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Uso de microorganismos antagonistas en el control de *Verticillium nonalfalfae* MLST2, agente causal de la verticilosis del kiwi, en diferentes portainjertos y su efecto en la fisiología del portainjerto Bruno

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RESUMEN

La marchitez por Verticillium en el kiwi de pulpa amarilla (*Actinidia chinensis* var. *chinensis*), causada por *Verticillium nonalfalfae* MLST2, supone una amenaza significativa para la producción de kiwi en Chile debido a la persistencia a largo plazo del patógeno en el suelo y a la limitada disponibilidad de alternativas de control eficaces. El estudio evaluó la capacidad de los agentes de control biológico (ACB) bacterianos y fúngicos para suprimir *V. nonalfalfae* MLST2 y mitigar el estrés fisiológico de las plantas en portainjertos de kiwi. Se evaluaron seis aislados de *Pseudomonas protegens* y *Bacillus* spp. contra *V. nonalfalfae* MLST2. La inhibición del crecimiento micelial alcanzó el 46,2 % con *Bacillus velezensis* BcB1 y el 40,2 % con *P. protegens* C025. Estos dos aislados bacterianos más prometedores y *Trichoderma harzianum* RGM 3510 se evaluaron en tres portainjertos de kiwi bajo inoculación del patógeno. Se observó una interacción significativa entre los tratamientos y los portainjertos, y el portainjerto Z5Z6 tratado con *P. protegens* C025 mostró el nivel de marchitamiento más bajo en comparación con el control inoculado con el patógeno ($P < 0,05$). Los ACB seleccionados también se evaluaron individualmente o en combinación en el portainjerto Bruno. Se registró el desarrollo de la enfermedad mensualmente durante tres meses y se determinó el área bajo la curva de progresión del marchitamiento (AUWPC). Se midieron las respuestas fisiológicas, incluyendo la conductancia estomática (gs), la SWP y los indicadores de eficiencia fotoquímica (Fv/Fm y PRI). El tratamiento que contenía *P. protegens* produjo la mayor reducción en la gravedad de la enfermedad según la AUWPC. Las combinaciones de ACB moderaron las disminuciones en Fv/Fm y PRI, lo que sugiere una protección parcial del rendimiento de la fotosíntesis. Los resultados sugieren que los portainjertos tolerantes y los ACB, en particular *P. protegens* y *T. harzianum*, pueden ser herramientas prometedoras para el manejo sostenibles de la marchitez por Verticillium en los huertos de kiwi chilenos.

Palabras clave: *Verticillium nonalfalfae* MLST2, portainjertos de kiwi, agentes de control biológico.

ABSTRACT

Verticillium wilt in yellow-fleshed kiwifruit (*Actinidia chinensis* var. *chinensis*), caused by *Verticillium nonalfalfae* MLST2, poses a significant threat to kiwifruit production in Chile due the long-term persistence of the pathogen in soil and the limited availability of effective control alternatives. The study evaluated the ability of bacterial and fungal biocontrol agents (BCAs) to suppress *V. nonalfalfae* MLST2 and mitigate plant physiological stress in kiwifruit rootstocks. Six isolates of *Pseudomonas protegens* and *Bacillus* spp. were evaluated against *V. nonalfalfae* MLST2. Mycelial growth inhibition reached 46.2% with *Bacillus velezensis* BcB1 and 40.2% with *P. protegens* C025. These two most promising bacterial isolates and *Trichoderma harzianum* RGM 3510 were evaluated on three kiwifruit rootstocks under pathogen inoculation. A significant interaction was observed between treatments and rootstocks, with Z5Z6 rootstock treated with *P. protegens* C025 showing the lowest wilting level compared with pathogen-inoculated control ($P < 0.05$). Selected BCAs were also evaluated individually or in combination in the Bruno rootstock. Disease development was recorded monthly for three months, and the area under the wilting progress curve (AUWPC) was determined. Physiological responses including stomatal conductance (gs), SWP, and photochemical efficiency indicators (Fv/Fm and PRI) were measured. Treatment containing *P. protegens* produced the greatest reduction in disease severity based on AUWPC. Combinations of BCAs moderated declines in Fv/Fm and PRI, suggesting partial protection of photosynthesis performance. Results suggest that tolerant rootstocks and BCAs, particularly *P. protegens* and *T. harzianum*, could be promising sustainable tools for managing Verticillium wilt in Chilean kiwifruit orchards.

Keywords: *Verticillium nonalfalfae* MLST2, kiwifruit rootstocks, biocontrol agents.

INTRODUCCION GENERAL

Chile se encuentra dentro de los principales exportadores de kiwi (*Actinidia deliciosa* Chev; Liang; Ferg.) a nivel mundial, ocupando el tercer lugar en exportación de esta fruta, detrás de Nueva Zelanda e Italia, dedicando una superficie cercana a las 7.750 ha para su producción, principalmente entre las regiones de O'Higgins a Ñuble (ODEPA & CIREN, 2024).

