PAL), peroxidasas (PO), superóxido dismutasa (SOD) y polifenol oxidasa (PPO). La proteína reguladora NPR1 (inexpresor de la proteína del gen 1 relacionada con la patogénesis) es crucial para la transducción de la señal SA (ácido salicílico) y funciona como coactivador transcripcional de la expresión génica relacionada con la PR (patogénesis relacionada). Se ha demostrado previamente que las quitinasas y las b-1, 3 glucanasas (ambas proteínas PR) de diferentes plantas restringen sinérgicamente los patógenos fúngicos (Wu *et al*., 2012). *Pseudomonas fluorescens* PICF7, endófito nativo de raíz de olivo y eficaz agente de biocontrol contra el marchitamiento del olivo por *Verticillium*, fue capaz de desencadenar una amplia gama de respuestas de defensa en los tejidos radiculares de esta planta. La inducción de los genes del olivo potencialmente codificantes para la lipoxigenasa (LOX-2) , catalasa (CAT), 1-aminociclopropano-1-carboxilato oxidasa (ACO) y fenilananina amonioliasa (PAL) fueron detectados y se confirmó así que la colonización de las raíces por esta bacteria endofítica no sólo desencadena respuestas de defensa en este órgano, sino que también monta una amplia gama de respuestas de defensa sistémica en tejidos aéreos (tallos, hojas); se concluyó que la bacteria endofítica *P*. *fluorescens* PICF7 induce respuestas de defensa sistémica en tejidos distantes tras la colonización de las raíces de olivo (Gómez *et al*., 2014).

ENDOPHYTIC BACTERIA CONTROLLING *Fusarium oxysporum* AND *Rhizoctonia solani* IN *Solanum tuberosum*

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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 phytoremediaton 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 50μL from a serial dilution up to 10-6 were plated on NA medium in triplicate. Petri dishes were incubated at 27 ±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±2 °C for 14 days. Each bacteria reaction mixture (20µL) contained 0.2µL of Taq DNA polymerase (1U/µL), 2µL of 10x PCR Buffer + $MgCl_2$, 0.32 of $MgCl_2$ (25mmol/L), 0.5µL of DMSO, 0.4µL dNTPs (10 mmol/L), 0.5µL (10µmol/L) of each primer, primers ITS1 (KIO Fw 3'-TAGAGGAAGTAAAAGTCGTAA-5') and ITS4 (KIO Rv 5'-TCCTCCGCTTWTTGWTWTGC-3'), 13.58µL of Milli-Q water and 2µL of template DNA at 40ng/µL. 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'-CTACGGRTACCTTGTTACGAC-3') primers. Each reaction mixture (20µL) contained 0.2µL of Taq DNA polymerase (1U/µL), 2µL of 10x PCR Buffer QIA, 0.5µL of DMSO, 0.4µL dNTPs (10 mmol/L), 0.8µL (5 µmol/L) of each primer, 14.3µL of Milli-Q water and 1µL of template DNA at 20ng/µL. 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®ExpinTM 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±2 ºC.

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 ± 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 $1x10^6$espores/mL and $1x10^8$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 $1x10^6$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 ± 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. Minitubers 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 $1x10^6$ 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 $1x10^6$ spores/mL, (FoC2, RsC2) Pathogen + consortium 2 $1x10^8$ spores/mL, (BC1) consortium 1 $1x10^6$ spores/mL, (BC2) consortium 2 $1x10^8$ 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*.

	Radial growth inhibition percentage (%)	
Strain	***Rhizoctonia solani***	***Fusarium oxysporum***
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

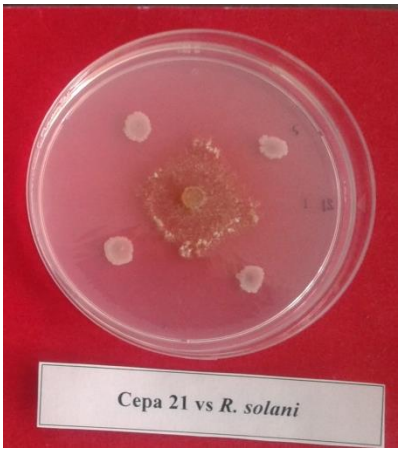
Strain 38	29.36 i	0. 00 h
Strain 41	28.75 j	0. 00 h
Strain 45	8.13 q	0. 00 h
Strain 47	2.50 s	0. 00 h
Strain 50	26.25 m	0. 00 h
Strain 52	35.00 d	28.75 d
Strain 53	72.71 b**	57.30 a**
Strain 54	36.25 c	0. 00 h
Strain 55	25.00 n	31.13 c
Strain 56	26.88 l	0. 00 h
Strain 57	25.00 n	18.75 f
Strain 59	26.88 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

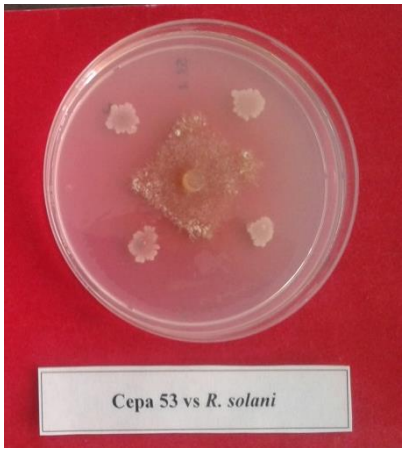
Note: Means with the same letter in the same column are not significantly different according to the least significant difference (LSD) tested at *P*=0.05. ****** Strains with better antagonistic capacity.

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.

