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Beauveria bassiana endófita: Agente de promoción de crecimiento vegetal y de biocontrol en tomate

Tesis para optar al grado de Doctor en Ciencias de la Agronomía

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RESUMEN

Solanum lycopersicum L. tiene gran importancia a nivel mundial por sus características alimentarias y sus contendidos de antioxidantes. Su cultivo se realiza en distintas zonas geográficas, al aire libre o bajo invernadero y su producción puede ser destinada a consumo fresco o procesado. Durante su crecimiento es afectado por factores bióticos y abióticos que repercuten negativamente en la producción y calidad de los frutos, por lo que es necesario tomar medidas de control adecuadas para evitar pérdidas económicas relevantes. Para enfrentar estos problemas, tradicionalmente se han utilizado agroquímicos; sin embargo, su uso indiscriminado ha traído consecuencias negativas en el medio ambiente y en la salud de las personas. El uso de microorganismos endófitos se presenta como una alternativa eficiente y sustentable para su cultivo, además de ofrecer una serie de beneficios adicionales más allá de su función objetivo.

El objetivo de esta investigación fue determinar la capacidad de colonización endofítica de cepas nativas de *Beauveria bassiana* en tomate, su actividad como agente promotor de crecimiento y de biocontrol. En la primera etapa, se utilizaron 50 cepas nativas de *B. bassiana* pertenecientes a la Colección Chilena de Recursos Genéticos Microbianos de INIA y se seleccionaron diez que colonizaron tomate en ensayos exploratorios. Estas cepas fueron evaluadas posteriormente en su capacidad de colonización endofítica de tomate y ají. Se realizaron inoculaciones del hongo mediante drench a las raíces, y luego se hizo su reaislamiento desde las distintas estructuras de la plantas (raíces, tallos y hojas). Además, se evaluó la actividad antagónica de ocho cepas frente al patógeno *Botrytis cinerea in vitro*, mediante enfrentamiento en placas; e *in vivo*, en macetas con aplicaciones del endófito a las raíces y del patógeno a las hojas. Las diez cepas evaluadas colonizaron de forma sistémica los distintos tejidos en tomate y ocho en ají, siendo la cepa RGM-557 la que alcanzó los mayores porcentajes de colonización en tomate y la RGM-547 en ají, con un 50 y un 35% respectivamente. A nivel *in vitro* todas las cepas mostraron algún porcentaje de inhibición del crecimiento radial del patógeno,

la cepa RGM-644 alcanzó el mayor porcentaje de inhibición (39%), sin diferencias estadísticas con RGM-547, RGM-570, y RGM-632. En los ensayos en maceta, todas las plantas tratadas con las cepas endófitas alcanzaron porcentajes de superficie foliar afectada por el patógeno significativamente inferior en comparación con el testigo tratado solo con *B. cinerea* (P≤0,05).

En la segunda etapa se evaluó el efecto de cinco cepas endófitas de *B. bassiana* en la reproducción de *Trialeurodes vaporariorum* y su efecto en la promoción de crecimiento de tomate. En la evaluación con mosquita blanca, el endófito se inoculó al sustrato y posteriormente las plantas se expusieron a poblaciones de adultos del insecto. Para la actividad promotora de crecimiento, se determinó el efecto de las cepas endófitas en la solubilización de fosfatos, producción de sideróforos de hierro, compuestos indólicos, altura de la planta y biomasa vegetal. *Beauveria bassiana* redujo el número de huevos por cm^2 de foliolo en comparación al control (agua destilada), comportándose de forma similar al compuesto químico (Insegar). Las plantas tratadas con las cepas RGM-557, RGM-644 y RGM-731 presentaron un número inferior de ninfas que el control y el tratamiento químico (P≤0,05). Por otro lado, la mayoría de las cepas alcanzaron algun grado de solubilización de fosfato, a excepción de la RGM-547; y de producción de sideróforos de hierro, a excepción de la RGM-570, mientras que ninguna cepa demostró capacidad de producir compuestos indólicos. Finalmente las plantas inoculadas con las cepas endófitas RGM-557 y RGM-731 alcanzaron las mayores alturas de planta y la RGM-731 obtuvo la mayor biomasa vegetal.

En este estudio se entrega evidencia del potencial que presentan los microorganismos endófitos, en particular los hongos entomopatógenos, para mitigar el estrés causado por factores bióticos. Sin embargo, es necesario aumentar el número de estudios orientados a entender los mecanismos de protección involucrados en la relación planta-endófito.

Palabras clave: *Beauveria bassiana*, endófitos, control biológico, promotores del crecimiento vegetal, hongos entomopatógenos.

ABSTRACT

Solanum lycopersicum L. has great importance worldwide for its nutritional characteristics and its antioxidant content. It is cultivated in different geographical areas, under field and greenhouse conditions, and its production can be used for fresh consumption or processing. During its growth it can be affected by biotic and abiotic factors that negatively affect the production and quality of its fruits, making adequate control measures necessary to avoid relevant economic losses. For these problems, traditionally chemical pesticides have been used; but their indiscriminate use has had negative consequences on the environment and human health. Biological control, based on the use of microorganisms, is thus presented as an efficient and sustainable alternative for crop protection and gives a series of additional benefits beyond their target function.

The objective of this research was to determine the endophytic colonization capacity of native *Beauveria bassiana* strains in tomato, its activity as a plant growth promoting agent and biocontrol. First, 50 native strains of *B. bassiana* belonging to the INIA Chilean Collection of Microbial Genetic Resources were evaluated, and ten strains that colonized tomato tissues in exploratory tests were selected. These strains were subsequently evaluated for their endophytic colonization ability on tomato and chili. Fungal inoculations were made by drenching the roots, and then the fungus was re-isolated from the different plant parts (roots, stems, and leaves). In addition, the antagonistic activity of eight strains against the pathogen *Botrytis cinerea* was evaluated *in vitro*, by in plate confrontation; and *in vivo*, in plants pots through applications of the endophyte to the roots and the pathogen to the leaves. The ten strains evaluated were able to colonize systematically the different tissues in tomato and eight strain colonized chili. The RGM-557 strain achieved the highest percentages of colonization in tomato and the RGM-547 in chili, with 50 and 35% respectively. In the *in vitro* evaluation, all the strains showed some percentage of radial growth inhibition of the pathogen, the RGM-644 achieving the highest percentage of inhibition (39%), without statistical differences with RGM 547, 570, and 632. In potted trials, all plants treated with endophytes reached a percentage of leaf

surface affected by the pathogen significantly lower than the control treated only with *B. cinerea* (P≤0.05)

In a second stage, the effect of five native endophytic strains of *B. bassiana* on the reproduction of greenhouse whiteflies and its effect on the growth promotion of tomatoes were evaluated. For the whitefly trial the endophyte was inoculated in the substrate, and plants were afterwards exposed to adult populations of the insect. For plant growth promoter activity, the effect of endophytic strains on phosphate solubilization, indole compounds, plant height, and plant biomass were determined. *Beauveria bassiana* reduced the number of eggs per cm^2 on leaflet in comparison to the control (detilate whater) and behaved similarly to the chemical insecticide (Insegar). Plants inoculated with strains RGM-557, RGM-644, and RGM-731 showed a lower number of nymphs than the control and insecticide (P≤0,05). On the other hand, most strains showed some degree of phosphate solubilization, with the exception of RGM-547; and for the production of iron siderophores, with the exception of RGM-570, while no strain shoewed the capacity to produce indole compounds. Finally, the plants inoculated with the endophytic strains RGM-557 and RGM-731 produced the greatest plant heights and RGM-731 obtained the greatest plant biomass.

This study provides evidence of the potential of endophytic microorganisms, particularly entomopathogenic fungi, to mitigate stress caused by biotic factors. However, it is necessary to increase the number of studies aimed at understanding the protection mechanisms involved in the plant-endophyte relationship.

Keywords: *Beauveria bassiana*, endophytes, biological control, plant growth promoters, entomopathogenic fungi.

I. CAPITULO 1: INTRODUCCIÓN GENERAL

1.1 Los problemas de la horticultura intensiva

La producción hortícola es fundamental para la alimentación de la población mundial, las hortalizas aportan gran cantidad de nutrientes, vitaminas y fibra. Dentro de este grupo destacan las que pertenecen a la familia *Solanaceae*, que incluye más de 3.000 especies, ocupando una amplia variedad de hábitats (Knapp, 2002). Esta familia tiene diversas especies de importancia económica como papa, tomate, pimiento, ají y berenjena.

El tomate (*Solanum lycopersicum* L.) es una de las hortalizas más producidas y consumidas a nivel mundial, destacando por su calidad nutricional y versatilidad de usos al igual que el ají *(Capsicum annuum* L.). Durante estas últimas décadas existe una tendencia hacia su cultivo forzado, principalmente bajo invernadero. Estos sistemas son altamente intensivos y sus condiciones ambientales se modifican para alcanzar altos niveles de producción; sin embargo, las plantas se ven sometidas a diferentes estreses bióticos y abióticos que pueden traducirse en pérdidas de calidad y rendimientos (López-Marín et al., 2011). Para mitigar el estrés biótico ocasionado por plagas y enfermedades tradicionalmente se hace uso de plaguicidas de síntesis química, los que pueden tener un impacto negativo en los humanos y en el medio ambiente (Geiger et al., 2010), por lo que han sido cuestionados y como consecuencia se han buscado alternativas más sustentables para el manejo fitosanitario de los cultivos. El uso de agentes microbianos se presenta como una alternativa concreta para cumplir con este fin, los microorganismos pueden apoyar en el crecimiento y desarrollo de las plantas mediante la disminución del efecto negativo causado por factores bióticos y abióticos (Glare et al., 2012). El reemplazo de los agroquímicos por productos biológicos obtenidos en base a microorganismos permitiría incrementar de forma significativa la producción de cultivos además de alcanzar producciones más sustentables (Dale, 2003).

1.2 Cultivo del tomate

El tomate es una de las especies de hortalizas más importantes económicamente a nivel mundial, fue previamente reconocido como *Lycopersicon esculentum* Mill., pero datos de su morfología y las secuencias moleculares apoyan su inclusión en el género *Solanum* (Peralta, 2008). Se ha establecido el origen de esta especie en las regiones andinas de América del Sur, sin embargo su origen aún sigue siendo un tema de debate (Colvine y Branthôme, 2016). Es una de las hortalizas más cultivadas, con una producción de aproximadamente 243 millones de toneladas en una superficie cercana a los 4 millones de hectáreas (FAOSTAT, 2018). La importancia económica de esta especie radica en su alto consumo, dado entre otros por su bajo contenido de grasas, excelente fuente de antioxidantes, altos niveles de fibra dietética, minerales y vitaminas (Erdinc et al., 2018). Es consumida en fresco o procesada en una gran diversidad de productos manufacturados (Sousa et al., 2008), siendo considerada como una de las principales hortalizas procesadas con un crecimiento sostenido de la superficie cultivada destinada al uso industrial (Colvine y Branthôme, 2016).

En Chile es una de las hortalizas más consumidas ocupando el tercer lugar en la canasta de consumo familiar después de lechuga y choclo. Su cultivo comercial se realiza principalmente desde la Región de Arica y Parinacota hasta la Región de La Araucanía. La superficie destinada al cultivo de tomate fresco es de 5.463 hectáreas, en el ciclo de producción 2019, siendo las regiones de Valparaíso (19,1%) y O'Higgins (17,6%) las que tienen mayor superficie (ODEPA, 2019). La producción de tomate se realiza principalmente bajo un sistema convencional que implica una alta demanda de agroquímicos. Durante su desarrollo, las plantas pueden ser afectadas por factores bióticos y abióticos simultáneamente que producen estrés, repercutiendo de manera negativa en el rendimiento (Atkinson y Urwin, 2012).

1.2.1 Factores bióticos que afectan la producción de tomate

Las altas temperaturas y altos niveles de humedad que se generan en los sistemas de producción intensiva del tomate, promueven el desarrollo de enfermedades que pueden progresar rápidamente (Punja et al., 2016). Entre las enfermedades ocasionadas por hongos que afectan a esta especie destacan *Fusarium oxysporum*, *Sclerotinia sclerotiurum, Rhizoctonia solani* y *Botrytis cinerea* (Amselem et al., 2011; Dean et al., 2012; Gwinn et al., 2010). Este último es el agente etiológico de la podredumbre gris, que afecta a más de 200 especies vegetales en todo el mundo, este hongo es capaz de penetrar a través de heridas y colonizar toda la parte aérea de la planta (Williamson et al., 2007), afectándola durante todo su desarrollo hasta la post-cosecha (Punja et al., 2016). En la producción bajo invernadero el principal daño provocado por *B. cinerea* se produce en los tallos, debido a su ingreso a través de las heridas de poda. Otro daño importante se produce en los frutos principalmente en la post-cosecha (Williamson et al., 2007). *Botrytis cinerea* es de difícil control debido a que presenta distintas formas de infección, tiene diversos hospederos como fuente de inóculo y puede sobrevivir como micelio, conidias y/o esclerocios por un largo tiempo en los restos de cultivos. Existe gran disponibilidad de fungicidas químicos en el mercado para el control de este hongo; sin embargo, su efectividad se ha visto afectada por la aparición de cepas resistentes (Leroux et al., 2010). Estimaciones del gasto en el que se incurre para su control señalan que alcanzan los 1,5 mil millones de dólares a nivel mundial (Dean et al., 2012). En este contexto el control biológico de enfermedades mediante el uso de agentes microbianos presenta ventajas, dado la disponibilidad de agentes como hongos, levaduras y bacterias (Williamson et al., 2007), su inocuidad y disminución de la probabilidad de producir resistencia en los patógenos.

El tomate también puede ser afectado por varias plagas que inciden negativamente en su productividad y obligan a hacer un uso intensivo de insecticidas químicos para su control. Entre las plagas que lo afectan destacan: mosquitas blancas, *Trialeurodes vaporariorum* (Westwood) y *Bemisia tabaci* (Gennadius); polilla del tomate, *Tuta absoluta* (Meyrick); trips de las flores, *Frankliniella occidentalis* (Pergande); pulgón verde del duraznero, *Myzus persicae* (Sulzer) y arañita roja,

Tetranychus urticae (Koch) (Polack, 2007; Baier et al., 2015). Dentro de este grupo las mosquitas blancas son consideradas plagas de importancia económica a nivel mundial (Hodges y Evans, 2005), tienen una amplio rango de hospederos que van desde especies de uso agrícola (como tomate, pepino y frutilla) hasta plantas ornamentales (Choi et al., 2003; Kim et al., 2014). *Trialeurodes vaporariorum* es una de las principales plagas en tomate especialmente bajo invernadero (Tsueda et al., 2014), se alimenta de los fluidos vegetales (celulares y savia) provocando el debilitamiento de toda la planta (daño directo), sus ninfas excretan sustancias azucaradas dando paso a la proliferación de hongos saprófitos que limitan la fotosíntesis y afectan la respiración de las hojas, lo que produce una caída de las mismas (Van Lenteren el at., 1990). Además, esta mosquita tiene la capacidad de transmitir enfermedades causadas por virus que provocan grandes pérdidas en la producción de tomate (Navas-Castillo et al., 2011). El método de control más usado se basa en insecticidas químicos, los que además de no alcanzar los niveles de eficiencia esperados (Kapantaidaki et al., 2018) presentan otras desventajas como: la destrucción de enemigos naturales, desarrollo de resistencia, incremento en los costos de producción, riesgo para la salud de las personas y contaminación ambiental (Singh et al., 2014). Lo anterior, ha estimulado el desarrollo de métodos alternativos de control como el biológico.

1.2.2 Factores abióticos que afectan la producción de tomate

Como todos los organismos vivos, el tomate está sometido de manera constante a condiciones ambientales cambiantes que pueden causar estrés. Condiciones como sequía, salinidad, viento, luz, temperatura y humedad, además del exceso o carencia de macro y micronutrientes, afectan negativamente el crecimiento, producción y calidad del cultivo (Grover et al., 2003; Djilianov et al., 2005). El efecto de una, o la combinación de estas condiciones, se traduce en una reducción en los rendimientos mayor al 50% en promedio en la mayoría de los cultivos (Rodríguez et al., 2005). Por ejemplo, plantas expuestas a una bajas temperaturas combinada con baja luminosidad tienen hojas más delgadas y grandes en comparación con las hojas

de las plantas que crecen en condiciones normales de crecimiento (Murchie et al., 2005; Sun et al., 2014).