Dentro de los principales cultivares de kiwi cultivados en Chile, destacan las de pulpa verde, cv. Hayward en su mayoría, seguido de variedades de pulpa amarilla (*A. chinensis* Liang), con una superficie cercana a las 205 ha, representando solo el 2,6% de la superficie total de este frutal en Chile (ODEPA & CIREN, 2024).

Una de las limitantes en la producción de kiwi amarillo es la bacteria *Pseudomona syringae* pv. *actinidiae* (Psa), la cual fue reportada en Chile por primera vez el año 2010 (Vanneste, 2013), siendo el kiwi amarillo la especie que presenta mayor susceptibilidad a esta enfermedad (cancro bacteriano del kiwi o Psa) disminuyendo el rendimiento y causando la muerte de las plantas (Cameron & Sarojini, 2014). Sin embargo, la producción de kiwi (verde y amarillo), se ha observado limitantes atribuida a un patógeno de suelo que ocasiona el marchitamiento y muerte de las plantas durante los primeros años de establecimiento de los huertos. Auger et al., (2009), describieron por primera vez esta problemática en Chile, indicando que durante el año 2003 comenzó la primera plantación de kiwi amarillo en Chile, alcanzando las 150 ha para el año 2006. Sin embargo, las plantaciones comenzaron a morir en un tiempo no mayor de 2 años, llegando a perder hasta el 80 % de las plantas de varios huertos en la temporada de cultivo 2007-2008. La enfermedad se caracterizó por un repentino marchitamiento y muerte regresiva que se puede observar como una declinación general de las plantas de kiwi, presentando entrenudos cortos, hojas pequeñas y cloróticas, caída prematura de frutos, y muerte de cargadores y brazos desde los extremos, observándose estos síntomas en cualquier momento de la temporada de crecimiento de la planta (Auger et al., 2009). Los autores identificaron como agente causal de la enfermedad, al hongo *Verticillium alboatrum* (Reinke & Berthier).

El género *Verticillium* spp.es un grupo de hongo fitopatógenos que habitan el suelo e infectan a más de 200 especies de plantas a través de las raíces, generando una infección sistémica

(Agrios, 2010), donde el hongo invade y bloquea los vasos del xilema impidiendo el transporte de agua y minerales desde la raíces hasta los órganos fotosintéticos (Zhang et al., 2020), afectando el estado hídrico de la planta y disminuyendo la actividad fotosintética, lo que lleva a la declinación de la planta (Pascual et al., 2010, Bowden and Rouse, 1991). La característica de *Verticillium* spp. de generar estructuras de resistencia como microesclerocios o hifas mielanizadas, les permite permanecer latentes en suelo durante años, dificultando su control

A nivel mundial existe sólo un reporte en kiwi, específicamente en un cultivar amarillo, donde este género de hongos ha causado la muerte de plantas, que corresponde al trabajo descrito de Auger et al., 2009, donde identificó a *V. alboatrum*, sin embargo, posterior a este reporte, la especie *V. alboatrum* se revisó taxonómicamente y se dividió en tres especies *V. alboatrum sensu stricto*; *V. alfalfae* y *V. nonalfalfae* (Inderbitzin et al., 2011).

De estas especies, *V. albo-atrum* sensu stricto ha sido descrita afectando kiwi amarillo en Nueva Zelanda (Mellow et al. 2019),

Kasson et al. (2014) determinó que los aislados obtenidos de *V. alboatrum* desde Chile fueran reclasificados como *V. nonalfalfae*. La caracterización molecular de aislamientos de *V. nonalfalfae* asociados con la marchitez de *A. chinensis* en Chile lo colocó en un grupo intraespecífico distinto, denominado secuencia multilocus tipo 2 (MLST2) (Lee et al., 2022, Kasson et al., 2014). Trabajos recientes proponen a *V. nonalfalfae* MLST2 como una nueva especie, que ha sido denominada *V. gasparssi* sp. nov. (García et al., 2020).

Con respecto al control, el uso de fumigantes químicos como la cloropicrina aplicados al suelo representa una alternativa para el control de *Verticillium* spp. y otros patógenos transmitidos por el suelo (Martin, 2003), sin embargo, dicha alternativa tiene un impacto ambiental, que además de presentar posibles riesgos a la salud de las personas, tiene un alto costo al establecer una plantación, ya que sobrepasa los US$ 4000 ha^{-1} (Boutaj et al., 2022). Por otra parte, actualmente no se dispone de ningún fungicida que cumpla con los estándares, (no afectar a microorganismos beneficos y/o saprofitos y no afectar la salud de las personas principalmente) de una agricultura sostenible para controlar *Verticillium* spp. (Depotter et al., 2016, Angelopoulou et al., 2014;). Dentro de los fungicidas recomendados tenemos fungicidas bencimidazoles usados en la desinfección de raíces (benomilo, carbendazima o

metiltiofanato), dado que han sido reportado controlando especies de *Verticillium* en distintas especies de frutales o berries (Moya-Elizondo et al., 2019).