A	B

C D





Biocontrol activity *in plant*

Results showed that the bacterial consortia reduce significantly the incidence and severity of the disease (P≤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

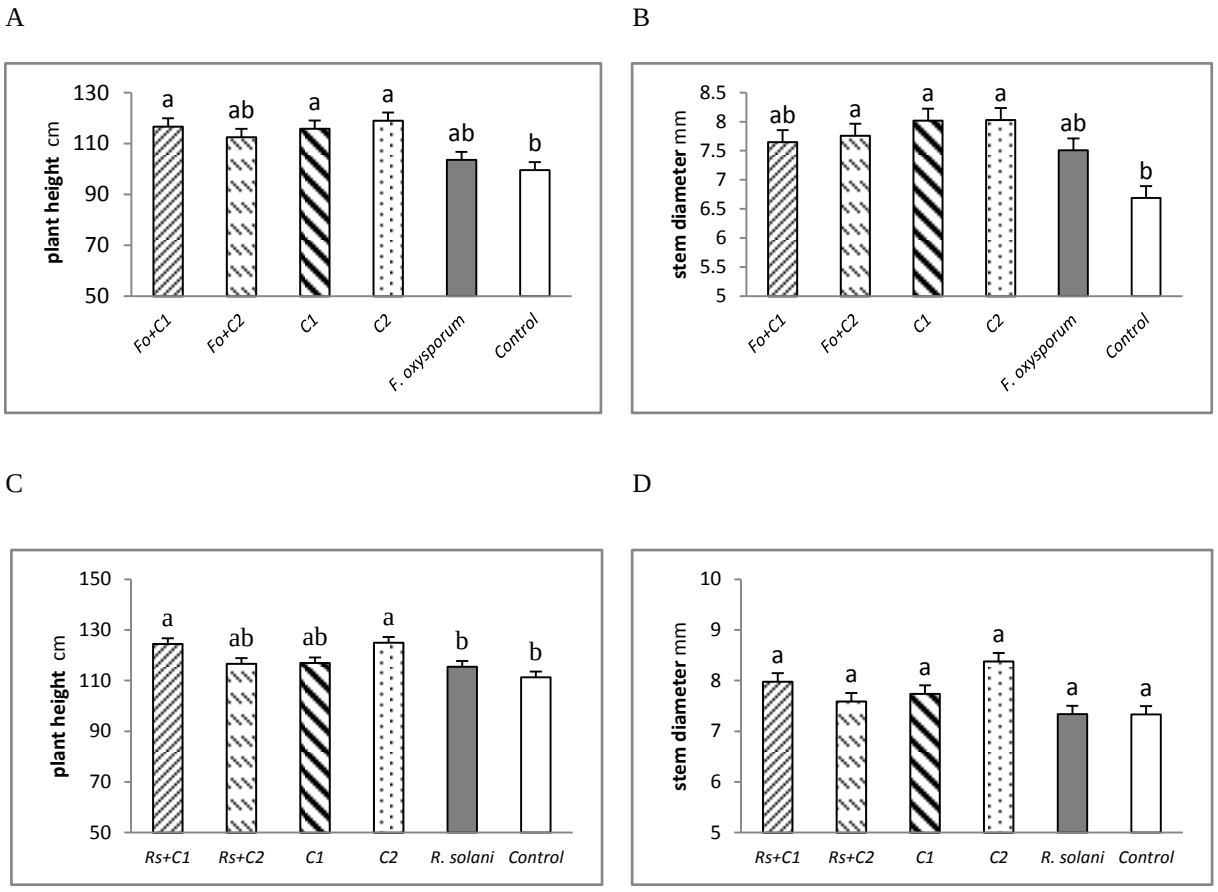
Rs+C1	50.00±4.6 ab	50.0	0.33±0.2 c	90.1
Rs+C2	33.33±18.3 b	66.7	0.50±0.0 c	85.0
Fo+C1	33.33±18.3 b	66.7	0.33±0.2 c	75.2
Fo+C2	33.33±18.3 b	66.7	0.33±0.2 c	75.2
C1	0.00±0.00 b	100.0	0.00±0.0 c	100.0
C2	0.00±0.00 b	100.0	0.00±0.0 c	100.0
Rhizoctonia solani	100.00±0.0 a	----	3.33±0.5 a	----
Fusarium oxysporum	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. ± are mean standard deviation. ---- Used to compare. Disease reduction (DR) was calculated using the following equation: DR = [1 − DT/DC] x 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.



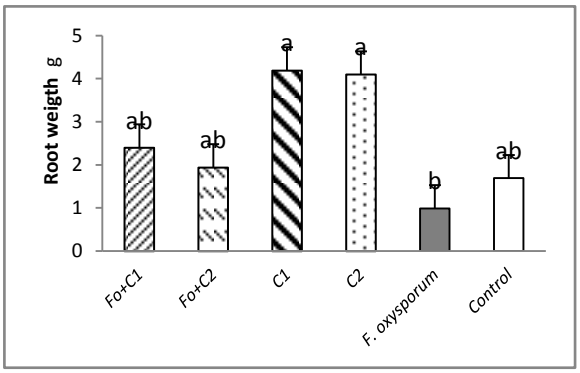
Means with the same letter are not significantly different according to the least significant difference (LSD) tested at *P*=0.05. Error bars are standard error of the mean.