En el ámbito nutricional el tomate puede sufrir estrés al estar expuesto a condiciones de déficit o exceso de micro y/o macronutrientes. El nitrógeno (N), es el principal factor limitante para el crecimiento y rendimiento de las plantas en la agricultura. La mayoría usa el N como iones de amonio o nitrato y la absorción de estas dos formas de nitrógeno está controlada entre otros por el genotipo, estado fisiológico y también por las propiedades del suelo como textura, estructura, pH contenido de agua y microorganismos (Hucklesby et al., 1992; Borgognone et al., 2013). Por ejemplo, bajo estrés abiótico la actividad de las enzimas asimiladoras de nitrato y amoníaco se inhibe significativamente (Debouba et al., 2006). Otro elemento limitante es el fósforo, este macronutriente es clave en el crecimiento del tomate debido a que participa en múltiples procesos biológicos básicos como la fotosíntesis, respiración, síntesis de ácidos nucleicos, activación e inactivación de enzimas, señalización y las reacciones redox (Herrera-Estrella y López-Arredondo, 2016). Su disponibilidad en suelos de uso agrícola se traduce en una creciente dependencia de los fertilizantes químicos. Como las plantas tienen un hábito de crecimiento sésil, se han adaptado para tolerar a las condiciones ambientales adversas, dentro de las estrategias usadas en el tomate para aumentar la tolerancia a estas condiciones destaca, la manipulación de genes que protegen y mantienen la función y estructura de los componentes celulares. En la actualidad, existe una creciente línea de investigación asociada al uso de microorganismos benéficos (hogos y bacterias), como aliados estratégicos en la protección del estrés abiótico (Berendsen et al., 2012; Caporale et al., 2014; Ruocco et al., 2015). Por ejemplo, un importante número de plantas podrían superar un suministro insuficiente de nutrientes mediante la formación de asociaciones simbióticas con bacterias del suelo, hongos micorrízicos y endófitos (Clark y Zeto, 2000). Como el tomate es relevante en la alimentación humana, la investigación y desarrollo de alternativas sustentables que permitan aumentar la tolerancia al estrés abiótico facilitará su producción, incluso en condiciones ambientales adversas.

1.3 Microorganismos endófitos

Los microorganismos endófitos son beneficiosos para las plantas e incluyen hongos y bacterias. El término endófito proviene de la palabra griega endon y phyton, donde endon significa dentro y phyton planta, fue acuñado por primera vez por De Bary en 1886 y se define como organismos que pasan la mayor parte o todo su ciclo de vida colonizando los tejidos de la planta hospedera sin causar daño evidente (Bascom-Slack et al., 2012). Su definición ha evolucionado notablemente, a medida que se fueron teniendo en cuenta las asociaciones con distintas partes de las plantas y las modalidades de asociación con sus hospederos (Carroll, 1988).

En la tierra existen aproximadamente 300.000 especies de plantas, cada una de ella se asocia a uno o más endófitos. Éstos se encuentran en forma natural en el ecosistema y tienen la capacidad de ocupar cualquier planta en cualquier ambiente, siendo considerados como socios extremadamente importantes para las plantas (Hallmann et al., 1997; Arnold et al., 2000; Strobel y Daisy, 2003; Suryanarayanan, 2013). Los endófitos cumplen una variedad de funciones de tipo simbióticas y ecológicas (Rodríguez et al., 2009). La planta hospedera recibe múltiples beneficios de la interacción con el endófito a cambio de recursos basados en carbono (Herre et al., 2007).

Dado lo anterior, el estudio de los microorganismos endófitos ha aumentado considerablemente durante los últimos años, sumado a las características benéficas que son capaces de conferir a sus hospederos (Aly et al., 2010). Dentro de los endófitos, los hongos entomopatógenos han despertado el interés de los investigadores debido a varias características que le podrían permitir ser usados con distintos objetivos en un sistema de producción agrícola.

1.4 Hongos entomopatógenos endófitos

Los hongos entomopatógenos son un grupo único y altamente especializado de agentes microbianos que poseen rasgos deseables que favorecen su desarrollo como bioplaguicidas (Lacey et al., 2015). Desde hace más de un siglo que Pasteur ya había pronosticado las ventajas de estos hongos por su papel como bioreguladores de plagas, al actuar como parásitos de insectos perjudiciales para las plantas. Actualmente, se conocen más de 700 especies de artrópodos parasitados por *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin y otras 200 por *Metarhizium anisopliae* (Metchnikoff), por lo que su uso como biopesticidas se ha incrementado durante las últimas décadas (Feng et al., 1994; Zimmermann, 2007). Varias especies de los géneros *Acremonium*, *Beauveria*, *Cladosporium*, *Clonostachys*, *Fusarium*, *Metarhizium*, *Paecilomyces*, *Trichoderma* y *Verticillum* se han reportado como endófitos naturales y también han sido inoculados de manera artificial en distintas especies de plantas cultivadas (Gómez-Vidal et al., 2006; Vega et al., 2008; Powell et al., 2009; Akello y Sikora, 2012; Akutse et al., 2014).

Dentro de este grupo destacan los géneros *Beauveria* y *Metarhizium*, ampliamente estudiados como controladores biológicos. Están constituidos por especies que se distribuyen en todo el mundo, las cuales generalmente habitan en el suelo y son patógenos de insectos. Especies como *B. bassiana* y *M. anisopliae* se han reportado como endófitos de un importante número de plantas destacando varios cultivos agrícolas (Jaber y Ownley, 2018). El rango de hospederos de las diferentes especies de hongos entomopatógenos endófitos (HEE) es variable y van desde especies con un único hospedero a otras muy generalistas con varios hospederos (Stone et al., 2004; Sánchez-Márquez et al., 2011). Establecen una relación de protocooperación con la planta que se traducen en mayor crecimiento, inhibición de organismos patógenos, remoción de contaminantes del suelo y aumento de tolerancia a condiciones extremas de temperatura, disponibilidad de agua y salinidad, entre otras (Quesada-Moraga et al., 2009; Ownley et al., 2010). Varios estudios han demostrado que la trasmisión de los HEE puede ser de tipo vertical, es decir, puede pasar de una generación a otra a través de la semilla u

horizontal pasando de un individuo a otro mediante la dispersión de sus conidias (Quesada-Moraga et al., 2014; Thakuria y Goyal, 2016).

La colonización de la planta por parte de los HEE puede ser sistémica, es decir, tienen la capacidad de ingresar por alguna de sus estructuras y desde ahí moverse internamente a través del sistema vascular para establecerse en sus tejidos (Gurulingappa et al., 2010; Gómez-Vidal et al., 2006; Quesada-Moraga et al., 2006). También puede ser localizada en ciertas partes de plantas (Wearn et al., 2012; Yan et al., 2015) o dividida dentro de partes de plantas (Behie et al., 2015; Zambell y White, 2015). Diversos estudios han demostrado que *M. anisopliae* se establece preferentemente en la raíz (Meyling et al., 2011; Liao et al., 2014; Barelli et al., 2016; Greenfield et al., 2016), mientras que *B. bassiana* ha sido reportada como un endófito de las partes de la planta que se encuentran sobre el suelo (Behie et al., 2015).

La forma de ingreso de los HEE a las plantas podría ser por aberturas naturales o a través de la cutícula (Landa et al., 2013), apoyada por la fuerza mecánica que es ejercida por la estructura de infección (Bidochka y Khachatourians, 1991), la disolución enzimática de la cutícula, o una combinación de ambos (Ferron, 1978; Kolattukudy, 1985), mecanismo similar al utilizado por el hongo para ingresar a los insectos. Se han estudiado diversas formas de inoculación artificial de los HEE en las plantas como aplicaciones de propágulos a las hojas (Tefera y Vidal, 2009; Russo et al., 2015; Rondot y Reineke, 2018), suspensiones a la raíz (Posada y Vega, 2005; Posada et al., 2007; Qayyum et al., 2015, Greenfield et al., 2016), recubrimiento e inmersión de semillas (Quesada-Moraga et al., 2009; Kabaluk y Ericsson 2007; Jaber, 2018) e inyecciones al tallo (Posada et al., 2007).

1.4.1 Factores que afectan el establecimiento de los HEE dentro de la planta como endófito

El establecimiento y persistencia de hongos endófitos pueden estar condicionados por factores bióticos (Vicari et al., 2002) y abióticos (Bultman y Bell, 2003). Dentro de los bióticos destaca los asociados a la planta como, especie y

variedad, edad, tipo de tejido y su microbiota endófita (Parsa et al., 2013; Posada et al., 2010). Estudios realizados por Vega et al. (2008) determinaron que la presencia de hongos endófitos en plantas de café pueden afectar el establecimiento de otro hongo endófito como *B. bassiana*. También pueden influir en su establecimiento aspectos asociados al hongo como la especie, cepa, concentración de inóculo (Tefera y Vidal, 2009; Parsa et al., 2013; Vidal y Jaber, 2015) e interacción con otros microorganismos antagonistas como hongos y bacterias (Fisher y Petrini, 1987).

Existen otros factores, que pueden influir en el establecimiento y persistencia de los HEE en la planta como los métodos de aplicación, lo que ha sido confirmado en diversos estudios (Posada et al., 2007; Quesada-Moraga et al., 2014). Por ejemplo, se ha demostrado que aplicaciones mediante aspersiones foliares dan paso a una persistencia temporal del endófito, ya que solo se ha logrado aislar temporalmente desde el área tratada desapareciendo posteriormente (Gurulingappa et al., 2010; Biswas et al., 2012; Batta, 2013; Landa et al., 2013). Sin embargo, este tema aún no está bien dilucidado debido a que en un estudio realizado por Tefera y Vidal (2009), confirmaron que *B. bassiana* coloniza las hojas, tallos y raíces de las plantas de sorgo, independientemente del método de inoculación utilizado (técnicas de inoculación foliar, semillas o suelo). Por otro lado, dentro de los factores abióticos que afectan el establecimiento de los HEE destaca el tipo de sustrato usado para las plantas. Por ejemplo, un sustrato estéril podría producir un efecto fungistático (Lingg y Donalson, 1981) y antagonista (Pereira et al., 1993) para el desarrollo del endófito. También influyen los factores ambientales como la temperatura, radiación y humedad relativa, a los cuales se expone a las plantas, junto con su estado nutricional (Bing y Lewis, 1991; Vidal y Jaber, 2015).

1.4.2 Hongos entomopatógenos endófitos para el control de plagas

Es ampliamente conocida la función en la regulación de poblaciones de insectos que cumplen los hongos entomopatógenos. Estos hongos en su acción directa parasitan al insecto, provocándole graves daños que pueden incluso ocasionar su muerte. Cuando las conidias toman contacto con la cutícula del insecto se forma el tubo de germinación y apresorio, mediante el cual se produce una acción

mecánica y enzimática para penetrar la cutícula y llegar al hemocele (Hajek y St. Leger, 1994). Una vez en el hemocele, el hongo tiene la capacidad de volver a atravesar la cutícula del hospedero y salir al exterior, donde puede continuar desarrollándose saprofíticamente sobre los cadáveres, esporulando y convirtiéndolos en nuevos focos de diseminación (Meyling y Eilenberg, 2007).

La acción de los hongos entomopatógenos como endófito es muy interesante, debido a que presentan distintos mecanismos de acción por los cuales podrían disminuir las poblaciones de insectos. Varios otros estudios informan efectos negativos de los HEE sobre diversas plagas de insectos (Powell et al., 2009; López et al., 2014; Lopez y Sword, 2015; Klieber y Reineke, 2016; Resquín-Romero et al., 2016; Sánchez-Rodríguez et al., 2018). Se reportan cuatro mecanismos de acción:

- Parasitismo: se produciría por ingestión, por parte del insecto, de hifas u otro propágulo del hongo que se encuentran en el interior de la planta al alimentarse de ella. Si bien esta acción ha sido mencionada por algunos autores (Akello et al., 2008; Klieber y Reineke, 2016; Barta, 2018), aún hay pocos estudios que pueden confirmarla.
- Antagonismo por la acción de metabolitos: varios estudios atribuyen la acción de control de insectos por parte de los HEE, a su capacidad de producir metabolitos secundarios que pueden ser tóxicos o presentar una acción de repelencia o anti alimentaria (Clay y Schardl, 2002; Vega et al., 2008; Menjivar et al., 2011; Resquín-Romero et al., 2016; Jaber y Ownley, 2018). La mayoría de estos estudios han atribuido la reducción del daño causado por insectos a la acumulación de micotoxinas producidas por los endófitos en los tejidos vegetales (Gurulingappa et al., 2011).
- Resistencia sistémica: se propone la hipótesis de que los HEE podrían indicar una respuesta de defensa sistémica indirecta en la planta, que se podría traducir por ejemplo, en una resistencia a la alimentación de insectos (Lopez y Sword, 2015). Pineda et al. (2010), propone que los microorganismos benéficos que incluyen hongos micorrízicos, endófitos y promotores de crecimiento pueden inducir la resistencia sistémica al interactuar con las raíces de la planta, produciendo una mayor expresión de los genes de defensa, lo que puede estar

relacionado con las hormonas vegetales como el ácido jasmónico y etileno. Si bien se han atribuido efectos adversos sobre los insectos como consecuencia de la inducción de resistencia por parte de los HEE (Wei et al., 2020, Rivas-Franco et al., 2020) aún no se estudian profundamente los mecanismos específicos involucrados.

- Interacción tritrófica: se ha informado que la colonización de los HEE induce efectos de atracción de miembros del tercer nivel trófico como los parasitoides los cuales podrían disminuir las poblaciones de insectos plagas. Esta acción podría estar asociada a la producción de metabolitos (Akutse et al., 2014; Gathage et al., 2016; Jaber y Araj, 2018).

1.4.3 Acción antagonista de HEE contra patógenos de plantas

Varios estudios demuestran la acción antagonista de los hongos entomopatógenos contra patógenos de plantas, al ser usados como endófitos en diversos cultivos (Ownley et al., 2008; Jaber y Salem, 2014; Jaber, 2018; Barra-Bucarei et al., 2020), incluso investigadores sugieren que estos hongos podrían tener la capacidad de controlar simultáneamente plagas y enfermedades (Kim et al., 2007; Ownley, et al., 2008).

Dentro de los géneros de HEE reportados con actividad antagónica de patógenos destacan *Beauveria*, *Lecanicillium* y *Metarhizium*. *Beauveria bassiana* es la especie con más reportes como endófito antagonista de diversos patógenos que incluyen hongos, bacterias y virus. Lo anterior, podría estar relacionado con su capacidad de producir una diversidad de metabolitos bioactivos, muchos de las cuales tienen propiedades antimicrobianas (Wagner y Lewis, 2000; Parine et al., 2010). Estudios realizados por Feng et al. (2015) determinaron que el genoma de *B. bassiana* presenta al menos 45 grupos diferentes de genes de biosíntesis de metabolitos secundarios. Dentro de los metabolitos que presentan propiedades antimicrobianas destacan, Beauveracina, Bassianolida, Bassianina, Beauveriolida, Bassiacridina y Ciclosporina, entre otros (Vining et al., 1962; Hamill et al., 1969; Suzuki et al., 1977; Logrieco et al., 1998). Se ha estudiado el efecto antagonista de

HEE frente a hongos como *Plasmopara vitícola* en *Vitis vinifera* (Jaber, 2015); *Pythium ultimum* en *Cucumis sativus* (Benhamou y Brodeur, 2001); *F. solani* f. sp. *phaseoli* en frijol (Sasan y Bidochka, 2013); *Rhizoctonia solani* en *Solanun lycopersicum* (Ownley et al., 2004), *Pythium myriotylum* en algodón (Ownley et al., 2008); *Fusarium oxysporum* en *Capsicum annuum*, *Fusarium culmorum* en *Triticum aestivum L*. (Jaber, 2018) y *Botrytis cinerea en S. lycopersicum y C. annuum* (Barra-Bucarei et al., 2020). También se han encontrado reportes de su acción frente a bacterias como *Xanthomonas axonopodis* pv*. malvacearum* en *Gossypium hirsutum*, (Ownley et al., 2008); y en menor cantidad su acción antagónica frente a virus como el Zucchini yellow mosaic virus en *Cucurbita pepo* (Jaber y Salem, 2014). La habilidad de los HEE como antagonistas de patógenos de plantas puede ser consecuencia de la acción de distintos mecanismos, sin embargo el conocimiento en este ámbito aún es escaso. Se les atribuyen mecanismos directos como la competencia, parasitismo y antibiosis, por la producción de metabolitos primarios, secundarios, enzimas o compuestos volátiles; e indirectos como la inducción de resistencias (Arnold et al., 2003; Herre et al., 2007; Ownley et al., 2008). Las respuestas antagonistas podrían contemplar una combinación de los mecanismos antes mencionados (Vega et al., 2009; Ownley et al., 2010).