Prácticas culturales como la utilización de material de propagación sano, uso de portainjertos y variedades que presenten tolerancia y/o resistencia al patógeno, el establecimiento de huertos en suelos libres del patógeno y la remoción de plantas afectadas, son considerados como manejos efectivos en el control de distintas especies de *Verticillium* (Fradin & Thomma, 2006; Morera & Páez, 2005).

Adicionalmente, la detección visual temprana ayuda a la toma de decisiones que permiten aislar la enfermedad y evitar su extensión dentro del huerto. En este aspecto, la detección del patógeno en plantas mediante técnicas moleculares como PCR o qPCR, tiene importantes ventajas, debido a la rapidez (1 a 2 días), sensibilidad, reproductibilidad y confiabilidad que presentan (Lee et al., 2022). En Chile, García et al., 2020 desarrolló un protocolo de identificación molecular basado en la caracterización genotípica de aislados chilenos de *Verticillium* sp., causantes de marchitez y muerte regresiva de kiwi amarillo, mientras en Nueva Zelanda se desarrolló un set de marcadores específicos para qPCR que permiten la identificación de *V. nonalfalfae* MLST2 (Lee et al., 2022).

El síntoma de marchitamiento causado por *Verticillium* influye en varios parámetros fisiológicos, como la tasa de transpiración, conductancia estomática, potencial hídrico a nivel de hoja (Sowik et al., 2016) y la fluorescencia de la clorofila (Ait Rahou et al., 2020). Sin embargo, el impacto que tiene *V. nonalfalfae* MLST2 en estos parámetros no ha sido estudiado en especies de kiwi.

El control biológico se considera una alternativa prometedora y en las últimas décadas se ha desarrollado una amplia gama de agentes de control biológico (BCA) para el manejo de enfermedades fúngicas y bacterianas (Bonaterra et al., 2022).

Bacterias pertenecientes a los géneros *Bacillus* y *Pseudomonas*, han sido reportadas con la capacidad de reducir el crecimiento de *Verticillium* spp. en especies frutales y hortalizas, además de presentar características funcionales como la promoción del crecimiento vegetal (Nesemann et al., 2018, Hollensteiner et al., 2017; Hayat et al., 2010). Sin embargo, no existen antecedentes reportados de control biológico sobre *V. nonalfalfae* MLST2 en kiwi.

Pseudomonas spp. han sido ampliamente estudiados como BCA, actuando como biocontrolador de varios patógenos del suelo, incluyendo *Verticillium* (Köhl et al., 2019,

Mishra et al., 2018 Deketelaere et al., 2017, Raaijmakers et al., 2012), y este género bacteriano ha sido asociado al fenómeno natural de suelos supresores (Jayaraman et al., 2021, Liu et al., 2021, Weller et al., 2002) y a la producción de compuestos antimicrobianos, como fenazinas, pirrolnitrina, fenazina-1-carboxamida, ácido fenazina-1-carboxílico, 2,4-diacetilfloroglucinol, ramnolípidos, pioluteorina, oomicina A, ecomicinas, viscosinamida, cepaciamida A, piocianina, butirolactonas, N-butilbencenosulfonamida, ácido pseudomónico, azomicina, cepafunginas y caralicina (Singh et al., 2022, Basu et al., 2021), además de presentar la capacidad de inducir resistencia sistémica para controlar enfermedades fungosas y bacterianas en cultivos como el trigo, algodón y frutales (Abo-Zaid et al., 2023, Kaur y Vyas, 2022; Nesemann et al., 2018; Maldonado González, 2015; Weller et al., 2012, Weller et al., 2007)

La mayoría de las cepas de *Pseudomonas* potenciales asociadas al biocontrol de enfermedades en plantas, pertenecen al grupo de *Pseudomonas fluorescens* (Nur Mawaddah et al., 2023, Saranraj et al., 2022, Deketelaere et al., 2017). Dentro de este grupo, *Pseudomonas protegens,* es una importante especie biocontroladora de enfermedades, ya que produce los antibióticos 2,4-diacetilfloroglucinol (2,4 DAPG), pioluteorina y pirrolnitrina (Ramette et al., 2011).

El género *Bacillus* representa el grupo más importante en el mundo que son utilizadas como bacterias promotoras del crecimiento de las plantas, así como agentes de biocontrol de plagas y enfermedades (Iwanicki et al., 2022, Khan et al., 2022, Hollensteiner et al., 2017). Las especies de *Bacillus* presentan un diverso metabolismo secundario y la capacidad de producir una amplia variedad de sustancias antagónicas, además de desencadenar respuestas de defensa de las plantas (Hollensteiner et al., 2017). Además, existen especies de *Bacillus* con la capacidad de aumentar la tolerancia de las plantas a condiciones de estrés abiótico, producir fitohormonas y facilitar la absorción de nutrientes por las plantas (Nunes et al., 2024, Iwanicki et al., 2022, Fira et al., 2018, Shafi et al., 2017). Dichas características han sido reportadas en distintas especies y cepas de Bacillus que se han reportado como controladora de distintas especies de *Verticillium* en diferentes cultivos (Poveda et al., 2022, Zhou et al., 2022, Deketelaere et al., 2017; Li et al., 2014).