Fresh root and biomass weight

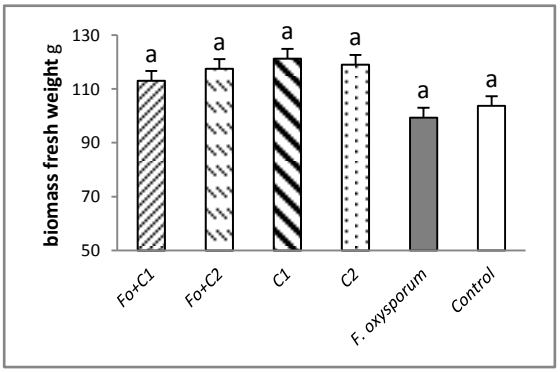
The consortia of endophytic bacteria promoted the highest fresh root and fresh biomass weight (Fig. 3). Plants exposed to consortium 2 without presence of *R. solani* showed maximum increases in fresh root weight by 132.92% over the control inoculated with the pathogen and 70.53% on uninoculated control 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.

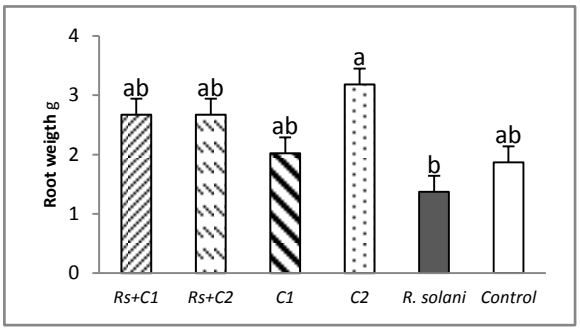
A



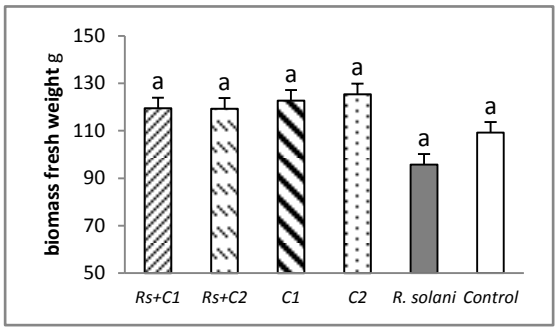
B



C



D

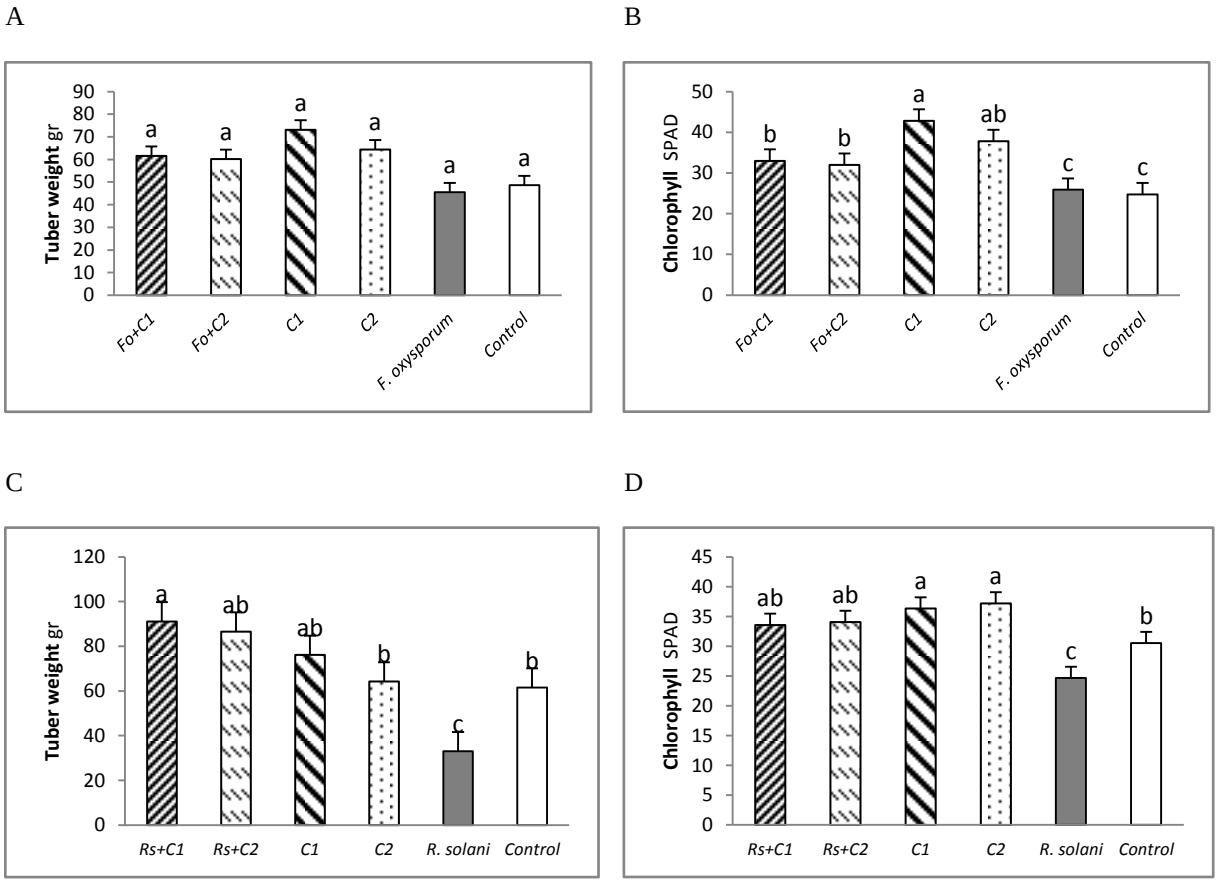


Means with the same letter are not significantly different according to the least significant difference (LSD) tested at *P*=0.05. Error bars are standard error of the mean.