La **competencia** entre el endófito y el patógeno en el interior de la planta puede ser por espacio y/o alimentos. Los HEE son capaces de colonizar con éxito los diversos tejidos y disponer de las fuentes de alimentos proporcionada por la planta lo que dejaría menos probabilidades para la colonización de los patógenos (Ownley et al., 2004). La inoculación temprana del endófito podría darle ventajas ya que al ser el primero en colonizar la planta, probablemente agote los recursos de ésta dejando pocos disponible para el patógeno. El **micoparasitismo** corresponde a una interacción antagónica entre un hongo parásito y un hongo hospedero que involucra generalmente la acción de enzimas líticas extracelulares que descomponen las paredes celulares del huésped. Si bien hay varios estudios de la acción de los HEE como micoparásitos, éstos solo presentan resultados a nivel *in vitro* sin encontrarse reportes de su acción en plantas (Ownley et al., 2010). La **antibiosis** es la acción directa de metabolitos tóxicos producidos por microorganismos sobre otro sensible a

éstos. Por ejemplo, Beauvericina es una micotoxina producida por *B. bassiana, Paecilomyces fumosoroseus* y *Fusarium* spp. (Bernardini et al., 1975). Este metabolito es interesante desde el punto de vista de la protección de los cultivos agrícolas, ya que presenta una importante actividad antibacteriana, antifúngica y antiviral (Xu et al., 2010; Wang y Xu, 2012). Bassionalida es otro metabolito producido por más de un género de HEE y ha sido reportado para *B. bassiana* y *Lecanicillium lecanii* (Suzuki et al., 1977). Por su parte hongos del género *Metarhizium* producen dextruxinas (Roberts y Leger, 2004), evaluadas con éxito en el control de los patógenos *F. oxysporum* y *Cladosporium herbarum* (Ravindran et al., 2014). Boucias y Pendland (1998), sugieren que los microorganismos oportunistas como los fitopatógenos podrían mantenerse a raya por las sustancias antimicrobianas que producen los hongos. Finalmente la **resistencia sistémica** es considerada como un mecanismo indirecto, puede ser un importante mecanismo de antagonismo frente a fitopatógenos y podría ser estimulada en la planta por la presencia de hongos no patógenos como los endófitos (Kavroulakis et al., 2007; Shoresh et al., 2010). Las plantas colonizadas endófitamente por hongos pueden desencadenar más rápido una respuesta, lo que puede estar asociado a su capacidad de producir metabolitos secundarios que actúan como elicitores para la producción de fitoalexinas (Gao et al., 2010). La resistencia sistémica permite reducir síntomas de la enfermedad en partes de la planta distantes del sitio en el que el agente inductor es activo.

1.4.4 Hongos entomopatógenos endófitos y su acción en la promoción del crecimiento de las plantas

Varios estudios han entregado evidencia del potencial que presentan los HEE para promover el crecimiento de las plantas en distintas especies cultivadas (Sasan y Bidochka, 2012; López y Sword, 2015; Jaber y Enkerli, 2017; Tall y Meyling, 2018). Dentro de los mecanismos que directamente promueven el crecimiento se han estudiado, la producción de fitohormonas, solubilización de nutrientes, aumento en la disponibilidad de nutrientes y producción de sideróforos (Joseph et al., 2012; Behie et

al., 2012; Behie y Bidochka, 2014; Jirakkakul et al., 2015). También se han estudiado mecanismos indirectos como la disminución de los efectos negativos causados por plagas y enfermedades, mencionados anteriormente, que podrían permitir una mayor expresión del potencial de crecimiento de las plantas y el aumento de la tolerancia al estrés abiótico como sequía (Swarthout et al., 2009), condiciones inadecuadas del suelo (Malinowski et al., 2004).

Los HEE inoculados artificialmente por distintas vías han mejorado parámetros como la altura de la planta, biomasa vegetal (tallos, hojas, raíces, frutos y semillas) entre otros parámetros, en cultivos como: maíz, tomate, algodón, soya, habas, poroto, pimiento dulce (Elena et al., 2011; Khan et al., 2012; Sasan y Bidochka, 2012; López y Sword, 2015; Jaber y Enkerli, 2017; Jaber y Araj, 2018; Russo et al., 2019). Estudios sugieren que algunos HEE originalmente fueron simbiontes de plantas y nunca dejaron este papel, y en consecuencia la patogenicidad de los insectos es una adaptación que les permitió acceder a una fuente especializada de nitrógeno y otros nutrientes (Elliot, 2000). Los HEE podrían intercambiar de manera efectiva nutrientes derivados de insectos para acceder a carbohidratos de las plantas (Barelli et al., 2016). De todos los nutrientes del suelo necesarios para el crecimiento de las plantas, el nitrógeno es el elemento más limitante (Vitousek et al., 1997), estudios realizados por Behie et al. (2012) demostraron que cepas de *M. robertsii* pueden transportar el nitrógeno derivado de insectos parasitados a las plantas, lo que plantea una novedosa vía de apoyo de los HEE en la adquisición de este nutriente por parte de las plantas. Por otro lado Krell et al. (2018). entregan evidencia de la capacidad que tienen cepas endófitas de *M. brunneum* para disminuir el estrés nutricional mediante la movilización del fósforo y su trasferencia hacia las plantas de papa.

Otros estudios han confirmado la capacidad que tienen los HEE de producir reguladores de crecimiento de las plantas. Liao et al., (2017) demostró que fitohormonas del grupo de las auxinas y citoquininas podían ser sintetizadas por estos hongos ya que cepas de *M. robertsii* inoculadas en plantas de *Arabidopsis* sp., estimularon la división celular por la producción de ácido indol-3-acético. En estudios realizados por Waqas et al. (2012), se confirmó la capacidad que tienen cepas endófitas de *Penicillium* sp. aislado de la raíz de pepino en la producción de

hormonas vegetales, ya que confirmaron que el hongo puede producir giberelinas y ácido indolacético en medios líquidos.

1.5 Perspectiva para el desarrollo de los HEE y su aplicación en la agricultura para alcanzar sistemas productivos sustentables

Dada la capacidad que presentan los HEE para mitigar los daños provocados a las plantas por factores bióticos y abióticos, se presentan como una alternativa prometedora en el desarrollo de bioproductos con aplicación en la agricultura, lo que permitiría reducir el uso de agroquímicos alcanzando producciones más sustentables.

Estos hongos son ubicuos en la naturaleza y muchos de ellos están presentes de manera natural en las plantas; sin embargo, también se ha demostrado su capacidad de colonizar un importante número de plantas cultivadas mediante inoculaciones artificiales (Gurulingappa et al., 2010; Jaber, 2015; Jaber y Enkerli, 2016; Sánchez-Rodríguez et al., 2018; Barra-Bucarei et al., 2020). La forma en como estos hongos colonizan la planta y las funciones que cumplen estando en su interior, permitiría superar problemas que actualmente presenta el uso comercial de los hongos entomopatógenos en su acción como epífito al ser aplicado al follaje, como es la baja persistencia de esporas en el medio ambiente afectadas por condiciones de temperatura, humedad, precipitaciones y la radiación UV (Jackson et al., 2010; Meikle et al., 2003; Lacey et al., 2015). La colonización interior de la planta puede ser una estrategia para aumentar su sobrevivencia, además su capacidad de persistir en su interior durante un tiempo considerable, proveería de un periodo de protección a la planta frente a diversos estreses (Posada et al., 2007; Brownbridge et al., 2012)

Los HEE desarrollan diversas funciones en asociación con las raíces y su capacidad de vivir como saprófito, independiente de la planta, podría permitir aumentar la microbiota benéfica del suelo y estar disponible para colonizar nuevos cultivos (Vega et al., 2008; Reay et al., 2010). Las esporas se producen generalmente en grandes cantidades y de forma continua, siempre que las

condiciones de crecimiento sean favorables, por lo que estos hongos pueden persistir durante mucho tiempo en el suelo. También puede ser una ventaja, desde el punto de vista de su integración en el manejo agronómico de los cultivos, el hecho que los HEE hayan sido ampliamente estudiados en su acción directa como entomopatógenos y se hayan desarrollado sistemas de producción a escala industrial, además de productos comerciales con licencias cuyas formulaciones contienen conidios y/o micelios que permiten su aplicación en el campo (De Faria y Wraight, 2007; Lacey, et al., 2015; Barra-Bucarei, et al., 2019). Actualmente estos hongos están incorporados en programas de manejo integrados de plaga de diversos cultivos con ventajas en términos de bioseguridad, ya que su manejo y aplicación no causan problemas ambientales y son considerados seguros para la salud humana y animal (Vega et al., 2008).

Una dificultad que debe enfrentar el desarrollo de productos en base a HEE es la capacidad que estos hongos tienen de producir metabolitos secundarios, los cuales podrían ser potencialmente dañinos para la salud de humanos y animales al ser ingeridos, inhalados o al entrar en contacto con la piel (Marin et al., 2013). Este es el caso de Beauvericina, considerado como micotoxina producida por hongos de los géneros *Beauveria* y *Fusarium* (Mallebrera et al., 2016); sin embargo, a la fecha no se han encontrado reportes de micotoxinas en plantas tratadas con HEE, por lo que es necesario realizar estudios en este ámbito, con el objetivo de descartar potenciales efectos dañinos ocasionados a consecuencia del uso de endófitos en la producción agropecuaria.

1.6 Hipótesis

- Cepas nativas de *B. bassiana* se establecen como endófito en plantas de tomate colonizándola de forma sistémica.
- Cepas nativas del endófito *B. bassiana* pueden promover el crecimiento del tomate.
- *Beauveria bassiana* endófita actúa como agente de biocontrol en tomate, entregándole protección contra *Botrytis cinerea* y *Trialeurodes vaporariorum.*

1.7 Objetivo

Determinar la capacidad de colonización endofítica de cepas nativas de *Beauveria bassiana* en tomate, su actividad como agente promotor de crecimiento y de biocontrol.

1.7.1 Objetivos específicos

1. Determinar la capacidad y tipo de colonización endofítica de cepas nativas de *B. bassiana* en plántulas tomate.
2. Evaluar el potencial de promoción de crecimiento del endófito *B. bassiana* en tomate y determinar posibles mecanismos de acción involucrados.
3. Determinar el efecto antagónico *in vivo* e *in vitro* del endófito *B. bassiana* frente al hongo fitopatógeno *Botrytis cinerea* en tomate.
4. Evaluar el efecto biocontrol del endófito *B. bassiana* frente a *Trialeurodes vaporariorum* en tomate bajo invernadero.

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II. CAPITULO 2: Antifungal Activity of *Beauveria bassiana* Endophyte against *Botrytis cinerea* in Two *Solanaceae* Crops

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2.1 ABSTRACT

Botrytis cinerea causes substantial losses in tomato and chili pepper crops worldwide. Endophytes have shown the potential for the biological control of diseases. The colonization ability of native endophyte strains of *Beauveria bassiana* and their antifungal effect against *B. cinerea* were evaluated in *Solanaceae* crops. Root drenching with *B. bassiana* was applied and endophytic colonization capacity in roots, stems, and leaves was determined. The antagonistic activity was evaluated using in vitro dual culture and also plants by drenching the endophyte on the root and pathogen inoculation in the leaves. Ten native strains were endophytes of tomato and eight of chili pepper. All strains showed significant in vitro antagonism against *B. cinerea* (30%–36%). A high antifungal effect was observed, strains RGM547 and RGM644 showed the lowest percentage of the surface affected by the pathogen. Native strains of *B. bassiana* colonized tomato and chili pepper tissues and provide important levels of antagonism against *B. cinerea.*

Keywords: entomopathogenic fungi; biological control; antifungal activity; *Solanum lycopersicum*; *Capsicum annuum*

2.2 INTRODUCTION

Among the phytosanitary problems affecting crops in the *Solanaceae* family, such as tomato (*Solanum lycopersicum* L.) and chili pepper (*Capsicum annuum* L.), fungal diseases that produce economic losses are highlighted. Intensive production systems, like greenhouse cultivation, high temperatures, and humidity, favor disease development and dissemination [1]. *Botrytis cinerea* is the etiological agent of grey mold, which affects more than 200 plant species and is the most frequent disease in the cultivation of tomato and chili pepper worldwide. This fungus is able to penetrate wounds and colonize the whole aerial part of the plant [2], affecting the whole growing cycle and at postharvest [1], and reducing fruit yield and quality. In greenhouse conditions, *B. cinerea* enters the tissue through pruning cuts, leaves, and flowers. In turn, damage to the fruits occurs largely at postharvest [3, 2].

Botrytis cinerea is difficult to control because it can infect using different strategies and use different hosts as its inoculum source because it can survive as mycelia, conidia, and/or sclerotia for long periods of time in crop residues and in the soil. Chemical fungicides are intensively used for its control; however, there is scientific evidence that highlights their detrimental effects on human health, the environment, and the economy [4,5]. Moreover, their effectiveness has been affected by the appearance of resistant strains [6]. In this context, the biological control of diseases using microbial agents has advantages over chemical fungicides, given the availability of agents such as fungi, yeasts, and bacteria [2] and their safety and decreased probability of producing resistant strains.

In recent years, world markets have shown a growing tendency towards the use of biological control agents as an alternative to chemical synthesis pesticides [7]. Many microorganisms are currently being used as biopesticides because they offer a series of additional benefits over and above their objective function [8]. Entomopathogenic fungi stand out in this group, and have been extensively studied for their effects as epiphytes for use in pest control and, on a smaller scale, against plant diseases [9,10]. However, in the last few years, there has been also a growing interest to study them as endophytes.

Endophytic fungi are defined as microorganisms that spend most or all of their lifecycle colonizing host plant tissues without causing any apparent damage to the host [11]. They are associated with the majority of plants, found naturally in the ecosystem, and considered an extremely important partner for plant development [12,13]. These microorganisms have been of interest in recent years because of the beneficial characteristics they are able to confer to their hosts [14]. Some of these benefits are plant growth promotion, inhibition of pathogenic organisms, removal of soil contaminants, and increased tolerance to extreme temperature, water availability, and salinity conditions [15,16,17], all of which are important features for future agri-food production.

Several studies have demonstrated that endophytic fungi can protect host plants against pathogens and herbivores [18,19,20,21,22]. The host plant receives multiple benefits from the interaction with the endophyte in exchange for carbon-based resources [23]. Endophytes can remain in plant tissues for long periods of their lifecycle, thus protecting them from pathogen attacks as well as potential environmental changes that could threaten their survival and biocontrol efficiency [24]. They can establish interspecific interactions, and the protection against pathogen attack is produced by direct mechanisms, such as competition, parasitism, antibiosis (production of primary and secondary metabolites, enzymes, or volatile compounds), and indirect mechanisms, such as induction of resistance [18, 25].

In the present study, we used native strains of the entomopathogenic fungus *Beauveria bassiana* to determine its ability to endophytically colonize tomato and chili pepper plants. We also investigated if its presence inside the plants enables the mitigation of the negative effects caused by *B. cinerea.*

La agricultura desempeña un papel vital en el suministro de alimentos. Se espera que la población mundial llegue a 9.730 millones de personas para 2050, lo que significa que la agricultura tiene que producir más alimentos para satisfacer esta creciente demanda y, por lo tanto, mejorar la productividad agrícola (Muller et al. 2017). El nitrógeno (N), el fósforo (P) y el potasio (K) son los principales macronutrientes esenciales para el crecimiento y la productividad de las plantas; sin

embargo, no siempre están disponibles para las plantas en el suelo (Meena et al., 2014).

Los diferentes géneros bacterianos son componentes vitales de los suelos. Están involucrados en diversas actividades bióticas del ecosistema del suelo haciéndolo más dinámico y sostenible para la producción de cultivos (Camelo et al., 2011). Las bacterias estimulan el crecimiento de las plantas mediante la movilización de nutrientes en los suelos (Rajkumar et al., 2010). Las bacterias que se alojan alrededor de las raíces de las plantas, las rizobacterias, son versátiles en la transformación, movilización, solubilización de los nutrientes (Hayat et al., 2010). Por ende, las rizobacterias son las fuerzas dominantes en el reciclado de los nutrientes del suelo y, en consecuencia, son cruciales para su fertilidad (Pii et al., 2015). Su aplicación en los sistemas productivos constituye una alternativa viable para reducir los costos de producción y el impacto ambiental asociado a la fertilización química (Alfonso et al., 2005). Con base en lo anterior, el objetivo de esta investigación fue seleccionar rizobacterias nativas de Chile por su capacidad de fijar N_2 y solubilizar fósforo y potasio *in vitro*.

2.3 MATERIALS AND METHODS

2.3.1 Source of fungal strains and seed

Native strains of *B. bassiana* were used in the assays, which were isolated by the methodology described by France et al. [26] and were identified by morphological and molecular methods. *Botrytis cinerea* strains isolated from tomato were also used. All strains form part of the Chilean Collection of Microbial Genetic Resources (CChRGM) (Table 1). For the seeds, tomato var. Limachino—INIA traditional cultivar and chili pepper seeds STa_01, from the Sweet Saten Company, were used.

Table 1. Fungal strains used in this study.

Code Strain*	Species	Collection location	Origin
RGM 393	*Beauveria bassiana*	Robinson Crusoe, Valparaíso Region, Chile.	Native forest soil
RGM 461	*Beauveria bassiana*	Cañete, Biobío Region, Chile.	Natural pasture soil
RGM 547	*Beauveria bassiana*	Santa Bárbara, Biobío Region, Chile.	Natural pasture soil
RGM 557	*Beauveria bassiana*	Los Lagos, Los Lagos Region, Chile.	Natural pasture soil
RGM 565	*Beauveria bassiana*	Portezuelo, Biobío Region, Chile.	Natural pasture soil
RGM 570	*Beauveria bassiana*	Molina, Maule Region, Chile.	Arable soil, *Vitis vinifera* fruit crop
RGM 632	*Beauveria bassiana*	Pencahue, Maule Region, Chile.	Natural pasture soil
RGM 644	*Beauveria bassiana*	Icalma, La Araucanía Region, Chile.	Natural pasture soil
RGM 657	*Beauveria bassiana*	Puerto Ibañez, Aysén del General Carlos Ibáñez del Campo Region, Chile.	Natural pasture soil
RGM 731	*Beauveria bassiana*	Río Cisnes, Aysén del General Carlos Ibáñez del Campo Region, Chile.	Natural pasture soil
RGM 2519	*Botrytis cinerea*	Colín, Maule Region, Chile	Tomato plant

* Accession number of microorganisms from the Chilean Collection of Microbial Genetic Resources—CChRGM. Fuente: Elaboración propia.