Las especies del género *Trichoderma* son abundantes en la naturaleza pudiendo encontrarse en el suelo, sobre la madera en descomposición, en la rizósfera y sobre otras formas de

materia orgánica (Howell, 2003), especies de este género han sido reconocidas como importantes agentes de control biológico los cuales son ampliamente utilizados en la agricultura (Alfiky & Weisskopf, 2021). Los mecanismos de biocontrol basados en *Trichoderma* se basan principalmente en la producción de antibióticos y/o enzimas hidrolíticas , así como en la competencia por nutrientes y la activación sistémica de las respuestas de defensa de las plantas (Carrasco et al., 2024, Abbas et al., 2022, Reghmit et al., 2021). Reportes en olivo (*Olea europaea)* demostraron que la aplicación de *Trichoderma asperellum* redujo la severidad de la enfermedad del marchitamiento del olivo, causado por *Verticillium dahliae* Kleb. (Carrero-Carrón et al., 2016).

La combinación de dos o más BCA es una estrategia para mejorar la eficacia del biocontrol o para controlar diferentes patógenos e incluso plagas. Sin embargo, la aplicación de los aislados específicos de BCA debe ser compatible sin reducir su efecto, Yang et al., 2013 demostraron que la aplicación combinada de tres cepas bacterianas endófitas resultó en mejor control de la marchitez por *Verticillium dahliae*en el algodón que las aplicaciones individual a la planta, lo que probablemente estuvo relacionado con el hecho de que las diferentes cepas como *Paenibacillus xylanilyticus, Paenibacillus polymyxa* y *Bacillus subtilis*predominaron en las diferentes etapas de desarrollo del algodón.

Basados en estos antecedentes, el uso de *Pseudomonas protegens, Bacillus* sp. y *Trichoderma* sp. aisladas desde distintos ecosistemas en Chile y que presentan características como la producción de compuestos antimicrobiales, la inducción de resistencia y la capacidad de competir por un nicho, sugieren que podrían ser una alternativa para el control de enfermedades como el marchitamiento del kiwi causado por *V. nonalfalfae* MLTS2, disminuyendo los efectos fisiológicos en la planta causados por el patógeno.

HIPÓTESIS

Aislados chilenos de *Pseudomonas protegens, Bacillus* sp. y *Trichoderma harzianum* inhiben la infección de *Verticillium nonalfalfae* MLST2 en diferentes patrones de kiwi, permitiendo el control de esta enfermedad, además de favorecer la fisiología de los portainjertos.

OBJETIVO GENERAL

Evaluar la capacidad de aislados de *Pseudomonas protegens, Bacillus* sp. y una *Trichoderma harzianum* en su capacidad para inhibir y controlar la infección de *Verticillium nonalfalfae* MLST2 de forma individual y conjunta en plantas de kiwi.

OBJETIVOS ESPECÍFICOS

- Seleccionar bacterias con actividad de antibiosis sobre *Verticillium nonalfalfae* MLST2 y la compatibilidad de uso en mezcla entre ellas y con *Trichoderma harzianum*

- Evaluar el control de *Verticillium nonalfalfae* MLST2 con agentes de biocontrol en forma individual o en consorcio, en plantas de kiwi bajo condiciones semicontroladas, además de evaluar su efecto sobre distintos parámetros fisiológicos.

- Evaluar el uso de biocontroladores seleccionados de forma individual sobre diferentes patrones de kiwi en el control de *Verticillium nonalfalfae* MLST2 bajo condiciones controladas**.**

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Capitulo 1

Use of antagonistic microorganisms for the control of *Verticillium nonalfalfae* MLST2, the causal agent of Verticillium wilt on yellow-fleshed kiwifruit, in different rootstocks, and its effect on the *p*hysiology of the Bruno rootstock.