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 whit *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 generus *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 whit *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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SYSTEMIC RESISTANCE INDUCTION BY PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) IN *Solanum tuberosum*

Artículo enviado a la revista *Indian Journal of Experimental Biology*

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Abstract

Currently many studies have been made on the use of natural products as substitutes for chemical control, due to possible ecological and economic advantages that many of these compounds offer, especially in crop protection. Many natural products have compounds with the ability to stimulate defense mechanisms in plants; defense reactions to the nearest tissue infection are restricted in some cases, resistance induction is associated to the expression of some defense genes, such as those coding for proteins linked to the pathogenesis. For example phenylalanine ammonia lyase and synthesis phytoalexins are highly toxic compounds to the pathogen. On the other hand the increase of peroxidases, can increase the mechanical strength of the host cells walls, inhibiting or possibly restricting a pathogen invasion. The objective of this study was to compare the effect of the PGPR consortium at 6, 12 and 24 hours after application, against *Fusarium oxysporum* and *Rhizoctonia solani*, determined by leaf tissue, quantification of protein and enzyme activities of phenylalanine ammonia lyase and peroxidase. The results presented in this paper clearly demonstrated the potential of the PGPR consortium strains to activate resistance against *F*. *oxysporum* or *R*. *solani*.

KEYWORDS*:* *Bacillus* spp. *Fusarium oxysporum, Rhizoctonia solani, Potato, Systemic resistance.*

Introduction

Solanum tuberosum (potato) crop it's known to be the most important humans source of food, the worldwide production comes to 326 million tons per year; this crop was designated by the United Nations (UN) in 2008 as a suitable food to combat world hunger (Anonymous, 2008). Potato crop requires more fungicides than any other crop in Mexico to prevent and to control several diseases caused by genres like *Verticillium, Fusarium, Colletotrichum* and *Rhizoctonia* generating losses among 7 to 64% (Hernández et al. 2008). One of the fungi that limit the production of potato in Mexico is *Rhizoctonia solani*, it produces serious damage to yield and tuber quality (Hernández et al. 2001); on the other hand dry rot of potatoes is caused by several species of *Fusarium* including *F. oxysporum* and *F. culmorum*, crop losses are attributed to dry rot and it have been estimated an average of 6% with reported losses up to 25% (Eken et al. 2000). The potato dry rot and black scurf can be controlled by a combination of storage technologies, physical methods and chemicals applications. An effective control has been obtained with the fungicide fenpiclonil and the mixture of thiabendazole and imazalil (Recep et al. 2009). Besides the high costs of chemical products and they have caused many problems to the environment, such as toxicity, in addition to resistance in certain pathogens (Hernández et al. 2005). In recent years, there has been an increase in studies related to the use of natural products instead of chemical fungicides, since they have offered ecological and economic advantages and also crop protection. Among natural elicitors products are compounds that are able to stimulate

defense mechanisms in plants (Rodríguez et al. 2005), these have promoted the consideration of biological disease control by using nonpathogenic plant-associated microorganism (Tjamos et al. 2005). A great number of reports indicated that certain bacterial strains are beneficial for plants growth, these are called Plant Growth Promoting Rhizobacteria (PGPR), root colonization o with PGPR can also induce resistance in parts of the plant that are spatially separated from inducing microorganism (Trotel et al. 2008), by activation of the plant defense mechanism through induced systematic resistance (ISR); in some cases ISR is associated to expression of some defense genes such as those encoding for pathogenesis-related (PR) proteins, as the activation of enzymes like phenylalanine ammonia lyase (PAL), itis crucial in the synthesis of phytoalexins, which are highly toxic pathogens compounds , PAL is concerned with the synthesis of salicylic acid and phenolic compounds (Shadle et al. 2003). Otherwise studies by PGPR showed evidence that the increased production of peroxidases (POD) and phenolic compounds maybe caused by the accumulation of structural substances (lignin) and may increase the mechanical strength of the host cell walls, inducing ISR would likely inhibit or at least restrict a pathogen invasion (Yedidia et al. 1999). Therefore, the objective of this study was to evaluate the effect of previously selected PGRP strains on potato dry rot and black scurf causing fungi species including *Fusarium oxysporum* and *Rhizoctonia solani*, under in vivo conditions.

Material and methods

PGPR strains

Bacterial antagonists were selected by testing antagonistic activity of *Bacillus* strains against *R. solani* and *F. oxysporum* using preliminary dual culture procedure. All PGPR strain were obtained from bacterial consortium, these mixtures contain *Bacillus* spp., with all these strains were prepared different conditioning forms to review their stability and biological viability.

Isolation and identification of pathogenic fungi

Virulent *F*. *oxysporum* and *R*. *solani* strains were isolated from potato that presented the characteristic symptoms. They were grown on potato dextrose agar (PDA) medium at 26±2 °C for 7 days. Their identity was determined by Leslie and Sumerell (2006) and Sneh (1991) techniques.