2.3.2 Surface seed disinfection

Seeds were disinfected according to the protocol adapted from Ownley et al. [21] and Griffin [20]. Seeds were submerged in a 95% ethanol solution for 1 min and then in 1.5% NaOCl for 3 min. They were washed three times for 1 min in sterile distilled water. Seeds were dried at room temperature on sterile absorbent paper for 3 h in a biosecurity cabinet and then used in all the assays involving plants. To test seed disinfection, a water sample from the third wash was taken with a bacteriological loop and streaked onto a Petri dish with PDA medium. On another dish with the same medium, the surface of one of the disinfected seeds was printed to rule out epiphytic colonization.

2.3.3 Plant inoculation with *B. bassiana* strains and endophytic colonization

Single conidia were sown on Petri dishes with 100% potato dextrose agar (PDA, Disfco^{Tm}) and 150 mg L^{-1} chloramphenicol. The dishes were incubated in the dark at 25 ± 2 °C for 10 days. Conidia were harvested and their viability was determined according to the methodology described by Moore et al. [27]. These were suspended in test tubes with 10 mL sterile distilled water and 0.01% (v/v) Tween 80% (Difco^{Tm}), and dilutions were performed until the concentration of 1 × 10^6 conidia mL^{-1} was reached. In the case of the *B. cinerea* pathogen, it was used at concentrations of 1 × 10^5 conidia mL^{-1}. The concentration of conidia was determined with a Neubauer counting chamber (BOECO, Germany), and the same inoculum was used for all the experiments.

Conidia suspension was inoculated in 100 mL test tubes containing 15 mL PDA [20]. One test tube was left without fungal inocula as a control. Test tubes were incubated in the dark at 25 ± 2 °C for 4 days. Once the apparent growth of the fungus in each tube was observed, 20 mL of the substrate was added, which consisted of a mixture of perlite, peat, compost, and vermiculite (2:2:2:1) sterilized twice in an autoclave at 120 °C and 115 psi for 1 h. Controls were incubated at 25 ± 2 °C for 5 days in the dark. Two seeds were sown in each test tube and, after emergence, one plant per tube was left. The tubes were incubated for 30 days in growth chambers at 25 ± 2 °C with 12 h/12 h light/darkness photoperiod and arranged in a randomized design with six replicates.

2.3.4 Assessment of endophytic colonization

To assess the endophytic colonization of *B. bassiana* trains, plants were extracted from the tubes, washed with tap water, and cuts were made to separate roots, stems, and leaves (n = 5). Each tissue was disinfected with 70% ethanol for 2 min, 1.5% NaOCl for 5 min, and rinsed three times for 1 min with sterile distilled water. Then they were left to dry on sterile absorbent paper, a modification of Resquín-Moreno et al. [28]. To control disinfection, the same procedure as used to test the seeds was conducted. Once disinfection was accomplished, 10 subsamples were cut from each plant part (roots, stem, and leaves), resulting in a total of 30

pieces per plant. Root and stem samples were 10 mm long, whereas leaves were cut in 6 mm discs. The cuttings were distributed in Petri dishes with Noble agar (Difco™) medium plus chloramphenicol and incubated in the dark at 25 ± 2 °C for 30 days. After the incubation, the pieces were evaluated by the presence of fungus growing from the border of the tissues, and the result obtained was the percentage of endophytic colonization (PEC). Strains that exhibited no endophytic colonization were discarded for use in the following evaluations.

2.3.5 Morphological and molecular identification of re-isolate strain

Agar samples (1 cm^2) with mycelia growing from inside the leaves inoculated with the endophyte were placed on a slide. Fungal structures were observed under an optical microscope with 40× magnification and were identified through taxonomic keys [29]. For molecular identification, samples were taken from mycelia coming out of the plant tissues and sown in the PDA medium. The polymerase chain reaction (PCR) method was used with specific markers for *B. bassiana* P1 (5′AAGCTTCGACATGGTCTG) and P3 (5′GGAGGTGGTGAGGTTCTGTT) according to the methodology described by Hegedus and Khachatourians [30]. Amplification products were separated in agarose gel and observed under ultraviolet light. Once the identity was confirmed, monoxenic samples were taken from the re-isolation of each strain and used as the inoculum in the next assays.

2.3.6 In vitro antifungal activity of endophytic *B. bassiana*

Mycelium discs (3 days, 5 mm diameter) from pure *B. bassiana* cultures obtained from plant tissues were placed 1.5 cm from the edge of a 9 cm Petri dish containing the PDA medium (20 mL). After 2 d, discs with *B. cinerea* pathogen (3 days, 5 mm diameter) were equidistantly placed opposite to the endophyte and incubated in the dark at 25 ± 2 °C for 10 d. Six replicates were included per treatment were placed in a completely randomized design. At the same time, control dishes containing only the pathogen were prepared [31]. When the pathogen colonized the whole Petri dish, the percentage of radial growth inhibition of the pathogen (PRGIP) was determined by Formula (1). The radio of the colonies was measured (mm) in each of the treatments using a digital caliper.

Formula (1) $PRGIP: [(\mathrm{R1} - \mathrm{R2})/\mathrm{R1}]\ \mathrm{x}\ 100$

where R1 is the radius of the pathogen colony growing alone (mm) and R2 is the radius of the pathogen colony competing against the endophyte (mm).

2.3.7 Evaluation of antifungal activity in the host plant

Previously disinfected tomato and chili pepper seeds were sown in individual 300 mL pots on a substrate consisting of a mixture of perlite, peat, compost, and vermiculite (2:2:2:1) sterilized twice in an autoclave at 120 °C and 115 psi for 1 h. Pots were left in growth chambers (at 25 ± 2 °C, 65% relative humidity, and 12 h/12 h light/darkness photoperiod) for 30 and 60 days for tomato and chili pepper, respectively. Plants were observed and watered with 3 mL of sterile distilled water every 5 days. Plants with 4–5 true leaves were inoculated with the endophyte by drenching the substrate with a solution of 5 mL sterile distilled water and 2 mL of the conidial solution (1 x 10^6 conidia mL^{-1}). The five strains that exhibited the best antifungal performance in the in vitro tests were selected. Pots were covered at their base with aluminum foil to prevent cross-contamination of the endophyte and maintained for another 10 days in the growth chambers under the same conditions previously described. A conidial solution of the pathogen (1 x 10^5 conidia mL^{-1}) was then prepared, using a modification of the method by Martin-Hernández et al. [32], and applied 14 days after the endophytes as a foliar spray on tomato and chili pepper leaves (leaves of the same age). For the analyses, three and two leaves located in the middle part of the chili pepper (eight weeks) and tomato plant (six weeks), respectively, were selected. The treatments were arranged in a randomized design, with five replications for each strain.

The disease incidence in both hosts was evaluated 10 days post-inoculation of the pathogen [33] by calculating the percentage of the surface affected by the pathogen (PSAP) by Formula (2). The total area (TA) and affected area (AA) per leaf were obtained with the ImageJ, an open source image processing software [34]. The software allows quantitatively determining the leaf area covered by sporulation,

damage, or chlorotic and necrotic symptoms. For both species, the assay was conducted at two times under the same conditions.

$$\text{PSAP: } (S2/S1) \text{ x } 100 \quad 1)$$

where S1 is the total leaf surface and S2 is the leaf surface affected by the pathogen.

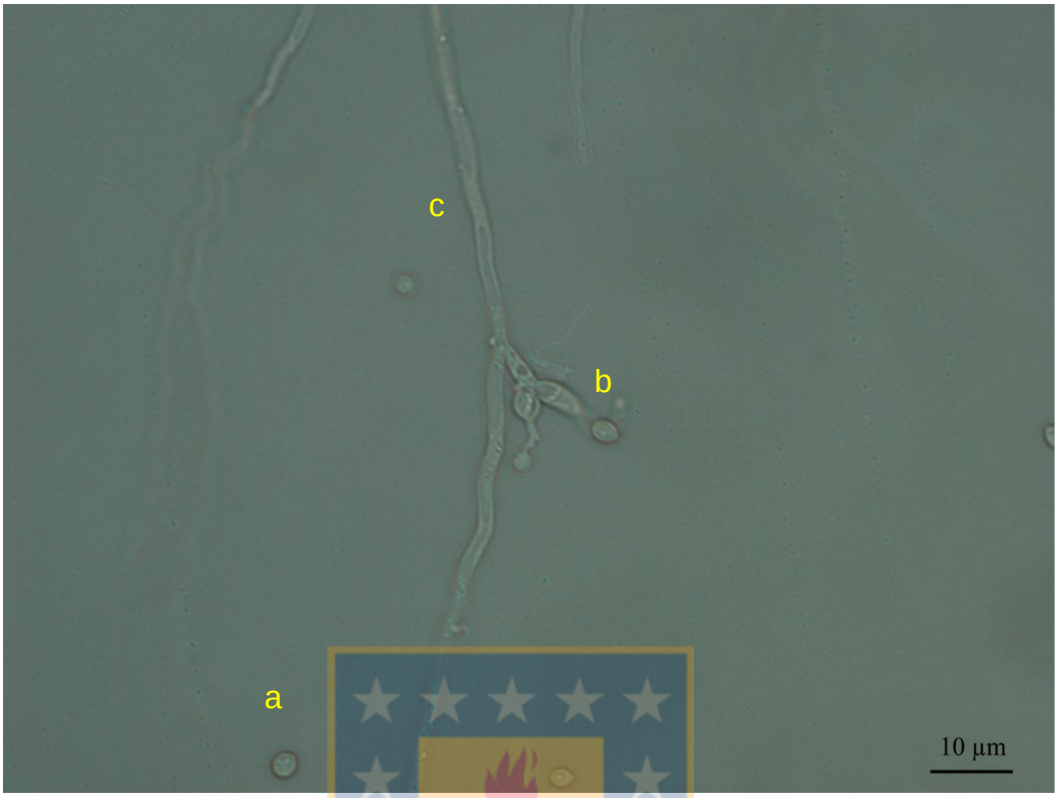
2.3.8 Statistical analyses

In all cases, a completely randomized design was used. Prior to the statistical analyses, data were used to determine the normality and homogeneity of the variance. The results were used to perform variance analyses and the means were compared with the least significant difference (F-LSD) test ($p < 0.05$) with the software InfoStat Version 2018 [35].

2.4 RESULTS

2.4.1 Endophytic colonization of *B. bassiana* strains in plants of tomato and chili pepper

The ten evaluated strains of *B. bassiana* colonized internally the different parts of the tomato plants, while eight strains colonized the chili pepper plants. White mycelium growing from the different plant tissues was observed with an optical microscope (Figure 1). Structures such as hyphae, conidiophore, and conidia of *B. bassiana* were observed in the different treatments, and confirmed by morphology and subsequent molecular analyses. The specific molecular markers gave positive amplification in all of the evaluated strains; consequently, the endophyte identity was confirmed as *B. bassiana*.



Fuente: Elaboración propia.

Figure 1. Morphological characteristics of endophyte *Beauveria bassiana* (RGM 644) on Noble agar (100×). (a) conidia, (b) conidiogenous cells, and (c) hyphae.

By using both disinfection control methods (water and tissue printing), epiphytic colonization could not be detected and, therefore, all the observed mycelium growth came from the internal tissues. No *B. bassiana* was re-isolated from the different tissue plants used as a control without fungal inoculation (Table 2).

In tomato, 100% of the evaluated strains exhibited some degree of endophytic colonization. Colonization fluctuated between 10% and 48% in the leaves and 8% and 46% in the stem and roots. Furthermore, 100% of the strains demonstrated a systemic mode of action, where the fungus inoculated in the roots was re-isolated from the leaves. The strain with the best performance, considering the sum of all plant tissue, was RGM 557 (50%), while the strain RGM 393 had the worst performance (10%) (F = 1.24; df = 10; P = 0.29). For chili pepper, colonization fluctuated between 0% and 68% in the leaves, 0% and 14% in the stem and roots 0% and 34% in the stem and roots. Strains RGM 393 and RGM 461 exhibited no endophytic colonization

ability. The strain with the best overall performance was RGM 547 with 35% (sum of all tissue plant colonized), and the worst performance (F = 14,76; df = 10; P < 0.0001) among the strains that achieved some degree of colonization was RGM 632 with 7%. The highest percentages of endophytic colonization, between the different tissues of tomato plant, are in stems. In the chili pepper plant, the highest percentages are in the leaves.

Table 2. Endophytic colonization (%) of *Beauveria bassiana* in tomato and chili pepper (n = 5).

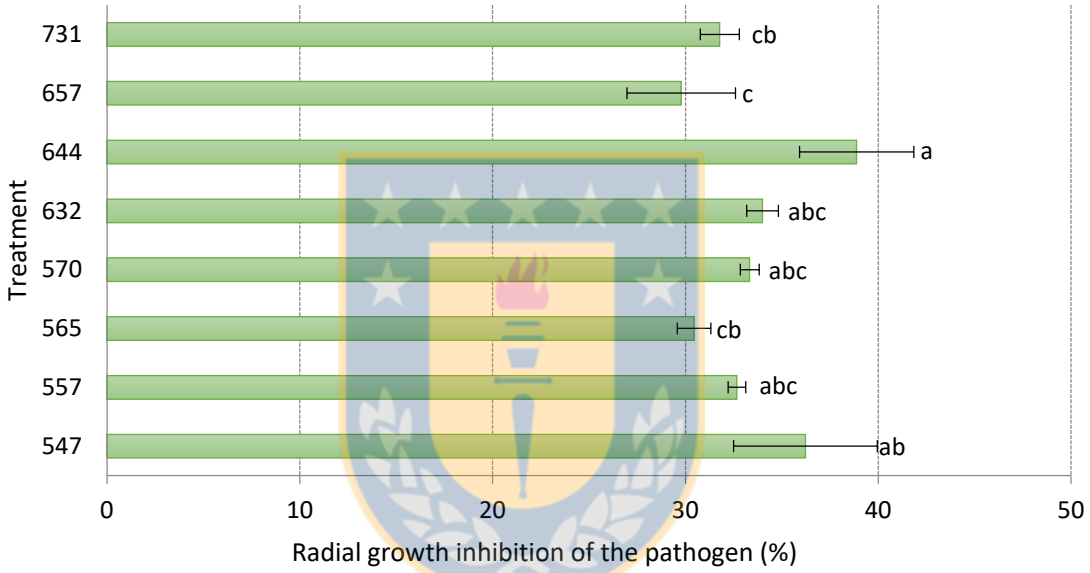
Species	Part plant	Treatments										
		RGM 393	RGM 461	RGM 547	RGM 557	RGM 565	RGM 570	RGM 632	RGM 644	RGM 657	RGM 731	Control[1]
Chili pepper	Leaves	0[2] ± 0.0[3] d[4]	0 ± 0.0 d	68 ± 13.6 a	28 ± 13.6 bc	36 ± 16.0 b	36 ± 7.5 b	10 ± 3,2 cd	36 ± 7.5 b	32 ± 8.0 bc	52 ± 8.0 ab	0 ± 0.0 d
	Stems	0 ± 0.0 b	0 ± 0.0 b	2 ± 2.0 b	12 ± 2 a	14 ± 2.5 a	14 ± 2.5 a	2 ± 2.0 b	14 ± 4.0 a	14 ± 2.5 a	14 ± 2.5 a	0 ± 0.0 b
	Roots	0 ± 0.0 d	0 ± 0.0 d	34 ± 6.8 a	18 ± 5.8 bc	18 ± 8 bc	18 ± 3.7 bc	10 ± 3.2 cd	16 ± 5.1 bc	24 ± 9.8 abc	28 ± 3.7 ab	0 ± 0.0 d
Tomato	Leaves	10 ± 6.3 ab	10 ± 3.9 ab	34 ± 17.2 ab	48 ± 23.1 a	24 ± 19.4 ab	22 ± 11.8 ab	20 ±12.3 ab	24 ± 19.4 ab	26 ± 3.7 ab	18 ± 8.0 ab	0 ± 0.0 b
	Stems	12 ± 8.0 abc	36 ± 19.8 abc	44 ± 21.1 ab	38 ± 19.4 ab	36 ± 16. abc	46 ± 6.9 a	12 ± 8.0 abc	12 ± 8.0 abc	8 ± 5.8 bc	36 ± 6.0 abc	0 ± 0.0 b
	Roots	8 ± 4.9 b	18 ± 11.9 ab	24 ± 19.4 ab	46 ± 22.3 a	34 ± 17.2 ab	16 ± 5.1 ab	20 ± 12.3 ab	28 ± 18.6 ab	18 ± 9.7 ab	8 ± 4.9 b	0 ± 0.0 b

Fuente: Elaboración propia

[1] Control represents plants without *Beauveria bassiana* applications. [2] Mean values of endophytic (%) colonization (n = 6). [3] Standard error. [4] Mean values of the same treatment followed by different letter are significantly different according to Fisher's LSD test ($p < 0.05$). Fuente: Elaboración propia.