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ABSTRACT

Verticillium wilt in yellow-fleshed kiwifruit (*Actinidia chinensis* var. *chinensis*), caused by *Verticillium nonalfalfae* MLST2, poses a significant threat to kiwifruit production in Chile due the long-term persistence of the pathogen in soil and the limited availability of effective control alternatives. The study evaluated the ability of bacterial and fungal biocontrol agents (BCAs) to suppress *V. nonalfalfae* MLST2 and mitigate plant physiological stress in kiwifruit rootstocks. Six isolates of *Pseudomonas protegens* and *Bacillus* spp. were evaluated against *V. nonalfalfae* MLST2. Mycelial growth inhibition reached 46.2% with *Bacillus velezensis* BcB1 and 40.2% with *P. protegens* C025. These two most promising bacterial isolates and *Trichoderma harzianum* RGM 3510 were evaluated on three kiwifruit rootstocks under pathogen inoculation. A significant interaction was observed between treatments and rootstocks, with Z5Z6 rootstock treated with *P. protegens* C025 showing the lowest wilting level compared with pathogen-inoculated control ($P < 0.05$). Selected BCAs were also evaluated individually or in combination in the Bruno rootstock. Disease development was recorded monthly for three months, and the area under the wilting progress curve (AUWPC) was determined. Physiological responses including stomatal conductance (gs), SWP, and photochemical efficiency indicators (Fv/Fm and PRI) were measured. Treatment containing *P. protegens* produced the greatest reduction in disease severity based on AUWPC. Combinations of BCAs moderated declines in Fv/Fm and PRI, suggesting partial protection

of photosynthesis performance. Results suggest that tolerant rootstocks and BCAs, particularly *P. protegens* and *T. harzianum*, could be promising sustainable tools for managing Verticillium wilt in Chilean kiwifruit orchards.

Keywords: *Verticillium nonalfalfae* MLST2, kiwifruit rootstocks, biocontrol agents

Introduction

Verticillium wilt in yellow-fleshed kiwifruit (*Actinidia chinensis* Planch var. *chinensis*), caused by *Verticillium nonalfalfae* MLST2, is one of the major diseases limiting the development of this fruit crop in Chile (Inderbitzin et al., 2014; Auger et al., 2011; Auger et al., 2009). The pathogen was first reported infecting a yellow-fleshed kiwifruit cultivar by Auger et al. (2009), who identified this pathogen as *Verticillium albo-atrum*. Subsequent taxonomic revision of *V. albo-atrum* resulted in its division into three distinct species: *V. alboatrum sensu stricto*; *V. alfalfae*, and *V. nonalfalfae* (Inderbitzin et al., 2011). Of these species, *V. albo-atrum sensu stricto* and *V. dahlie* have been reported affecting yellow-fleshed kiwifruit in New Zealand (Mellow et al., 2019). However, Kasson et al. (2014) determined that isolates previously identified as *V. alboatrum* from kiwifruit in Chile should be reclassified as *V. nonalfalfae*. Molecular characterization further revealed that *V. nonalfalfae* isolates associated with infections in *A. chinensis* in Chile constitute a distinct intraspecific group, termed multilocus type 2 (MLST2) (Lee et al., 2022; Kasson et al., 2014). Species of the genus *Verticillium*, such as *V. nonalfalfae* MLST2, infect the vascular system of plants, causing progressive wilting that, in severe cases, leads to plant death (Auger et al., 2009; Fradin and Thomma, 2006; Pegg and Brady, 2002). The hyphae produced by these pathogens from their survival structures (micro-sclerotia or thick-walled mycelium) present in the soil can penetrate the root directly through wounds caused by insects, nematodes, or cultural practices (Fradin and Thomma, 2006). Upon reaching the xylem and increasing its biomass, the pathogen induces vascular occlusion, leading to plant wilting (Yadeta and Thomma, 2013). Furthermore, pathogen colonization combined with the host defensive responses, such as the formation of gums and tylosis, further obstruct xylem vessels, restricting water and nutrient transport due to a reduction in hydraulic conductivity induced by the disease (Gharbi et al., 2017). The resulting symptoms in leaves and stems include

wilting, chlorosis and leaf necrosis, and defoliation. These symptoms may be associated with severe water stress even under conditions of adequate soil moisture (Duniway, 1973).

Verticillium wilt has caused severe and widespread outbreaks in Chile. However, existing literature has focused primarily on pathogen identification and disease symptoms (Auger et al., 2009), with comparatively limited attention to the impact of the disease on plant water status and management strategies.

Kiwifruit species, and especially its leaves, are particularly sensitive to water deficit. Native from China, this species originates from a tropical center and exhibits high transpiration rates (Calderón-Orellana et al., 2025; Hussain et al., 2021) but is poorly adapted to withstand water stress levels considered moderate for many other fruit crops. In addition, kiwifruit has a poor embolism repair system (Clearwater and Clark, 2003) and low to moderate stomatal sensitivity to water stress (Calderón-Orellana et al., 2021). Under Mediterranean climatic conditions characterized by high evaporative demand, occlusion of xylem vessels in infected kiwifruit plants may accelerate leaf dehydration due to reduced hydraulic conductivity of the xylem, thereby promoting wilting and senescence (Baldi et al., 2024). Kiwifruit is an economically important fruit crop in several Mediterranean-climate regions that frequently experience droughts during the growing season, such as Chile and Italy. Therefore, understanding the relationship between *V. nonalfalfae* MLST2 incidence and changes in plant water relations is essential for maintaining the profitability of kiwifruit production.