DNA extraction, amplification and sequencing

DNA Extraction

Fungal cultures were grown on PDA at 26±2 °C for 14 days, mycelium was ground (approximately 4.0 g) to a fine powder under N_2(l) using a pre-cooled pestle and mortar. The grounded mycelium was placed in a 35 mL centrifuge tube and resuspended at 0 °C in 10 mL of DNA extraction buffer (10 mM Tris buffer pH 8.0, 10 mM EDTA, 0.5% SDS). Phenol:chloroform:isoamyl alcohol (25:24:1, 10 mL) was added to the aqueous mycelium solution and mixed slowly for 15-30 min on an horizontal cylindrical rotor. The phases were separated by centrifugation at 6000 rpm for 15 min at 4°C. The aqueous layer was removed and the phenol extraction/aqueous layer separation procedure was repeated until the interface between the two layers was clear. Any traces of phenol were

removed by treating the aqueous layer with a chloroform isoamyl alcohol mixture (24:1) and the phases were separated as they were before. Ribonuclease A (bovine pancreas) solution (10 Wl, 20.0 g l31) was added to the aqueous layer and incubated at 37 °C for 30 min. Phenol extraction, followed by chloroform extraction was repeated once more and the DNA precipitated from the aqueous solution with 2.5 volumes of 100% ethanol and 1/10 volume of LiCl solution (4 M) at 320 °C overnight. The DNA was recovered by centrifugation at 13 000 rpm for 10 min, the pellet was washed with 70% ethanol at 4 °C, air-dried and resuspended overnight in approximately 1 mL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Genomic DNA used as a template in PCR reactions was stored at 4 °C (Nicholson et al*.* 2001and Peters et al*.* 2008).

Polymerase chain reaction (PCR)

The 5.8S nuclear rDNA unit together with its flanking internal transcribed spacer (ITS) sequences were amplified using primers ITS1 (5´TCC GTA GGT GAA CCT GCG G 3´) and ITS4 (5´TTC TTC GCT TAT TGA TAT GC3´) (1O µM), using a mixture of amplification with final volume of 20 µL, ((13.58 µL MQ-H_2O, 2 µL $MgCl_2$ (10 X), 0.32 µL buffer (32 mM) $MgCl_2$, 0.4 µL dNTP's (10mM), 0.5 µL ITS (10 M), 0.2 µL taq DNA -polymerase 1U and 1 µL DNA (40 ng / µL)).

Sequencing of PCR products

The PCR products were increased and purified by using a GeneAll®ExpinTM SV PCR purification kit; these were sequenced by the laboratory Macrogen (Rockville Maryland, USA). The sequences were deposited in the NCBI

GenBank database and were stripped from initial and final part to increase the sensitivity of the analysis.

Establishment and application of treatments

Source of potato minitubers

The potato minitubers *cv*. Fianna were selected free of wounds and rots and as homogeneous as possible in size, and were stored at 0-1 °C until use.

In vivo assay in greenhouse conditions

Based on the antifungal activity on Petri plates, PGPR were selected as potential biocontrol agents; cell suspensions of PGPR were tested for activity against pathogenic fungi on potato minitubers under greenhouse conditions. Potato minitubers were planted in pots of 5 kg with pasteurized soil. At the time of sowing the inoculation of plant pathogens was performed; in case of *F. oxysporum* conidia suspension $1x10^6$ / mL was used; for *R. solani* mycelial fragments at $1x10^6$ / mL, prepared by macerating 4 petri dishes with PDA and *R. solani* growth of 14 days in 1L of sterile distilled water. The treatments were defined as follows (T1) pathogen + bioformulation 1 - $1x10^6$, (T2) pathogen + bioformulation 2 - $1x10^7$, (T3) bioformulation 1 - $1x10^6$, (T4) bioformulation 2 - $1x10^7$, (T5) pathogen (*F. oxysporum* or *R. solani*), (T6) control (water). 3 applications of bioformulations were made, the firs one at sowing time, the second when plant were 15 cm of length and the third at 45 days old. The bioformulations contained a mixture of *Bacillus* spp., in different formulations conditions to review the stability and biological viability. The experiment was

arranged in a randomized block design with seven replicates per treatment and kept in a greenhouse; each repetition had two crop plants under study.

Preliminary test for the extraction and enzymatic quantification

For enzymatic extraction was required 1 g of potato leaf, sampling was performed at day 45 of potato crop in green house conditions, the leaves were processed immediately, cleaned with sterile water and macerated with phosphate buffer solution of pH 7.3, 0.1 M in 1:2 ratio (w/v); subsequently samples were centrifuged at 12 500 rpm for 15 min at 4 °C, the supernatant was separated from the residue, to use in the enzymatic determinations.

Determination of plant defense reactions

Protein determination (PR)

Protein quantification for this assay was performed by the method Bradford, using bovine serum albumin as patron (Bradford, 1957).

Phenylalanine ammonia lyase (PAL)

PAL activity was assayed by measuring the formation of cinnamic acid at 290 nm according to the method of Tanaka et al. (1974). The reaction mixture (0.8mL) containing the enzyme extract (150 µL), 40 mM phenylalanine (200 µL), and 0.1 M Tris-HCL (pH 8.8), was incubated at 37 °C for 30 min the reaction was stopped by adding 200 µL of trichloroacetic acid (25%). The assay mixture was centrifugated at 10000 x g for 15 min (4 °C) and cinnamic acid formed during enzymatic reaction was quantified by using a molar extinction coefficient of 17.4/mM/cm.

Peroxidases (POD)

POD activity was assayed spectrophotometrically at 610 nm with phenol red as a substrate. The complete reaction mixture (1 mL, 37 °C) contained 10 to 20 mL of a crude enzyme preparation, 50 mL of 0.2% (w/v) phenol red, and 50 mM sodium citrate (pH 4.2). Reactions were initiated with 10 mL of 1 mM hydrogen peroxide and stopped after 3 min with 40 mL of 2N sodium hydroxide. The optical density was detected at 610 nm as described above. The absorbance was recorded at 610 nm and calculated with a molar extinction coefficient of 22,000/M/cm for the oxidized product. Peroxidase activity was expressed as millimoles of phenol redoxidized per gram of fresh tissue per minute (Yedidia et al*.* 1999).