3.4.2 In vitro growth inhibition of the pathogen

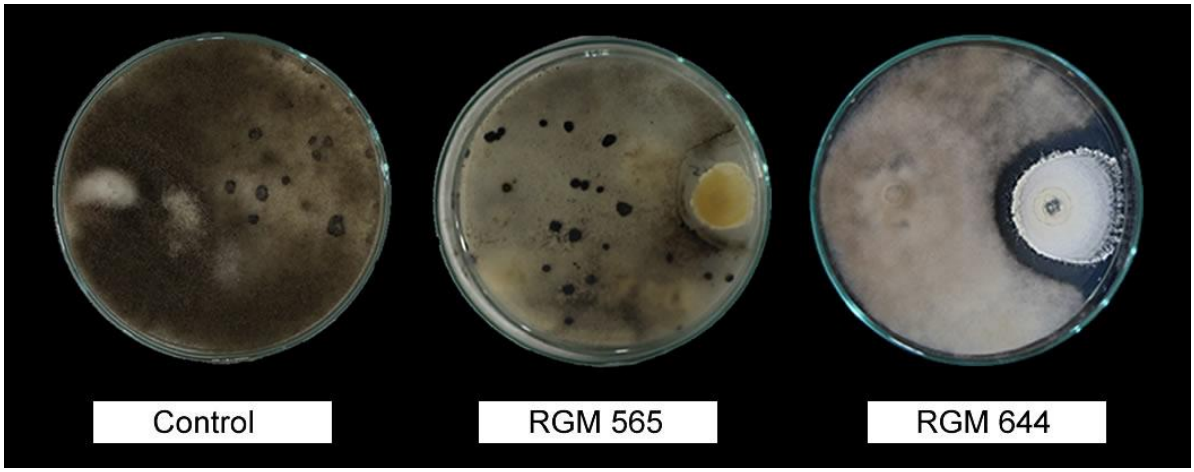
The monoxenic culture of the pathogen was able to totally colonize the Petri dish after 7 days in a 75 mm radius. When the advance of the pathogen against the endophytic strains was measured, all the cases showed a level of radial growth inhibition of the pathogen, the PGIP fluctuated between 30% and 39%. The pathogen was only able to grow 45 mm against strain RGM 644 (F = 2.13; df = 7; P = 0.062), while the greatest advance (53 mm) was against the strain RGM 657 (Figure 2).



Fuente: Elaboración propia

Figure 2. Radial inhibition growth of *Botrytis cinerea* (%) against strains of endophytic *Beauveria bassiana* at 7 days (n = 6). Data represent the mean ± standard error. Different letters over the bars represent significant differences among the treatments according to the Fisher's LSD test (p < 0.05).

It was observed that some strains did not allow mycelia to achieve the density and tonality found in the control plate. Moreover, in strains with the best performance in terms of PGIP, almost no sclerotia were detected (Figure 3).

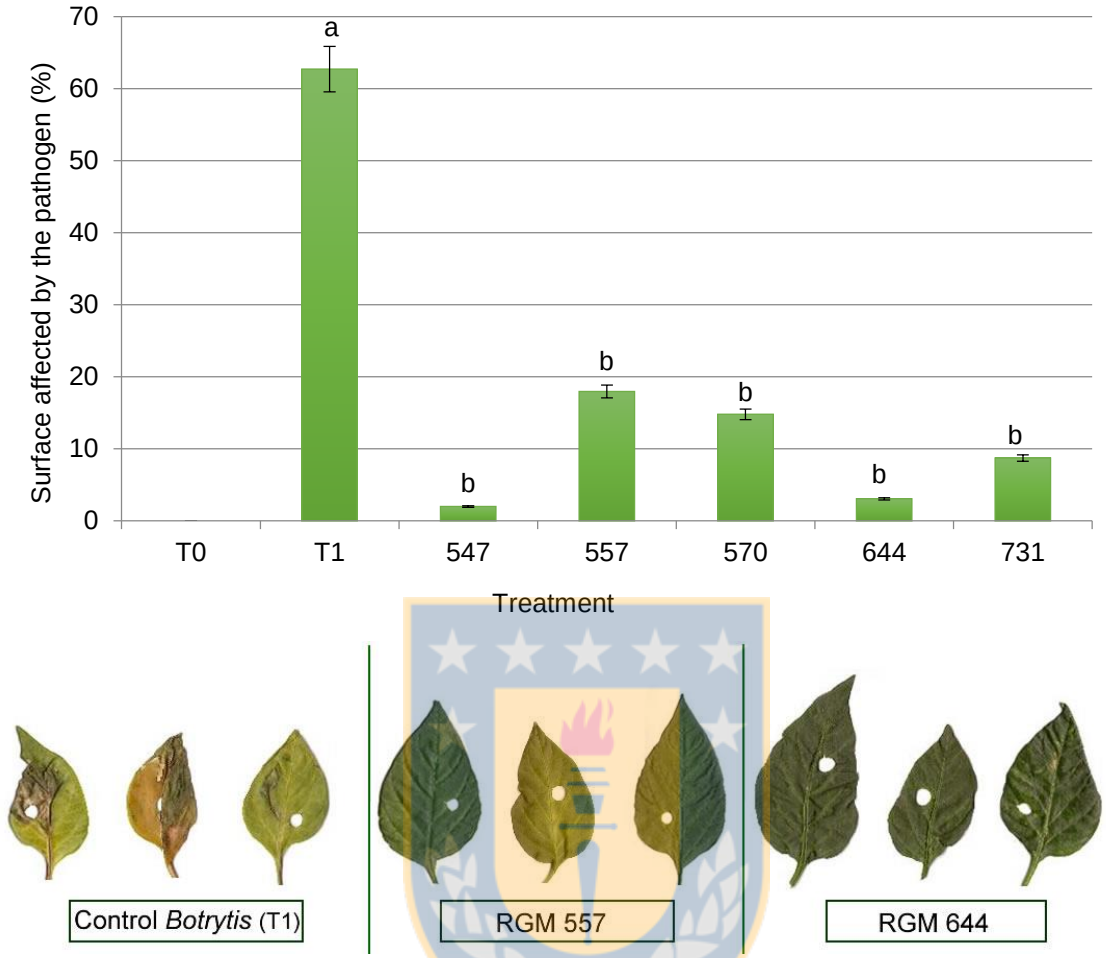


Fuente: Elaboración propia

Figure 3. Dual cultures of *Botrytis cinerea* against different endophytic strains of *Beauveria bassiana.* (a) *Botrytis cinerea* (control) 7 d after inoculation, (b) pathogen against endophytic strains RGM 565, and (c) RGM 644, showing different mycelia density and inhibition of sclerotia.

3.4.3 Antifungal activity in the host plant

The PSAP in chili pepper leaves was noteworthy lower in plants (F = 11.46; df = 6; P < 0.0001) inoculated with endophytes compared to plants only inoculated with the pathogen. The plants inoculated with endophytes ranged from 2% to 18% of PSAP; with the lowest for the RGM 547 strain and the highest for RGM 557. Meanwhile, treatment only with the pathogen exhibited early symptoms of the disease (during the first week of inoculation) with a PSAP of 63%, while some symptoms were observed after the second week in plants with endophytes (all endophyte treatment). Leaves of plants that were not inoculated with the pathogen showed no symptoms of the disease (Figure 4).

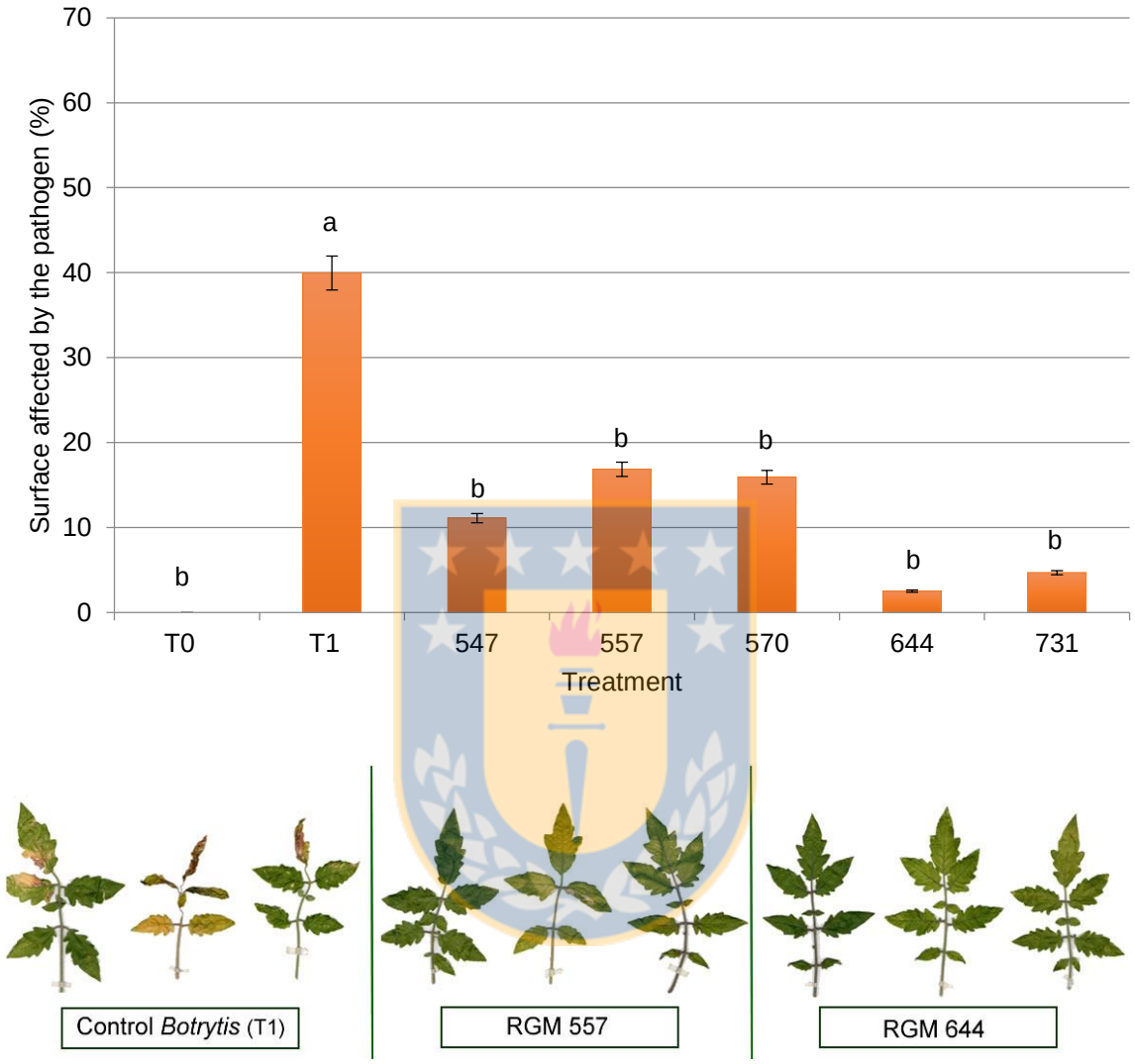


Fuente: Elaboración propia

Figure 4. Surface affected by the pathogen (cm2) in chili pepper leaves 10 days post-inoculation (n = 5). The leaves of plants not inoculated with *Botrytis cinerea* (T0) were asymptomatic. The leaves with endophytic strains showed a low level of symptoms (RGM 547, RGM 557, RGM 570, RGM 644, and RGM 731), while the leaves of plants inoculated with *B. cinerea* RGM 2519 (T1) exhibited chlorotic and necrotic spots. Values are expressed as means ± standard error. Means with different letters are significantly different at $p < 0.05$ by Fisher's LSD test ($p < 0.05$).

In the case of tomato, leaves of plants inoculated with the pathogen had the highest PSAP (40.0%), which was notably higher ($F = 4.51$; $df = 6$; $P = 0.0007$) than the percentages obtained for plants inoculated with endophytes. This percentage is similar to the result obtained in chili pepper. The range of PSAP for plants with endophytes was 2.5% to 16.9%, and significant differences existed among strains. The strains with the lowest percentage were RGM 644 and 731 with values of 2.5% and 4.7%, respectively. The strains with the highest pathogen incidence were RGM

557 (16.9%) and 570 (15.9%). Leaves of plants that were not inoculated with the pathogen exhibited no symptoms of the disease (Figure 5).



Fuente: Elaboración propia

Figure 5. Surface affected by the pathogen (cm^2) in tomato leaves at 10 days post-inoculation (n = 5). The leaves of plants not inoculated with *Botrytis cinerea* (T0) were asymptomatic. The leaves with endophytic strains showed a low level of symptoms (RGM 547, RGM 557, RGM 570, RGM 644, and RGM 731), while the leaves of plants inoculated with *B. cinerea* RGM 2519 (T1) exhibited chlorotic and necrotic spots. Values are expressed as means ± standard error. Means with different letters are significantly different at $p < 0.05$ by Fisher's LSD test ($p < 0.05$).

2.5 DISCUSSION

2.5.1 Endophytic colonization of *Beauveria bassiana* strains

The present study reports on the colonization of *B. bassiana* in tomato and chili pepper as endophytes. Every evaluated strain was able to endophytically colonize tomato plants; these results concur with those shown by other studies in tomato [20,21,36]. As for chili pepper, 80% of the strains endophytically colonized the plants. Studies conducted by Paul et al. [37] reveal an assorted diversity of endophytic microorganisms in chili pepper, including some entomopathogens as *Paecilomyces*, *Cordyceps*, *Cladosporium*, and *Penicillium*; however, the authors do not report the natural presence of *B. bassiana*. Recent studies on chili and sweet pepper demonstrated the endophytic colonization of *B. bassiana*, these are some examples of the limited numbers of reports about the colonization on this species [38,39].

The results of endophytic colonization in tomato leaves (10%–48%) in this study were lower than those presented by Klieber and Reineke [40], who obtained the highest colonization (60%). This may be due to several factors, such as the disinfection technique, which could kill the endophyte [36], the inoculation techniques [21], the type of leaf used for sampling (tissue age and location in the plant), and the time between inoculation, re-isolation [40], and the plant´s endophytic microbial community because actinobacteria and yeasts were also found in the re-isolation. Several studies have provided evidence of the endophytic colonization of *B. bassiana* in plants inoculated by different methods through the seeds, leaves, and roots [41,42,21,43]. This fungus has been reported as a facultative endophyte of several plants [44], suggesting that *B. bassiana* is not a specific endophyte for a plant species or particular cultivar. This characteristic can be related to the specificity exhibited by entomopathogenic fungi with the host insect; this can be very limited in the case of obligate pathogens or very broad in the case of facultative pathogens [45].

It was observed that *B. bassiana* showed a systemic colonization pattern for both plant species and, thus, the fungus was re-isolated from the roots, stem, and leaves. Previous studies conducted with other *Solanaceae* have also demonstrated a systemic colonization pattern for these fungi [43,46,39]. Behie et al. [47] in *Phaseolus*

vulgaris, demonstrated that *B. bassiana* is able to colonize plant tissues on and below the soil, while endophytes of the genus *Metarhizium* prefer to colonize the roots. This colonization pattern is very interesting from the viewpoint of the development of biocontrollers, because it would allow access to places in the plant that most chemical products cannot reach.

The important colonization levels obtained for both species in the present assay can be related to the evolution of these fungi over time. Barelli et al. [48] suggest that some entomopathogenic fungi evolved from fungi related to plants (symbionts) and that the pathogenicity in insects is part of a more recent adaptive process. In other words, these fungi were endophytes at first and then were able to become independent of the plants and survive as insect pathogens. The same authors also propose that these fungi have never left their symbiotic relationship with the plant and that pathogenicity towards insects could be a strategy by which the endophyte has access to nitrogen sources in exchange for carbohydrates provided by the plant; this mechanism was reported by Herre et al. [23] and Behie [49].

Although there is an important number of reports about the endophytic colonization of *B. bassiana* and its action as a biological control agent, most of them have been carried out under controlled conditions, like the present study. However, field conditions could affect its action [50]. *Beauveria bassiana* is classified as saprophytic and exhibits poor competition in the soil [51, 52]. Therefore, soil inoculations could be inefficient because of the poor survival of the endophyte when encountering more competitive microbiota, hindering its arrival to the roots and limiting its endophytic colonization [53]. On the other hand, in foliar applications these fungi spend a significant amount of time on the leaf surface and their germination could be affected by radiation, temperature, and humidity conditions [54].