The use of resistant rootstocks is one of the most efficient strategies for controlling verticillium wilt caused by *Verticillium dahlie* in watermelon (Devi et al., 2021), pistachio (Antón-Domínguez et al., 2024), tomato (Rysin et al., 2014), and olive (Díaz-Rueda et al., 2022; Montes-Osuna et al., 2020; Trapero et al., 2015). This approach allows susceptible cultivars grafted onto resistant rootstocks to exhibit resistance, preventing the development of disease symptoms. Conversely, when a resistant cultivar is grafted onto a susceptible rootstock, the plant may lose this resistance. Although no studies have reported this phenomenon in kiwifruit, research on *Ceratocystis fimbriata*, the causal agent of the disease known as Ceratocystis wilt, has led to the development of resistant rootstocks capable of suppressing this soil pathogen (Oliveira et al., 2021). These findings suggest that evaluating rootstocks may represent a promising tool for reducing damage caused by *V. nonalfalfae* MLST2.

Fungicides such as fludioxonil and fluopiram (Stoddard et al., 2018) and soil fumigants such as calcium cyanamide ($CaCN_2$), chloropicrin (trichloronitromethane; CCl_3NO_2), and dazomet, have been widely used to control soilborne diseases in high-value agricultural production systems (Li et al., 2021; Wu et al., 2019). The application of some of these disinfectants through irrigation, mainly drip irrigation, in a practice known as chemigation (Gómez-Gálvez et al., 2020; 2019) has shown some effectiveness. However, the use of synthetic compounds has been increasingly questioned due to their environmental impact and potential risks to human health.

Biological control is considered a promising alternative, and in recent decades a wide range of biological control agents (BCAs) has been developed for the management of soilborne pathogens (Alqahtani et al., 2025; Bonaterra et al., 2022; Palmieri et al., 2022). Bacteria belonging to the genera *Bacillus* and *Pseudomonas* have been reported to suppress the growth of *Verticillium* spp. in several fruit and vegetable species, while also exhibiting functional characteristics such as plant growth promotion (Nesemann et al., 2018; Hollensteiner et al., 2017; Hayat et al., 2010). However, no studies have reported the biological control of *V. nonalfalfae* MLST2 in kiwifruit. Among BCAs, *Pseudomonas* spp. have been extensively studied for their ability to act against a variety of soilborne pathogens, including *Verticillium* (Köhl et al., 2019; Mishra et al., 2018; Deketelaere et al., 2017; Raaijmakers et al., 2012). This bacterial genus has been associated with the natural phenomenon of suppressive soils (Jayaraman et al., 2021; Liu et al., 2021; Weller et al., 2002) and the production of antimicrobial compounds, such as phenazines, pyrrolnitrine, phenazine-1-carboxamide, phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol (2,4-DAPG), rhamnolipids, pyoluteorin, oomycin A, ecomycins, viscosinamide, cepaciamide A, piocianin, butyrolactones, N-butylbenzenesulfonamide, pseudomonic acid, azomycin, cepafungins, and caralicin (Singh et al., 2022; Basu et al., 2021). In addition, these bacteria can induce systemic resistance, contributing to the control of fungal and bacterial diseases in crops such as wheat, cotton, and fruit trees (Abo-Zaid et al., 2023; Kaur & Vyas, 2022; Nesemann et al., 2018; Maldonado-González, 2015; Weller et al., 2012, Weller et al., 2007). Most *Pseudomonas* strains associated with biocontrol of plant diseases belong to the *Pseudomonas fluorescens* group (Nur Mawaddah et al., 2023; Saranraj et al., 2022; Deketelaere et al., 2017). Within

this group, *P. protegens* is an important disease biocontrol species due to its ability to produce the antibiotics 2,4-DAPG, pyoluteorin, and pyrrolnitrin (Ramette et al., 2011).

The genus *Bacillus* represents the most important group of plant growth-promoting bacteria and effective BCAs against plant pests and diseases (Iwanicki et al., 2022; Khan et al., 2022; Hollensteiner et al., 2017). *Bacillus* species has a diverse secondary metabolism and can produce a wide variety of antagonistic substances while also triggering plant defense responses (Dimkić et al., 2022; Wu et al., 2018). In addition, several *Bacillus* species are able to enhance plant tolerance to abiotic stress conditions, produce phytohormones, and facilitate nutrient absorption by plants (Nunes et al., 2024; Iwanicki et al., 2022; Fira et al., 2018; Shafi et al., 2017). These traits have been reported in different *Bacillus* species and strains that have shown the capacity to control different *Verticillium* species in different crops (Poveda et al., 2022; Zhou et al., 2022; Deketelaere et al., 2017; Li et al., 2014).