Statistical analysis

Data from protein quantification and enzymatic assay was subject to ANOVA using SAS 9.0 software and means were separated by Tukey's multiple range test ($P<0.05$) to analyze statistical differences.

Results

Isolation and identification of pathogenic fungi

Fungal strains isolated from diseased potato plants were identified by morphological features such as *F. oxysporum* (Leslie and Summerell 2006) and *R.* solani (Sneh et al*.*1991).

Confirmation of morphological identification of species was obtained by sequencing the ITS1 and ITS4 intergenic region, the sequences obtained in BLAST showed 99% homology to *F*. *oxysporum* and *R*. *solani*.

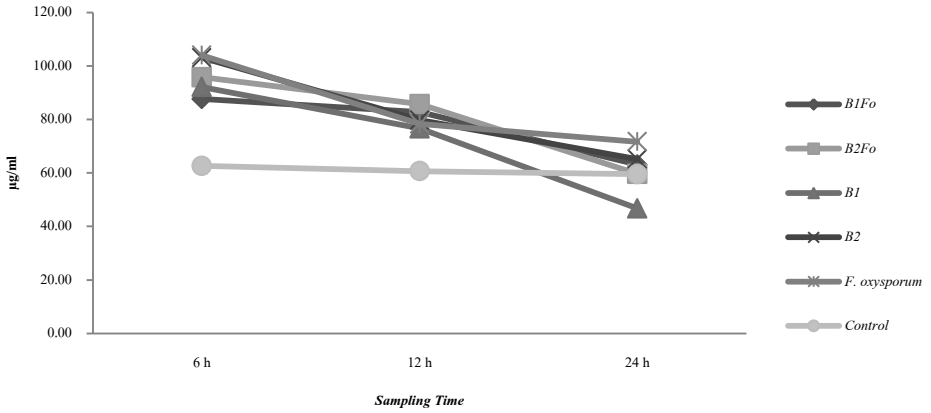
Determination of plant defense reactions

Protein quantification (PR)

The treatments elicited induction of protein activity in *S*. *tuberosum* plants against *F*. *oxysporum* and *R*. *solani*. They showed activity in potato plants as response to *F*. *oxysporum* was increased at 6 h in all treatments except control, as soon as time increased these levels decrease with significant differences (fig.1). At 6 h Fo treatment showed high protein activity, maybe *F*. *oxysporum* activated defense mechanisms on plants, at 12 h B1Fo and B2Fo showed high protein activity by increasing the percentage from 36.54% and 41.38% as compared to control (fig. 1A). It is noteworthy that control remained constant the three times.

Meanwhile average of protein activity on potato plants at different times of treatment against *R*. *solani* on plants with application of B1Rs and B2Rs was significantly higher than control plants (110.20% increased to 113.64%) at 12 h and 24 h (46.98% increased to 91.81% respectively) (fig.1B) as compared to control.

A



B

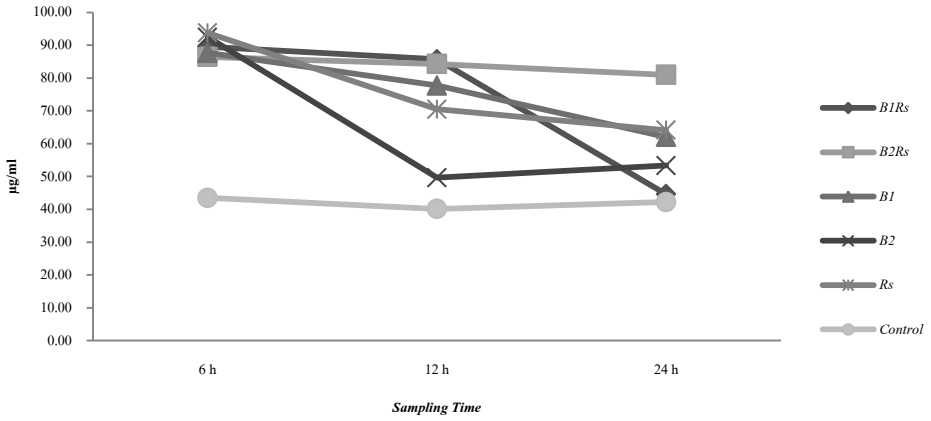


Fig. 1. Protein quantification of treatment applying (A) *F. oxysporum* (Fo), Bioformulation 1 and 2 (B1 and B2) and (B) *R. solani* (Rs) on *Solanum tuberosum*.

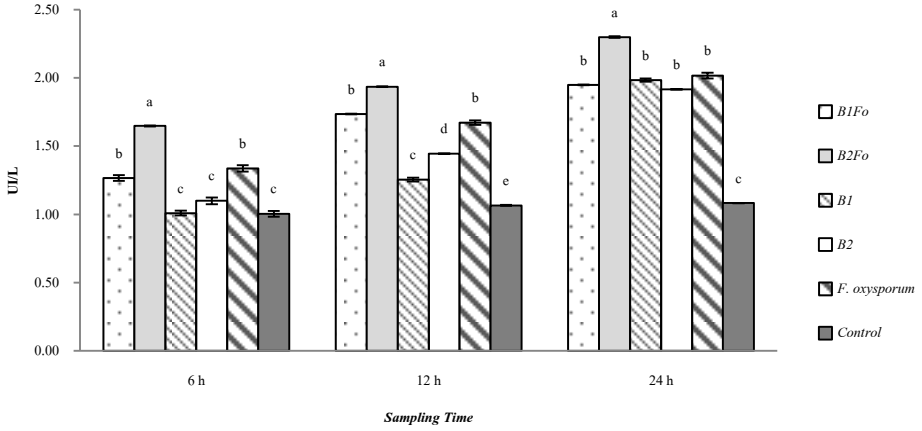
Phenylalanine ammonia lyase (PAL)