2.5.2 In vitro growth inhibition of the pathogen *Botrytis cinerea*

The important levels of pathogen inhibition by these nine strains of *B. bassiana* could be due to the ability of these fungi to produce a great variety of bioactive metabolites, which have antimicrobial properties [37,56]. Oosporein, beauvericin, bassianolide, bassianin, beauveriolide, bassiacridin, and cyclosporine are highlighted

among the metabolites produced by this fungus [57,58,59,60], and, of these, oosporein and beauvericin have antifungal activity [61,62]. Studies conducted by Feng et al. [63] determined that the genome of *B. bassiana* has at least 45 different groups of secondary metabolite biosynthesis gene clusters. The dissemination of these compounds in the medium affects the growth of pathogenic fungi. Other in vitro evaluations have also demonstrated the antifungal effect of the endophyte against pathogens such as *Botrytis cinerea*, *Cladosporium herbarum* [64], *Fusarium* spp. [65,64], *Gaeumannomyces graminis* var. *tritici* [66], and *Rhizoctonia solani* [67]. Antibiosis and/or competition are highlighted among the mechanisms used by the endophyte to inhibit pathogen growth. Mycoparasitism was not detected when observing the advance zone under the microscope; this suggests that the evaluated strains have no ability to parasitize *B. cinerea* hyphae.

2.5.3 Antifungal activity in the host plant against *Botrytis cinerea*

The results obtained in this study suggest that the action of *B. bassiana* as an endophyte would increase the plant's ability to resist the attack of pathogens such as *B. cinerea.* Thus, the endophyte exhibits a relevant antifungal activity against *B. cinerea* on both tomato and chili pepper. *Beauveria bassiana* was applied to and subsequently colonized roots, moving up to the stem, presumably through the vascular system [55], and reaching the leaves where the pathogen was inoculated. Several plants inoculated with different strains were asymptomatic, while the control was affected and PSAP was greater than 39%. A growing number of studies provide evidence of the protective action that *B. bassiana* confers on different pathogens [67,21,68,69,70]; the present study is the first report that demonstrates the protective effect of *B. bassiana* endophyte against *B. cinerea* on chili pepper and tomato.

The lowest PSAP obtained in chili pepper and tomato leaves could be the result of a direct or indirect effect of the endophyte in the plant. Direct effects would occur when the endophyte is able to enter through the roots, move through the vascular system until reaching the leaf tissues, and compete for space and food with the pathogen, reducing its colonizing ability [67]. Another possibility could be when

pathogen hyphae enter the leaves and are parasitized by the endophyte hyphae (mycoparasitism), which weaken and decrease their damage potential [21].

The indirect effect could firstly be due to the action of secondary metabolites, as mentioned for dual cultures that could act in distant plant tissues from which they are produced. There would be enzymes degrading the cell wall within these metabolites, which is an important mechanism involved in controlling phytopathogenic fungi [70]. Secondly, indirect action could occur by activating the systemic resistance in the plant, an action mechanism used by the endophyte *B. bassiana* against zucchini yellow mosaic virus in pumpkin and against *Xanthomonas axonopodis* pv. *malvacearum* in cotton, as reported by Jaber and Salem [71] and Ownley et al. [21], respectively. According to Vega et al. [68] and Ownley et al. [16], the antifungal action exhibited by endophytes could be due to a mixture of the previously mentioned mechanisms rather than to the action of a single mechanism.

2.6 CONCLUSIONS

The results of the present study provide evidence of the potential exhibited by endophytic strains of *Beauveria bassiana* to control *Botrytis cinerea* in chili pepper and tomato; eventually, they could be used to control diseases in other species of the *Solanaceae* family. Future research should focus on conducting assays under field conditions because of the possible effects of the environment on soil and leaf inoculations with the endophyte. It is also necessary to perform complete sequencing of the strains, aimed at identifying possible genetic relationships with their antifungal activity.

The use of endophytic fungi could overcome some of the challenges faced in controlling plant diseases, such as chemical fungicide toxicity, the appearance of resistance of some pathogens, and food safety. The studied isolates represent excellent candidates for the development of biocontrol tools to control not only insects but also pathogens. They could be used preventively within an integrated management strategy due to the diversity of mechanisms by which they could act

against the attack of different pathogens. However, as *Beauveria bassiana* produces a considerable amount of secondary metabolites which could act as mycotoxins, it is necessary to increase the number of studies to determine the potential damage that could cause mycotoxins of this fungus in health to the people and animals in its action as endophytes.

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III. CAPITULO 3: *Beauveria bassiana* multifunction as an endophyte: growth promotion and biological control of *Trialeurodes vaporariorum*, (Westwood) (Hemiptera: Aleyrodidae) in tomato.

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3.1 ABSTRACT

Solanum lycopersicum L. is one of the most consumed vegetables in the world; nevertheless, it is affected by biotic and abiotic factors that reduce its productivity. The whitefly is globally considered as the main pest under protected crop conditions, where biological control using endophytic fungi emerges as a sustainable alternative. We evaluated the indirect effects of five native endophytic strains of *Beauveria bassiana* on the reproduction of greenhouse whiteflies and the growth of tomatoes. The plant growth substrate was inoculated with five strains of this endophyte and the resulting plants were then exposed to whiteflies afterwards. The effect that endophytic strains had on phosphate solubilization, iron siderophore production, indole compounds, plant height, and plant biomass were evaluated. The evaluated endophytes reduced the number of eggs per cm^2 on leaflets compared to the control and behaved similarly to the commercial synthetic insecticide. Leaflets inoculated with strains RGM-557, RGM-644 and RGM-731 showed fewer nymphs than the control and those treated with insecticide. RGM-557 and RGM-731 produced the greatest plant heights and RGM-731 obtained the greatest plant biomass. Our study provides evidence that native endophytic strains of B. bassiana have a biocontrol effect on whitefly and could be used to promote tomato growth.

Key words: entomopathogens, endophytes, biocontrol, *Solanun lycopersicum*, greenhouse whitefly.

3.2 INTRODUCTION

Solanum lycopersicum L. is one of the most cultivated vegetables worldwide due to its low fat content and excellent source of dietary fiber, minerals, vitamins and antioxidants [1]. It can be consumed fresh and/or processed into a wide variety of manufactured products [2]. Plant development can be negatively affected by biotic and abiotic factors, provoking decreased yields [3]. Among the biotic factors, several pests negatively affect tomato production; the greenhouse whitefly (GWF) *Trialeurodes vaporariorum* Westwood stands out due to its prolificacy and the important production losses it can cause in both greenhouse and field production. Moreover, the costs for its control are considerable if early measures are not taken. The damage produced consists of perforating the plant tissues and sucking the sap directly from the vascular bundles, which leads to a decrease in photosynthetic activity, reduced vigor and loss of fruit quality (indirect damage) due to the presence of sooty mold [4]. Traditionally, synthetic insecticides from different groups have been used for GWF control, which has generated a selection of resistant populations [5,6]. Therefore, insecticides are not only relatively expensive, they have also decreased their effectiveness over time. Furthermore, the excessive and irrational use of insecticides has led to negative consequences for the environment, with especially negative effects on the soil [7,8]. Microbial agents, such as entomopathogenic fungi, have emerged as a sustainable alternative for GWF control, some of which are available at a commercial level as a substitute for chemical insecticides [9]. Studies in both the laboratory and the field have provided evidence that the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. has potential to control *T. vaporariorum* [10,11,12]. It is also worth noting that in recent years entomopathogenic fungi have

received attention due to their ability to colonize the tissues of a number of plants, in an endophytic association of mutual benefit [13,14].

Many plants live in association with endophytic fungi [15], which live inside their tissues causing no apparent damage [16]. They are inherent organisms to plants, as they establish a symbiotic association of protocooperation with their host for mutual benefit [17]. Endophytes present important phylogenetic and lifestyle diversity traits such as: colonization, dissemination, specificity of plant host, and location in the interior of diverse plant tissues [18]. Within this group, the entomopathogenic fungal endophytes (EFEs) have received much attention due to their agronomic importance [19,14]. Studies have demonstrated that these fungi have the ability to colonize diverse cultivated plants, including species like tomato, bean, corn, and coffee [20,21,22]. These fungi are not obligate plant symbionts and can thus survive without plants [23]. Over recent years, there has been a considerable increase in research on EFEs due to the multiple benefits they provide to plants, including arthropod control, phytopathogen antagonism, and plant growth promotion [24,25,18,13,17,26]. In terms of the mechanisms involved in growth promotion, EFEs have proven to produce phytohormones (auxins), improve water transport, increase the availability of nutrients (solubilization of phosphate, potassium, and siderophores production) as well as acting indirectly by activating biological protection mechanisms and inducing systemic resistance to phytopathogens[18,27]. Within the auxins group, the indole-3-acetic acid is related to plant cell elongation, division and differentiation, and is important in regulating plant defense responses [28,29]. Different species of *Beauveria* can produce organic acids, such as oxalic and citric acids in the case of *B. caledonica*, and formic, lactic, orotic, oxalic and citric acids in the case of *B. bassiana*; these organic acids change the pH of the medium and inorganic phosphorus is released [30]. *Beauveria bassiana* also produces siderophores, which play an important role against the cellular stress caused by iron deficiency; moreover, iron is required for fungal cell growth and metabolism [31].

Some studies have demonstrated that *B. bassiana* can become established as an endophyte in the leaves, stems and roots of sorghum and tomato through the inoculation of leaves, seeds or soil [32,33,34]. This fungus can provide the plant with a greater competitive ability, allowing for the expression of its genetic potential, expressed in higher rates of germination, more biomass accumulation, and greater seed production [35]. Some of the most important mechanisms used by *B. bassiana* for insect control include pathogenicity, antagonism, systemic resistance, and the tritrophic action associated with natural enemies, such as parasitoids [21,36]. Provided the proven abilities of EFEs to control pests and diseases, in addition to promoting plant growth, the objective of this study was to evaluate the potential of native strains of endophytic *B. bassiana* as growth-promoters and their indirect antagonistic effect against *T. vaporariorum* in tomato.

3.3 MATERIALS AND METHODS

3.3.1 Genetic material

Tests were completed at the Instituto de Investigaciones Agropecuarias, INIA-Chile. The tomato plants used were a Mykonos Seminis company variety. Five strains of endophytic fungi were evaluated for growth promotion and their biocontrol effect on GWF (Table 1). These strains form part of the Chilean Microbial Genetic Resource Collection of INIA, and they were morphologically and molecularly identified and selected for their ability to endophytically colonize tomato and chili pepper tissues [34]. In the case of GWF, individuals were collected from a greenhouse tomato crop located in the town of Colín, Maule region, Chile. Once identified according to their morphological features [37], uniform populations were obtained from tomato plants grown under controlled conditions (26 ± 2 ° C, HR = 65 ± 5%, photoperiod= 14:10). A hundred neonatal adults (24 hours) were placed on tomato plants with 4 to 5 true leaves inside 50 x 50 x 50 cm cages covered with anti-aphid mesh (300 µm), according to the methodology described by Oreste et al. [12]. After 20 days, the plants showed leaves with 80-100 nymphs on average.

Table 3. Fungal strains assessed in this study

Code Strain*	Species	Origin	Habitat
RGM-547	*Beauveria bassiana*	Santa Bárbara, Biobío Region, Chile.	Natural pasture soil
RGM-557	*Beauveria bassiana*	Los Lagos, Los Lagos Region, Chile.	Natural pasture soil
RGM-570	*Beauveria bassiana*	Molina, Maule Region, Chile.	*Vitis vinifera*, vineyard soil
RGM-644	*Beauveria bassiana*	Icalma, La Araucanía Region, Chile.	Natural pasture soil
RGM-731	*Beauveria bassiana*	Río Cisnes, Aysén del General Carlos Ibáñez del Campo Region, Chile.	Natural pasture soil

Fuente: Elaboración propia.

* Accession number of microorganisms from the Chilean Collection of Microbial Genetic Resources—CChRGM.

3.3.2 Fungal inoculum

The fungal inoculum was prepared by cultivating each strain in Petri dishes (90 mm in diameter) with potato dextrose agar (PDA) incubated at 25 ± 2°C in the dark for 7 days. After incubation, conidia were harvested from the dishes in a biosecurity cabinet and were then added to a sterile distilled water solution with Tween 20 at 0.01% (Difco^{Tm}). The conidia concentration was estimated using a Neubauer chamber (BOECO, Germany) and was later adjusted to 1×10^{7} conidia mL^{-1} for the assays with GWF and 1×10^{6} conidia mL^{-1} for the growth promotion assays. The conidia viability was evaluated with the methodology proposed by Goettel and Inglis [38]. The suspensions were used for both the GWF biocontrol and growth promotion assays.

3.3.3 Endophyte effect on greenhouse whitefly

Tomato seeds were disinfected for 1 minute in 95% ethanol, then 3 minutes in 1.5% sodium hypochlorite, and 1 minute in 95% ethanol, finally the seeds were rinsed five times for 1 minute each in sterile distilled water. The seeds were then dried on sterile absorbent paper for 1 hour in a biosecurity cabinet. Ten µL of water were taken from the fifth rinse jar and cultured in Petri dishes with PDA plus chloramphenicol to verify the quality of the disinfection process. Afterwards, the seeds were sown in seed trays with a substrate composed of a mixture of perlite, peat, compost and vermiculite (1:1:1:0.5) which was sterilized twice in autoclave at 121°C and 793 kPa for 1 hour.

The plants were placed in a growth chamber with conditions of 24 ± 2°C, 65 ± 2% relative humidity, and a photoperiod of 14 hours of light and 10 hours of darkness. When the plants had two true leaves they were transplanted to 300 mL pots with a sterile substrate similar to the seed trays. They were then placed in 50 x 50 x 50 cm cages covered in anti-aphid mesh (300 µm) located in greenhouses with controlled temperatures (16 ± 3°C at night and 26 ± 3°C during the day), a photoperiod of 14 hours of light and 10 of darkness, and a humidity of 65 ± 5%. Three days later, the plants were inoculated with five strains of *B. bassiana* endophytes. These endophyte strains were applied to the substrate through a 10 mL solution with a concentration of $1x10^{7}$ conidia mL^{-1}; afterwards, the substrate was covered with aluminum foil. An absolute control was inoculated with the same quantity of sterile distilled water, 10 mL, in addition to 0.01% Tween 20 (Difco^{Tm}). A control with a commercial insecticide, whose active ingredient is Fenoxycarb (INSEGAR® 25 WG, Syngenta Crop Protection, Monthey AG, Monthey, Switzerland), was also established. In this case, a 0.6 mL solution of the insecticide was prepared in 1 liter of water and 10 mL of this solution was applied to the foliage of each plant. The following day, the treated plants were placed in cages; each cage contained a plant with an average population of 80 to 100 neonatal nymphs of GWF. Plants were kept in the greenhouse for 45 days and were watered daily with 50 mL of sterile distilled water. Samples of five GWF adults were collected from the leaves and placed in humid chambers to determine the direct pathogenic effect of the five *B. bassiana* endophyte strains. Endophytic colonization was confirmed by taking 10 leaflets from each plant per treatment. These were disinfected and each leaflet was cut into 6 mm discs; these were placed in Petri dishes with Noble agar (Difco™) medium plus chloramphenicol and incubated in the dark at 25 ± 2 °C for 30 days using the method described by Barra-Bucarei [34]. The presence of fungus on the border of the disc was considered as positive, and the obtained result was determined to be the percentage of endophytic colonization.

A completely randomized block design with five replicates per treatment was used. The number of eggs and nymphs (instars III and IV) per cm^2 of leaflet located in the middle part of the leaf was evaluated, considering two leaflets per leaf and two leaves in the midsection of each treated plant.

3.3.4 Analysis of Plant Growth-Promoting Attributes

A qualitative evaluation of the phosphate solubilizing activity of the five strains of *B. bassiana* was carried out. The ability of these strains to solubilize inorganic phosphorus [$Ca_3(PO_4)$] was determined using the phosphate medium from the National Institute of Botanical Research (NBRIP), which contains insoluble glucose, 10 g of $Ca_3(PO_4)_2$; 5 g of $MgCl_26H_2O$; 0.25 g of $MgSO_47H_2O$; 0.2 g of KCl and 0.1 g of $(NH_4)_2\ SO_4$, as well as 5 g of TRP and 15 g of agar. The pH was adjusted to 7.0 and dissolved in one liter of sterile distilled water [39]. Six mm mycelium discs were placed in 8 cm Petri dishes with the NBRIP culture medium. These Petri dishes were then incubated for 10 days at 25 ± 2°C in the dark. A completely randomized design with 10 replicates for each strain was used and the solubilization was determined by the phosphate solubilization index that corresponds to the ratio of the total diameter (colony + solubilization halo) and the diameter of the colony [40].

The production of indole compounds was evaluated. The determination of the hormone indoleacetic acid (IAA) was carried out with the Salkowski methodology [41]. However, due to the possibility of a cross-reaction with other similarly structured compounds, the results were considered a production of indole compounds [42]. The different strains were cultured in a potato dextrose broth (DifcoTm), supplemented with 0.2 mg mL^{-1} of tryptophan and were incubated at 25 ° C for 5 days with 100 rpm of agitation. The cultures were centrifuged for 10 min at 6,000 rpm. Afterwards, 250 µL of the supernatant were taken and mixed with 1 mL of the Salkowski reagent (50 mL of 35% $HClO_4$ and 1 mL of a 0.5M $FeCl_3$ solution). The samples were incubated in the dark at 25°C for 30 minutes, according to the modified method of Bose et al. [43]. The absorbance of the solution was measured with a spectrophotometer at 535 nm (Epoch, Biotek Inc.). The concentration of indole compounds was calculated after interpolating the absorbance in a linear regression performed with known concentrations of commercial IAA (number CAS 6505-45-9, Sigma-Aldrich®, EE. UU.) as a standard. A completely randomized design with three replicates per strain

was used and the development of a pink color was considered as an indicator of the production of indole compounds.