Trichoderma species have been recognized as important BCAs and are widely used in agriculture (Alfiky and Weisskopf, 2021). The biocontrol mechanisms of *Trichoderma* are mainly based on the production of antibiotics and/or hydrolytic enzymes, mycoparasitism, competition for nutrients, and the systemic activation of defense responses in plants (Carrasco et al., 2024; Abbas et al., 2022; Reghmit et al., 2021). Reports on olive trees (*Olea europaea* L.*)* have shown that the application of *T. asperellum* can reduce the severity of olive wilt disease caused by *Verticillium dahliae* Kleb. (Carrero-Carrón et al., 2016).

To our knowledge, no previous studies have evaluated cultural and biological alternatives for controlling *V. nonalfalfae* MLST2 in kiwifruit. The integration of alternatives such as the use of BCAs and rootstocks with varying levels of resistance to *V. nonalfalfae* MLST2 may represent a viable option for suppressing this disease in kiwifruit. Therefore, the objective of this research was (i) to evaluate the ability of isolates of *P. protegens, Bacillus* sp., and *T. harzianum,* applied individually and in combination, to inhibit and control *V. nonalfalfae* MLST2 infection in different kiwifruit rootstocks, and (ii) to assess the magnitude of the effects of these biocontrol strategies on stem water potential (Ψ_{stem}) in response to *Verticillium* infection in the Bruno kiwifruit rootstock.

Materials and methods

Selection of antagonists

Six bacterial strains of *Pseudomonas protegens* (strains C019, C023, C025, C028, C033, and C116), six isolates of *Bacillus* sp. (isolates BcB1, BcB8, BcB9, BcB11, BcB12, and BcB13), together with the *T. harzianum* strain RGM 3510, were provided by the Plant Pathology Laboratory of the Faculty of Agronomy at the Universidad de Concepción, Chile. *Pseudomonas protegens* strains were isolated from soils that suppress take-all disease caused by *Gaeumannomyces tritici* in wheat (Moya-Elizondo, 2013) and have been shown to be effective in controlling soil fungi (Doussoulin et al., 2022; Quezada et al., 2022; Castro-Tapia et al., 2020; Vera et al. 2019). The *Bacillus* strains were obtained from walnut fruits (San Martín et al., 2023).

Since the six *Bacillus* isolates were not identified at the species level, their identification was carried out using the 16S rRNA, *glpF, ilvD,* and *ptA* genes and phylogenetic analysis based on the methodology described by Mora et al. (2011). Additionally, the isolates were evaluated for the presence of genes associated with the biosynthesis of antimicrobial peptides (AMPs), including surfactin, bacylisin, fengycin, bacyllomycin, subtilin, and iturin. For this purpose, the bacterial isolates were cultured in Luria Bertani (LB) medium, and genomic DNA was extracted from pure cultures using the CTAB method with modifications proposed by Doyle & Doyle (1987). Briefly, 500 µL of CTAB buffer were added to each sample, and DNA precipitation was carried out using 7.5 M ammonium acetate and 100% isopropyl alcohol. Amplification of the six AMP genes was performed using the primers and protocols described by Mora et al. 2011. In addition, *Bacillus* isolates were biochemically characterized using the BIOLOG® GEN III Microplate system (Biolog Inc., USA). For this purpose, the *Bacillus* strains were cultured on tryptic soy agar (TSA) for 6–8 hours at 33°C in darkness. Colonies approximately 3 mm² in diameter were then suspended in IF-B buffer according to the manufacturer's instructions, and the optical density was adjusted to 0.0022 (λ = 590 nm). Subsequently, the plates were inoculated (100 µL per well) and incubated at 33 °C in darkness. Absorbance readings were taken at 8 and 22 h, with the latter considered as the endpoint for evaluation.

In addition to the aforementioned bacterial microorganisms, the *Trichoderma harzianum* strain RGM 3510 (BioHarz®, Bioprotegens Innovations SpA., Chillán, Chile) was included. This strain is a biological product developed at the Universidad de Concepción and is

available in the Chilean Collection of Microbial Genetic Resources (https://www.cchrgm.cl/).

In vitro antagonism assay

The six isolates from each bacterial genus and *T. harzianum* RGM 3510 were used for dual cultures with *V. nonalfalfae* MLST2 strain RGM 3750.

To evaluate the antagonistic activity of *T. harzianum*, actively growing mycelial discs (7 mm in diameter) from both the pathogen and the antagonist fungus were placed 8 cm apart on the midline of 90-mm-diameter Petri dishes containing 20 mL of PDA medium. due to its slower growth under co-culture conditions, discs of *V. nonalfalfae* MLST2 RGM 3750 were placed on the plates three days prior to the placement of *Trichoderma* strain. The plates were incubated in darkness in a growth chamber at 24°C. Antagonistic activity was evaluated according to the scale proposed by Bell et al. (1982), which relates the growth of *Trichoderma* to that of the pathogen after six days of co-culture.