PAL determination in potato crop against *F. oxysporum* presented significant differences ($p < 0.05$), in (fig 2A) it is shown that B2Fo presented the highest PAL levels from 6 h at 24 h, activity was increased in relation to time, B1Fo

presented the same behavior than B2Fo but in a lower level. Treatments B1Fo and B2Fo at 12 h and 24 h were capable of stimulating PAL responses by increasing the percentage from 27.00% and 65.00% respectively. Moreover B1 and B2 treatments also increased by 35.85% and 83.33% as compared to control (fig. 2A). Control was kept constant during the 3 periods where the assay was conducted.

On the other hand when plants were inoculated with B1Rs and B2Rs, the levels of PAL increased markedly at 12 h and 24 h postinoculation, compared to control (fig. 2B). The results of activity of PAL by application of bioformulations against *R*. *solani* shown that B1Rs and B2Rs increased from 12 h at 24 h. The PAL activity was increased by 38.1% to 55.95% over B1Rs and B2Rs, also B1 and B2 treatments increased by 54.76% and 143.18% as compared to control (fig. 2A).

A



B

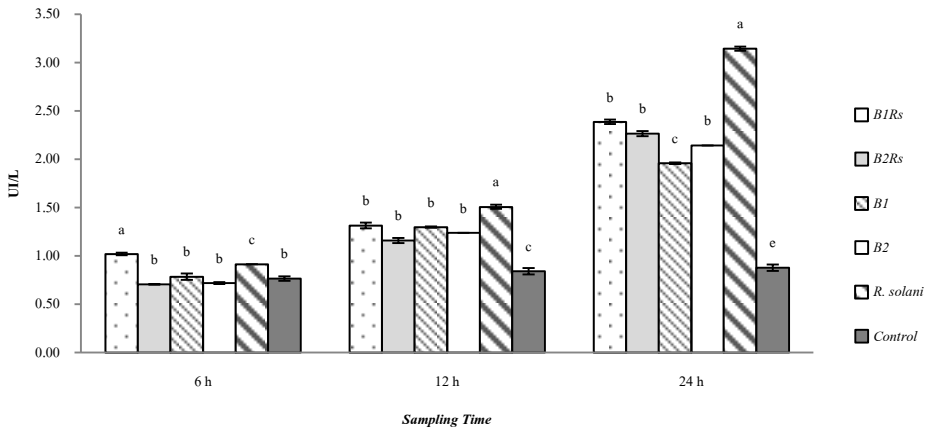
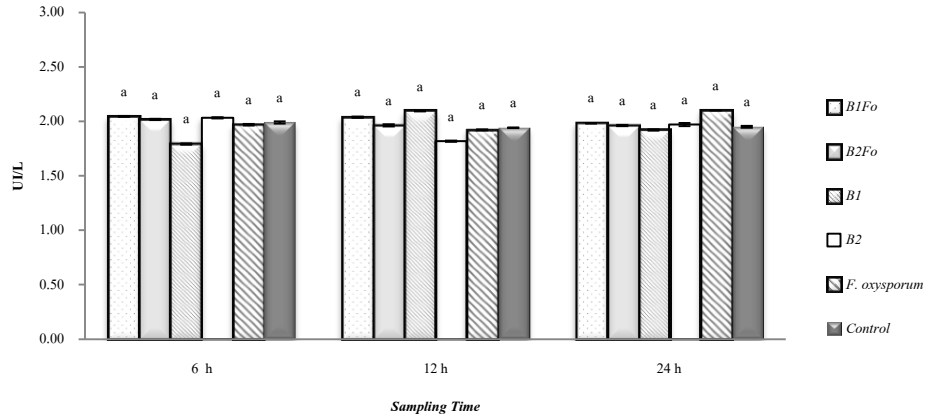


Fig. 2. Phenylalanine ammonia lyase activity at different application times on *Solanum tuberosum* with (A) *F. oxysporum* (Fo), Bioformulation 1 and 2 (B1 and B2) and (B) *R. solani* (Rs). The error bars are mean standard deviation. Values followed by a common letter are not significantly different at $P < 0.05$, according to Tukey's test.

Peroxidases (POD)

Results of peroxidases induction in potato plants are shown in fig. 3A and 3B. There were no significant differences between treatments, however this activity was increased by 1.03% to 5.15% over B1Fo and B2Fo; in B1 and B2 as compared to the control 1.03% to 4.64%, meanwhile the treatments B1Rs and B2Rs were increased by 2.87% to 3.47% at 24 h. Treatment B2 only shown 3.85% activity of POD more than control at 24 h.