The production of iron siderophores was also evaluated. The chrome azurol S (CAS) technique was used to determine iron mobilization. Six mm mycelium discs of the five different strains were placed in 8 cm Petri dishes with the CAS culture medium. The Petri dishes were left in the incubator for 10 days in the dark at a temperature of 28°C [44], five replicates were carried out for each strain. The production of iron siderophores was determined according to the modified method of Ghosh et al. [45], in which a change in color of the medium, from blue to orange implies a reduction of Fe^{+3} to Fe^{+2}. After subtracting the surface area occupied by the colony of each Petri dish, a value of two was assigned to those strains that showed a change in coloration of more than 50% of the surface of the dish, while a value of 1 was assigned in the case that the coloration change was < 50%. The strains whose mediums did not change color were assigned a value of zero.

3.3.5 Growth promotion *in vivo*

Tomato seeds were disinfected in a similar manner to the methodology previously described when evaluating the endophyte effect on GWF. Afterwards, they were submerged for 4 hours in a sterile distilled water solution with Tween 20 (0.01%) and a concentration of $1x10^6$ conidia mL^{-1} of each strain. In the case of the control, seeds the plants were submerged in a sterile distilled water solution with Tween 20 (0.01%). They were then sown in 300 mL pots with a sterile substrate composed of a mixture of perlite, peat, compost and vermiculite (1:1:1:0,5). The pots were maintained in growth chambers at 24 ± 2°C, 65 ± 2% relative humidity, and a photoperiod of 14 hours of light and 10 hours of darkness; they were watered daily for 30 days. A completely randomized design with five replicates for each treatment was used and measurements of the total plant height (cm) and the stem diameter (mm), measured at the base, were taken. To determine plant biomass, the plants were dried for 48 hours at 60 ± 2 °C separating aerial growth from roots. The assays were carried out twice.

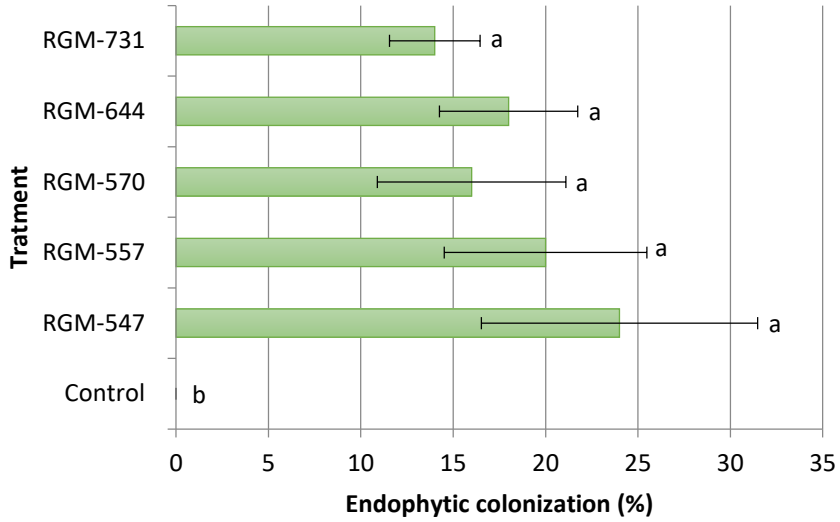
3.3.6 Statistical analysis

In the case of the growth promotion *in vivo* and the phosphate solubilization, the data was analyzed with a one-way analysis of variance (ANOVA) and the measurements were compared with the LSD-Fisher test ($P < 0.05$). For the greenhouse study, as the assumptions of normality and homogeneity in the number of eggs and nymphs variables were not met, non-parametric statistics were used, applying the Kruskal-Wallis test [46]. The statistical program InfoStat version 2011 was used for all cases [47]. All assays were conducted twice under the same conditions.

3.4 RESULTS

3.4.1 Endophyte effect on *T. vaporariorum*

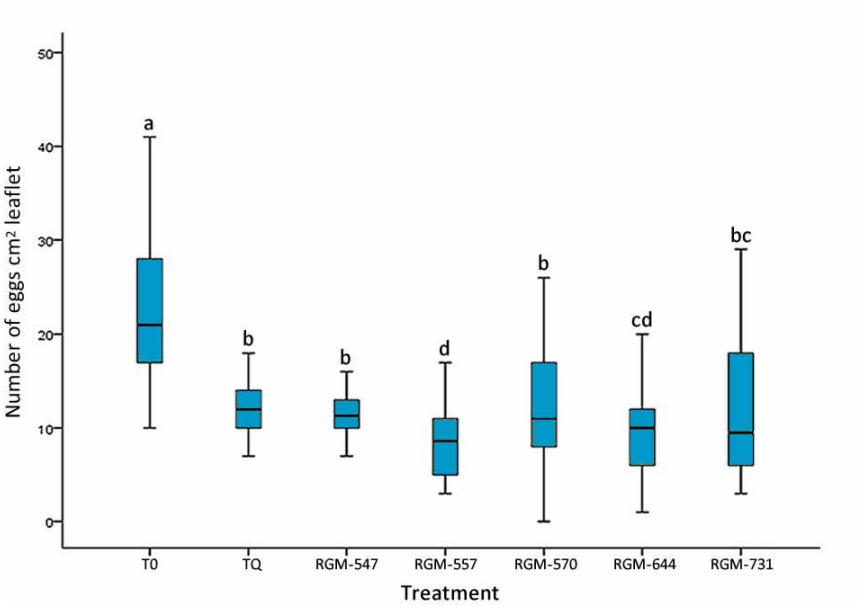
Significant differences were found between the plants treated with endophytes and the control plants ($F = 3.1$; $df = 5$; $P < 0.026$). The five evaluated strains internally colonized tomato leaves. White mycelium growing on leaf discs obtained from inoculated plants and placed in culture media was observed with an optical microscope and confirmed as B. bassiana by its morphology while in the control, the leaf discs obtained from plants showed no mycelium. The endophytic colonization fluctuated between 14% and 24%, but no significant difference was observed among the tested strains. Furthermore, 100% of the strains demonstrated a systemic mode of action, where the fungus inoculated in the roots was re-isolated from the leaves (Figure 6).



Fuente: Elaboración propia.

Figure 6. Colonization percentage of tomato by *Beauveria bassiana* 45 days after the inoculation (n=5). Bars with different letters differ according to Fisher's LSD test ($p < 0.05$).

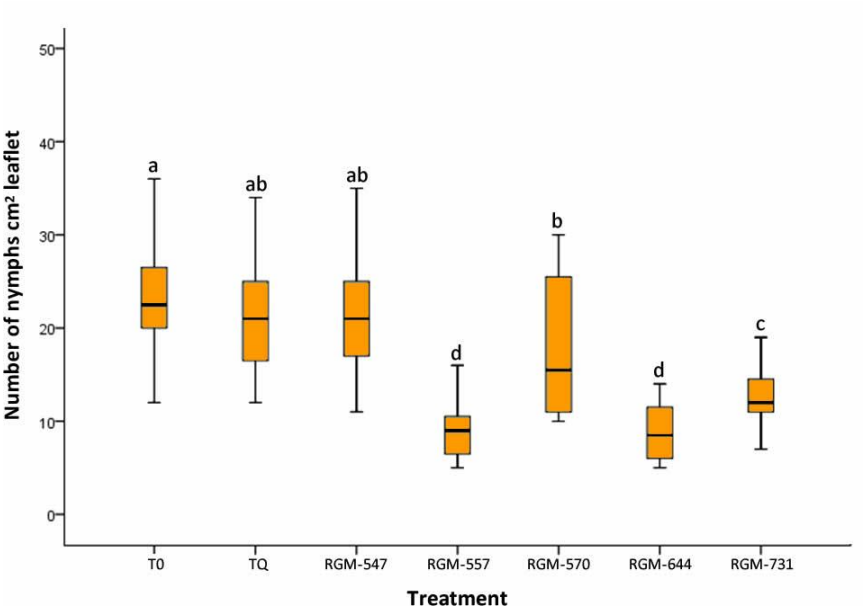
Root inoculation of tomato plants with native endophyte strains of *B. bassiana* significantly reduced the number of eggs on the leaflets after 45 days of incubation in comparison to the control treated with water (T0). Pairwise comparisons (Kruskal-Wallis=84.37, df =40, $p<0.0001$) showed that the plants treated with the RGM-557 & RGM-644 strains presented the least amount of eggs ($p<0.05$), with means of 8.18 and 9.68 ($N°cm^2$ leaflet) respectively, when compared to plants treated with the INSEGAR® 25 WG (TQ) insecticide and those treated with the RGM-547 and RGM-570 endophytes (Figure 7).



Fuente: Elaboración propia.

Figure 7. Effect of the tomato root inoculation with native strains of the *B. bassiana* endophtye on the number of eggs of *T. vaporariorum* on tomato leaves, 45 days after the inoculation (n=40). Treatments with a common letter are not significantly different according to the Kruskal-Wallis test at *P* = 0.05. The data corresponds to means (±SE).

In the case of the endophyte effect on the number of nymphs, a significantly lower number was also observed with respect to T0 (control) and TQ (chemical treatment) (Kruskal-Wallis =172, df =40, *P*<0.0001), with the RGM-664 and RGM-557 strains again presenting the lowest number of nymphs, with means of 8.9 and 9.4 (N° cm^2 leaflet), respectively. T0 reached a means of 25.4 and TQ 21.3 nymphs per leaflet (cm^2) (Figure 8). It is worth mentioning that the decrease in the number of nymphs observed as a result of the RGM-644 strain with respect to the TQ treatment was 58% and compared to the T0 was 65%.

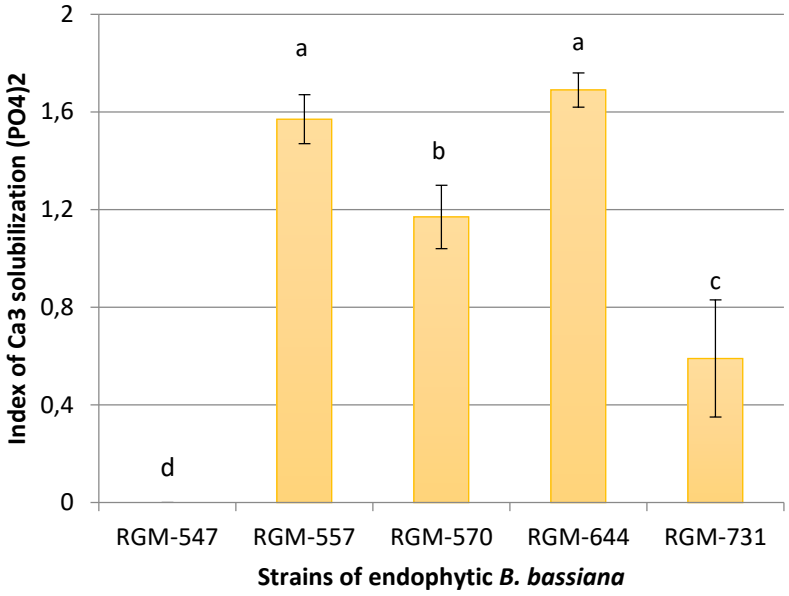


Fuente: Elaboración propia.

Figure 8. Effect of the tomato root inoculation with native strains of *B. bassiana* endophytes on the number of nymphs (instars III & IV) of *T. vaporariorum* on leaves, 45 days after the inoculation (n=40). Treatments with a common letter are not significantly different according to the Kruskal-Wallis test at *P* = 0.05. The data corresponds to means (±SE).

3.4.2 Phosphorous solubilization, production of iron siderophores and indole compounds per *B. bassiana* endophyte strain

In the *in vitro* studies, four strains of *B. bassiana* showed some degree of Ca_3 solubilization $(PO_4)_2$. The phosphate solubilization index fluctuated between 0 and 1.69. The only strain that showed no solubilization was RGM-547. In the analysis of the strains that presented some degree of solubilization, the strains RGM-644 and RGM-557 presented solubilization indices of 1.57 and 1.69, significantly superior to the strains RGM-570 and RGM-731 (Figure 9).



Fuente: Elaboración propia.

Figure 9. P-solubilization of $Ca_3(PO_4)_2$ by endophytic *B. bassiana* in the agar medium NBRIP. Different letters over the bars represent significant differences among the treatments according to the Fisher's LSD test ($p < 0.05$).

In the Indole compound production, none of the endophytic strains evaluated presented the ability to produce indole compounds. For the iron siderophore production variable, the dishes inoculated with the endophytes RGM-547, RGM-557, RGM-644 and RGM-731 presented a coloration change which was considered positive by the CAS method. The dishes with the strain RGM-570 showed no coloration changes and were therefore considered negative (Table 2). The endophytic *B. bassiana* strains RGM-644 and RGM-731 produced the greatest amount of siderophores (Figure 10).

Table 4. *In vitro* detection of siderophore production on CAS agar plate by endophytic *B. bassiana* strains.

Code Strain*	Species	Siderophore production
RGM-547	*Beauveria bassiana*	1
RGM-557	*Beauveria bassiana*	1
RGM-570	*Beauveria bassiana*	0
RGM-644	*Beauveria bassiana*	2
RGM-731	*Beauveria bassiana*	2

Fuente: Elaboración propia.

* (2) indicates a high amount of siderophore production, (1) indicates an intermediate amount and (0) indicates no siderophore production.

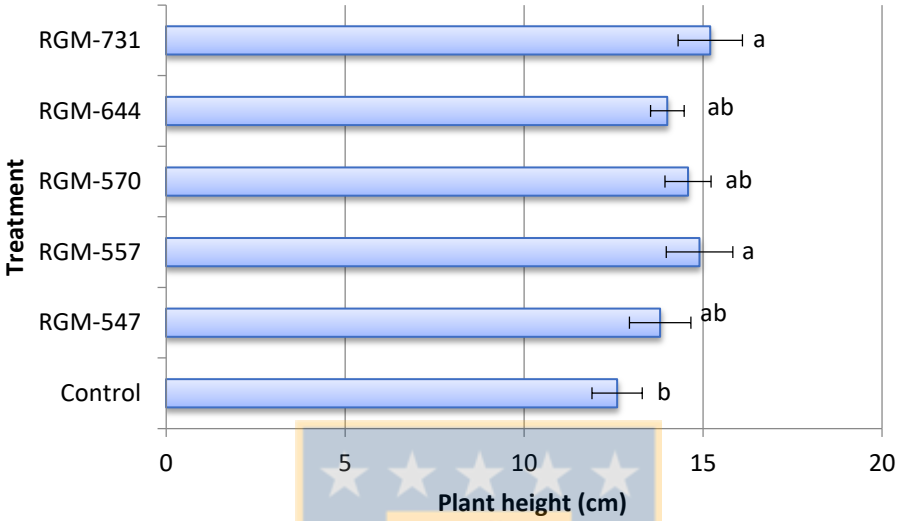
Fuente: Elaboración propia.

Figure 10. *In vitro* production of iron siderophores. a) Absence of coloration change in medium, strain RGM-570 did not produce siderophores. b) Strain RGM-557 presented a medium production of siderophores. c) Strain RGM-644 presented a high production of siderophores.

3.4.3 Growth promotion *in vivo*

The inoculation of tomato seeds with native strains of endophytic *B. bassiana* had significant effects on the growth parameters studied. Thirty days after inoculation with strains RGM-557 and RGM-731 tomato plants presented a total height more than 21% and 18%, respectively, in comparison with the control that reached a height of

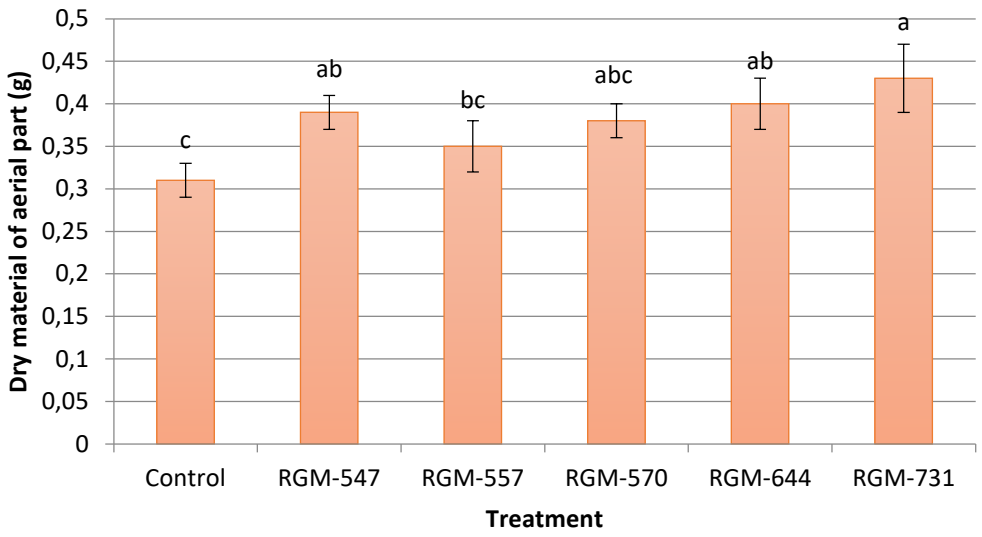
12.6 cm (F=1.49; df=5; P=0.23). No significant differences were found among the treatments inoculated with diverse endophytic strains (Figure 11).