A



B

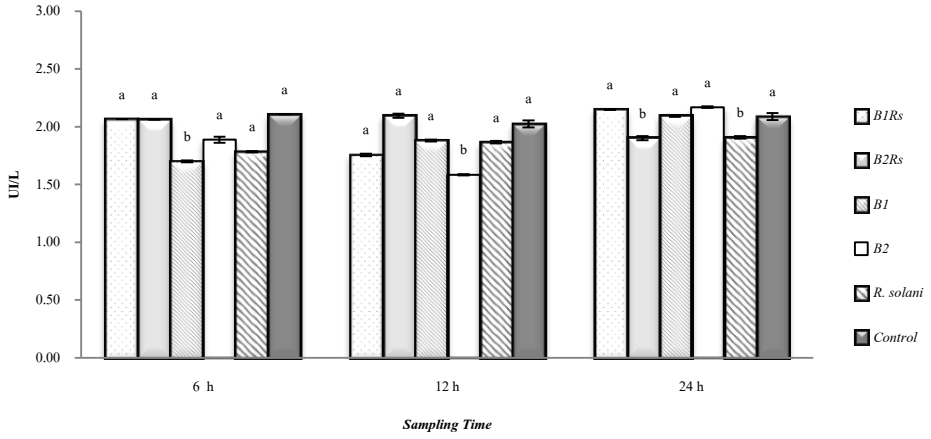


Fig. 3. Peroxidase activity at different application times to *Solanum tuberosum* with (A) *F. oxysporum* (Fo), Bioformulation 1 and 2 (B1 and B2) and (B) *R. solani* (Rs). Bars are mean standard deviation. Values followed by a common letter are not significantly different at $P < 0.05$, according to Tukey's test.

Discussion

Results presented in this paper clearly demonstrated the potential of PGPR strains bioformulations to activate resistance against *F. oxysporum* or *R. solani*.

Current research on mechanisms of biological control by PGPR revealed that several PGPR strains protect plants against pathogen infection through induction of systemic resistance, without causing any symptoms themselves (Devendra and Bhavdish, 2009). In most cases induction of resistance is associated with the expression of some defense genes, such as encoding specific protein (PR) related pathogenesis. This study shows an initial protein content increase in the first 6 h, as time passes by it has a tendency to decrease, this could be due to the initial protein synthesis by high response of the defense system, depending on the crop and pathogen proteins, metabolic pathways that activate protection are targeted. Many plants are invaded by pathogens that impair their growth and reproduction. The plants have a defense system ranging from physical barriers to molecular and systemic signals (Ramos and Portal, 2010), that is why it is so important to know what is occurring in the host-pathogen interaction at the metabolic level. The PAL enzyme is one of the key enzymes in the phenylpropanoids pathway which results in the synthesis of secondary metabolites such as phytoalexins, lignins, benzoic acid and salicylic acid (Hammerschmidt, 1999; Ferraz et al. 2014). Ardila (2011) determined that during *Fusarium* infection, tolerant varieties of carnation present induction of PAL activity in the root at 48 h after inoculation, whereas in susceptible varieties may increase this at 6 h, as occurred in the present study thus determining PAL represents an important route of plant defense against a pathogen. These results confirm that the phenylalanine ammonia lyase enzyme is activated either by plant-pathogen (*F*. *oxysporum* or *R*. *solani*) interaction, as by the application of treatments in other stress conditions. It is postulated that the peroxidase

activity might be important in penetration resistance of pathogens in plants to be involved in the deposit extension cell wall mediate cross-linking in the presence of H_2O_2 and participate in the oxidative polymerization of hydroxicinnamylic alcoholto form lignin, processes that lead to stiffening the cell wall and placing barriers to pathogen infection. Yedidia (1999) reported the POD activity by inducing resistance in plants of *Cucumis sativus* through biocontrol applications of *Trichoderma harzianum*, where she found that the peak of POD activity was at 72 h post-inoculation. Studies by Cuervo (2009) analyzed the induction of POD in *Dianthus caryophyllus* during exposure to *F*. *oxysporum f. sp*. *dianthi* showing high enzyme activity at 48 h, and associated the increase of lignin within 48 h, confirming a relationship between increased PAL and lignin. Otherwise Chai (2005) reported low activity of SOD associated with increased oxidative damage, appeared in banana seedlings studies, jute plants (*Corchorus* sp*.)* under water stress showed the ratio of the low activity of all enzymes SOD and other antioxidant enzymes, plant senescence is corroborated by several studies (Synkova and Valckeb, 2001) Results showed that the *Bacillus* spp*.* bioformulations have potential as elicitors of defense mechanisms in *S*. *tuberosum,* they promote resistance to *F*. *oxysporum* and *R*. *solani* under greenhouse conditions but it is necessary to continue making contributions to scientific information of host - pathogen interaction and the interaction of formulations against a threat of plant pathogens, research shows that the bioformulations stimulate protein production, consequently, PAL is also activated, however data through more hours should be seek to confirm the activity of POD.

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CONCLUSIÓN

Los resultados de la presente investigación demostraron que las bacterias endófitas cepa 21 y 53 identificadas como *Bacillus amyloliquefaciens* redujeron significativamente el crecimiento micelial *in vitro* de *Fusarium oxysporum* y de *Rhizoctonia solani* cuando se utilizaron solos y en consorcio, sin embargo, bajo estas condiciones no se consiguió un efecto aditivo o sinérgico cuando se usó el consorcio microbiano. Bajo condiciones de invernadero, las cepas de *B. amyloliquefaciens* fueron capaces de proteger las plantas de papa a la infección de *F*. *oxysporum* y *R*. *solani* aumentando la sanidad de planta en 909.09% contra *R. solani* y 303.03% frente a *F. oxysporum*. El consorcio microbiano también tuvo la capacidad de promover el crecimiento y desarrollo del cultivo, induciendo mayor altura de planta, diámetro de tallo, peso fresco de la biomasa aérea y de la raíz, aumento del contenido de clorofila alcanzando un mayor rendimiento en comparación a donde no fueron aplicados los consorcios microbianos. Finalmente los microorganismos en consorcio mejoraron la activación de vías metabólicas como inducción de resistencia sistémica en plantas de papa, elevando los niveles de peroxidasa, fenilalanina amonioliasa y proteínas totales.

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