Fuente: Elaboración propia.

Figure 11. Effect of endophytic strains of *B. bassiana* on the mean height (± SE) of tomato plants (*Solanun lycopersicum*), 30 days after inoculation (1x10^{6} conidia mL^{-1}). Different letters over the bars represent significant differences among the treatments according to the Fisher's LSD test (p < 0.05).

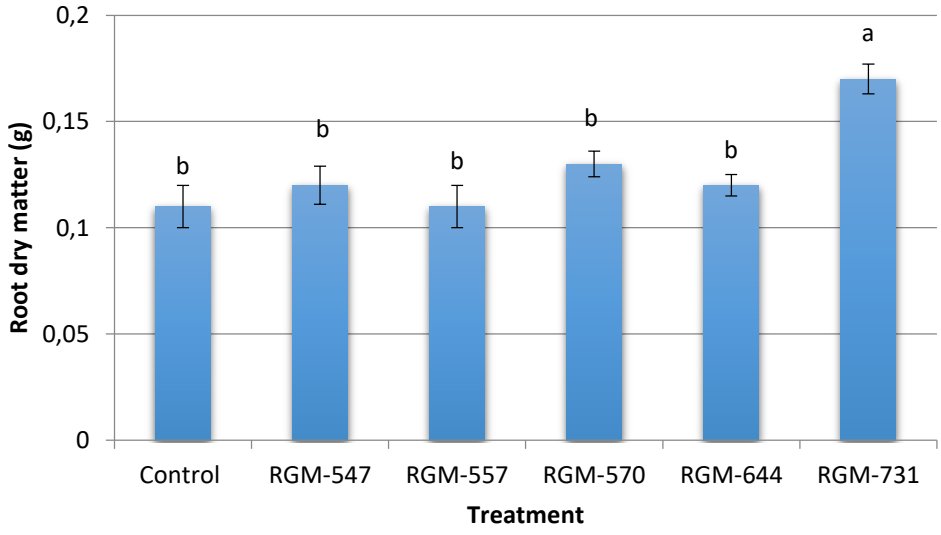
For the plant biomass production parameter that included the dry material from both the aerial (leaves and stems) and underground (roots) parts, significant differences were also found between the plants obtained of seed treated with endophytes and the control plants. The aerial dry biomass reached significantly higher mean values when seeds were treated with *B. bassiana* endophytes in comparison to the control (F = 2.58; df = 5; P = 0.052). Plants from treated seeds with the strains RGM-547, RGM-644 and RGM-731 reached the highest aerial dry weight, while no significant differences were found among strains (p>0,05), (Figure 12).



Fuente: Elaboración propia.

Figure 12. Effect of the endophytic strains of *B. bassiana* on the dry material of the aerial part (g) (± SE) of tomato plants (*S. lycopersicum*), 30 days after inoculation (1x10^6 conidia mL^{-1}). Different letters over the bars represent significant differences among the treatments according to the Fisher's LSD test ($p < 0.05$).

In the case of root dry weight, the mean weight fluctuated from 0.11 to 0.17 g. Plants from treated seeds with the strain RGM-731 reached a mean weight 55% higher than that of the control, ($F = 5.2$; $df = 5$; $P = 0.002$), while plants from treated seeds with the other four of *B. bassiana* strains presented no significant differences compared to the control (Figure 13).



Fuente: Elaboración propia.

Figure 13. Effect of endophytic strains of *B. bassiana* on root dry matter (g) (± SE) of tomato plants (*Solanun lycopersicum*), 30 days after inoculation (1x10^6 conidia mL^{-1}). Different letters over the bars represent significant differences among the treatments according to the Fisher's LSD test ($p < 0.05$).

3.5 DISCUSION

The five evaluated strains presented evidence of endophytic colonization in tomato leaves, which persisted until the end of the trial (day 45). These results thus provide evidence of the growth-promoting activity of the native strains of endophytic *B. bassiana* in addition to their biocontrol action, as they were able to decrease the numbers of GWF eggs and nymphs on tomato plants.

3.5.1 Endophyte *effect on T. vaporariorum*

Previous studies have provided evidence regarding the direct (entomopathogenic) effect of *B. bassiana* against *T. vaporariorum* in tomato. It has been shown that strains of this fungus (Naturallis, $ATCC_{74040}$, AL_1, ALB_{55} y OF_{13}) are pathogens to GWF, with a nymph mortality above 85% [12]. Meanwhile, Quesada-Moraga et al. [10] evaluated the effect of various strains of entomopathogenic fungi on nymphs of GWF in *Cucumis melo*, reaching mortality values over 50%. Although previous evidence has shown that *B. bassiana* can control GWF, this research is novel because it provides further details regarding the effects this fungus has as an endophyte against this pest [12; 10]. Here, we demonstrated that the endophytic strains achieved a significant decrease in the number of eggs and nymphs in comparison to the uninoculated control, with strains RGM-557 and RGM-644 showing similar effects to the insecticide in relation to the decreased number of GWF eggs, and strains RGM-557, RGM-644 and RGM-731 showing a significantly higher reduction in the number of nymphs compared to the synthetic insecticide.

Powell et al. [48] demonstrated a reduction in the survival of *Helicoverpa zea* larva when fed on tomato plants inoculated with strains of endophytic *B. bassiana*. The population reduction of *H. zea* as a consequence of the endophytic action of *B. bassiana* and *Purpureocillium lilacinum*, was also reported by Lopez and Sword [49] in cotton (*Gossypium hirsutum*). On the other hand, it has been shown [50] that *Vicia faba* L. seeds treated with endophytic fungi induced systemic changes in the plant, negatively affecting the behavior of aphids *Acyrthosiphon pisum* and *Aphis fabae*. Although a significant number of studies have reported the antagonist effects of endophytic fungi against various insects, the mechanisms involved in their action have not yet been clearly demonstrated and reported.

The decrease in the number of eggs on plants inoculated with endophytic strains in our study could be explained by GWF adults preferring to lay their eggs on uninoculated plants instead of inoculated plants [51]. On the other hand, this study showed that the GWF is most susceptible to the negative effects of the evaluated endophytic strains in its nymph stage. Studies by Mascarin et al. [52], have also shown that entomopathogenic fungi are more effective in controlling GWF in their nymph stage; they related this effect with the direct contact between the nymphs and fungi conidia in foliar applications, considering their limited mobility. Nevertheless, in our study, the epiphytic action of the fungus was discarded because the conidia of the fungus did not come into direct contact with the insect when were applied (substrate inoculation). Furthermore, there was no evidence of *B. bassiana* growth in GWF adults under incubation, suggesting that the decrease in nymph population could be attributed to the presence of the fungus within the plant. In a review of several studies by Vega [14], where entomopathogenic fungi were used for insect control, 93% of these provided evidence of the absence of mycosis in insects, demonstrating its effect as an endophyte. Studies by Menjivar et al. [51] showed a decrease in GWF in tomato plants (50 to 70% less insects) when roots were inoculated with the endophytic fungus from the genus *Fusarium*. Several authors have affirmed that the negative effect in the early stages of insect development due to entomopathogenic fungal endophytes, as in our study, are related to the production of toxic substances

(secondary metabolites) in the plant tissues [53,54,55,56] and/or due to the changes in bioactive compounds induced in plants by the endophytic fungus [50].

Studies by Xu et al. [57,58] with *B. bassiana* have demonstrated that bassianolide and beauvericin metabolites function as virulence factors against various insects. It has also been reported that *Beauveria* produces the Bassicridin metabolite which has a toxic action in insects [59]. The above suggests that the production of compounds in the plants also could inhibit the insect from searching for plants [60].

On the other hand, the decrease in the number of eggs and nymphs could also be related to a plant response against insects mediated by endophytic fungi, resulting in the production of secondary metabolites with toxic, repellent or anti-feeding effects for insects [51,61,14]. It is possible that the endophytic fungi negatively affect the insect population as a result of the plant induced systemic response [62], which can occur far from where the elicitor was inoculated [63].

According to the manufacturer's recommendations, INSEGAR® 25 WG should be applied at the beginning of the tomato crop, and repeat its application three times. If a 130-day tomato crop cycle is considered, this product could provide protection for periods of 40 days. In the first period of time, the endophytic strains RGM-557 and RGM-644 presented better results than this insecticide in reducing the number of eggs and nymphs. The endophytic fungi could complement or substitute the use of this chemical insecticide in the control of whiteflies.

Our study provided evidence of the negative effect of the endophytic fungi strains on the number of GWF eggs and nymphs on tomato leaves. Nevertheless, future studies must further examine the mechanisms that cause these responses.

3.5.2 Growth promotion

Our results showed that the endophytic strains used in this study exerted a growth promotion effect, which led to taller plants and greater aerial and root dry weights, in most cases superior to the uninoculated control. This confirms the protocooperation between the plant and the endophytic fungus, which improved the

growth of the plant. Various studies have confirmed the positive effects of strains of entomopathogenic fungal endophytes in the growth of different crops, such as tomato, wheat, corn, cotton, and bean [64,32,20,17,65,49,27]. Our results coincide with those presented by Sánchez-Rodríguez et al. [65], where they demonstrated the ability of *B. bassiana* to internally colonize tomato plants without negatively affecting plant height and biomass production. Tall and Meyling [27] also provided evidence of the growth promoting effect of the endophytic strains of *B. bassiana*, which when applied to *Zea mays* seeds had a positive effect on plant growth in a substrate with a high nutrient content. Studies by Sánchez-Rodríguez et al. [35] in *Triticum aestivum* demonstrated that seed inoculation with the endophytic *Beauveria* showed no significant differences in comparison to the uninoculated control in terms of plant height during the first 17 days after inoculation, which could explain the energy cost the plant must pay in order to tolerate the endophyte. Nevertheless, 23 to 31 days after the inoculation they found significant differences in height. A significant increase in the dry weight of the spikes was also registered. It is important to mention that, similar to a study by García et al. [20] with *M. anisopliae* endophytes, our study observed that the growth promoting effect is dependent upon the fungal strain, as significant differences were observed among strains. In our study, the strain RGM-731 showed the most promising results in terms of plant height and root and shoot dry matter.

In relation to the mechanisms associated with plant growth promotion activity by endophytic fungi, we first ruled out the growth promotion effect through indirect mechanisms, since the plants were not affected by pests or diseases during the completion of the assays. Therefore, the growth promoting activity could be explained by direct mechanisms, such as the production of phytohormones or growth regulators (metabolites), in addition to the bioavailability of the necessary nutrients for the growth of plants. In this sense, this study discards, in part, the effect associated with the production of phytohormones, specifically indole compounds such as indoleacetic acid, provided the evaluated strains were unable to produce them. Our results could be more related to the increase in the bioavailability of nutrients and could be explained in part by the production of iron siderophores and phosphate solubilization.

It cannot be ruled out that other types of hormones could have been produced, such as cytokinins or gibberellins, which have been widely reported in association with bacteria and some fungi linked to plants as endophytes [66, 67, 68].

In the present study, only the endophytic strain RGM-547 showed no ability to solubilize phosphate, while the other four strains were able to form solubilization halos, which, according to Perez et al. [69], could indicate different degrees of efficiency of phosphate solubilization. In this case, the strains RGM-731 and RGM-557 reached the highest efficiency. Studies by Pal and Ghosh [70] have also provided evidence that *B. bassiana* has the ability to solubilize phosphate. The strain used in their study produced phosphate solubilization halos of 1.4 cm at day 14, while the endophytic strain RGM-644, used in the present study, reached halos of 1.24 cm; though, these measurements were taken after only 10 days. The endophytic strains that solubilize phosphate could provide an important quantity of available phosphate for plant growth, which could explain the growth promoting activity enduced by the endophytic strains found in the present study in tomato.

On the other hand, the ability to produce iron siderophores by entomopathogenic fungi has been reported by various authors and in all cases it has been related with siderophores of the hydroxamate type [71, 72, 73]. The present study confirmed that the evaluated native strains of entomopathogenic endophytes can indeed produce siderophores, which could be associated with the ability that these fungi have to produce secondary metabolites of diverse types. Studies by Jirakkakul et al. [31] relate the siderophore production of *Beauveria* with Tenellin, a metabolite produced by this fungus that could act as an iron chelator, associated with the accumulation of Ferricrocin in the fungus hyphae. The significant siderophore production by the strain RGM-731 could leave iron available for the plant, which could lead to a higher dry biomass. Studies by Sánchez-Rodríguez et al. [65] in tomato plants demonstrated that the endophytic *B. bassiana* is capable of increasing the bioavailability of iron in calcareous substrates, which stimulated both shoot and root growth.

Our results, in terms of the growth promotion and decrease in the GWF population in tomato plants, is concurrent with that presented by Jaber and Ownley [74] in the sense that the entomopathogenic fungi can live as endophytes and contribute to insect pest suppression, as well as to plant growth promotion.

3.6 CONCLUSIONS

The results obtained in this study suggest that tomato seed inoculation with endophytes could be used as a growth-promoting alternative. Furthermore, the evaluated strains show potential for GWF biological control and could replace or be used in conjunction with insecticides, with consequent environmental, social and economic benefits. However, it is necessary to validate these results in the field since endophyte behavior could be modified by uncontrolled environmental variables. It is also necessary to deepen our understanding of the mechanisms involved in the effect that endophytes have on GWF. On the other hand, it is necessary to explore the results that could be obtained by combining the action of *B. bassiana* as an epiphyte and as an endophyte against the GWF, since its antagonist action could be enhanced by other control mechanisms.

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V. CONCLUSIÓN GENERAL

Beauveria bassiana es un conocido hongo entomopatógeno y presenta un gran potencial para ser usado como endófito con distintos objetivos, ya que se asocia a diversas especies cultivadas. Nuestra investigación entregó evidencia de la capacidad que tienen cepas nativas de este hongo para colonizar los distintos tejidos de tomate y ají, debido a que ocho de las diez cepas evaluadas pudieron colonizar ambas especies de forma sistémica. Los resultados se obtuvieron gracias al uso de técnicas de microbiología convencional; sin embargo, esta metodología podría entregar falsos negativos, que no permitiría conocer el real potencial de colonización del hongo. El uso de las herramientas moleculares se proyecta como una herramienta efectiva para el estudio de colonización endofítica, pero aún son pocos los estudios en esta materia.

En el ámbito de la mitigación del estrés causado por plagas y enfermedades, las cepas nativas de *B. bassiana* evaluadas obtuvieron un buen desempeño en los ensayos de antagonismo frente al patógeno *Botrytis cinerea*, importante enfermedad que afecta a diversos cultivos. Tanto *in vitro* como en planta, se observó que todas las cepas evaluadas presentan algún grado de antagonismo, destacando los resultados obtenidos con los endófitos RGM-547 y RGM-644. Este es el primer reporte de la acción antagónica de *B. bassiana* endófito frente a *B.cinerea* en tomate y ají. Los resultados obtenidos podrían estar relacionados con las diversas

estratégias que utilizan estos hongos en el control de patógenos, por lo que es necesario realizar futuras investigaciones para profundizar en el o los mecanismos involucrados.

El presente estudio demostró además, que las cepas endófitas evaluadas tienen la capacidad para controlar poblaciones de *T. vaporariorum* en el cultivo de tomate bajo invernaderos, ya que su inoculación en etapas tempranas del crecimiento de la planta lograron disminuir el número de huevos y ninfas en hojas, alcanzando resultados similares al tratamiento con insecticida químico en el caso de las cepas RGM-557 y RGM-644. El uso de los endófitos podría entregar mayores ventajas para el control de insectos que la acción convencional de los hongos entomopatógenos, debido a que presentan diversos mecanismos de acción que incluyen desde la acción directa por la ingestión de alguna de sus estructuras presentes en la planta, hasta un efecto indirecto asociado a una interacción tritrófica que permite atraer parasitoides para que puedan controlar las poblaciones del insectos. Dado lo anterior, la incorporación de hongos endófitos en el manejo fitosanitario del tomate se presenta como un método más sustentable al uso de los químicos convencionales.

Finalmente queda demostrado el potencial que presenta *B. bassiana* endófita para promover el desarrollo de las plantas. Aplicaciones en las semillas permitieron alcanzar una biomasa vegetal en la mayoría de los casos superior al testigo. La asociación con el hongo podría tener un costo en el desarrollo inicial del tomate, sin embargo éste podría ser compensado en etapas posteriores al obtener un mayor crecimiento y fructificación. En la medida que el endófito pueda acceder a recursos del medio que rodea a la planta, su acción en la promoción de crecimiento puede ser superior, dada su capacidad para solubilizar y/o hacer más disponible los nutrientes. En este ámbito aún queda mucho por investigar, en este trabajo sólo se entrega evidencia de la capacidad que tienen esta cepas de solubilizar fósforo y producir sideróforos, sin embargo son solo algunos de los mecanismos mediados por los endófitos que podrían estar involucrados en el proceso de crecimiento de las plantas.