In the case of bacterial isolates, a 7-mm-diameter disc of actively growing mycelium from *V. nonalfalfae* MLST2 strain RGM 3750 was placed in the center of Petri dishes containing PDA supplemented with K_2HPO_4 and $MgSO_4$ x $7H_2O$ (PDA+KB agar medium) for *P. protegens* isolates, and PDA medium alone for *Bacillus* sp. isolates. The selected bacterial isolates were inoculated 7 days after the pathogen, at a distance of 3 cm from the disc, using a 10 µL aliquot of each bacterium at a concentration of 10^7CFU mL^{-1}. The bacterial concentration was standardized to 10^7 CFU mL^{-1} by measuring the optical density (0.1) at 600 nm (Sezonov et al., 2007). Antagonistic activity was evaluated 30 days post-inoculation (dpi) by measuring the growth radius of the pathogenic fungus in the direction of the BCA (R2) and the growth radius of the pathogen in the absence of BCA (R1). These readings were used to calculate the percentage of radial growth inhibition (PRGI) using the formula PRGI = ((R1-R2) / R1) x 100 (Skidmore *et al*., 1976).

From the six strains of *P. protegens* and *Bacillus* sp, the isolate from each genus showing the greatest *in vitro* inhibition was selected for further studies in plants.

Compatibility assays

In vitro compatibility assays were performed between the selected bacterial isolates of *P. protegens*, *Bacillus* sp., and *T. harzianum* RGM 3510 to evaluate their use as a consortium in plant treatments. For this purpose, a 5-mm-diameter disc of actively growing *T. harzianum* mycelium was placed in the center of a Petri dish containing PDA culture medium. The two bacterial isolates selected in the previous assays were seeded separately by applying a 10 µL aliquot of a bacterial suspension (10^7 CFU mL^{-1}) that was transformed into two L-shaped lines using a sterile loop at 4 cm equidistant from the mycelium disc. A control treatment consisting of PDA medium only with *T. harzianum* was included. The growth of *T. harzianum* was measured at 3, 5, and 9 dpi with the bacterial isolates. Fungal growth on the bacterial strain colonies was considered evidence of compatibility between the two microorganisms; conversely, the presence of an inhibition zone was considered as incompatibility between the bacterial isolates and the fungus.

The compatibility between the selected antagonistic bacteria was determined using the methodology described by Navarro (2023) with modifications. *Bacillus* sp and *P. protegens* were seeded in LB and King's B culture media, respectively, and both microorganisms were adjusted to a concentration of 10^7 CFU mL^{-1}. Once the bacterial lawn was dry, a 10 µL aliquot of *Bacillus* sp was inoculated onto the *P. protegens* lawn, and vice versa. Each treatment was performed in triplicate. The aim was to rapidly evaluate potential inhibitory effects of one bacterium on the growth of the other. Plates were incubated at 25–° C and assessed after 5 and 9 days.

Evaluation of the activity of antagonistic microorganisms for the control of *V. nonalfalfae* MLST 2 in three different kiwifruit rootstocks

In this experiment, five treatments were evaluated: a control root inoculated with *V. nonalfalfae* MLST2 (CI), a non-inoculated control (CNI), and roots individually treated with *P. protegens* strain C025 (PpC025), *B. velezensis* strain B1 (BcB1), and *T. harzianum* RGM 3510 (Th3510). These treatments were applied to rootstocks of cultivars Z5Z6 (*A. deliciosa*), Hayward (*Actinidia chinensis* var. *deliciosa* (A. Chev.) Liang and Ferg.) and Bounty (*A. macrosperma*), which were inoculated with the isolate of *V. nonalfalfae* MLST2 RGM 3750

at a concentration of 10^6 conidia mL^{-1}. Z5Z6 and Hayward plants were kindly provided by the Jingold Chile S.A. nursery, while Bounty plants were supplied by the Meyer S.A. nursery. The plants were less than one year old, had a stem diameter of at least 5 mm, and were grown in substrates based on peat and pine bark. Z5Z6 and Hayward plants had 3 - 4 true leaves, while Bounty plants had at least 12 leaves.

Individual plants were removed from the substrate, washed with sterile distilled water, and the root tips were cut with sterile scissors prior to inoculation. Inoculation was performed by immersing the roots in a suspension of 10^6 conidia per mL^{-1} of *V. nonalfalfae* MLST2 (RGM 3710), previously cultured, either alone (inoculated control) or together with the respective BCAs for 30 min. Prior to inoculation, strains of *P. protegens* and *Bacillus* sp. were adjusted to a concentration of 10^7 CFU mL^{-1}, while *T. harzianum* was adjusted to 10^6 conidia mL^{-1}. Antagonistic bacterial isolates were cultured in King's B and LB broth for *P. protegens* and *Bacillus* sp., respectively, at 25 °C for 48 h and bacterial concentrations were adjusted to 10^7 CFU mL^{-1} using a spectrophotometer (Epoch™, Biotek), considering an optical density (OD) equal to 0.1 as equivalent to this concentration (Sezonov et al., 2007).

Verticillium nonalfalfae MLST2 (RGM 3750) and the *T. harzianum* strain (RGM 3510) used for the tests were incubated at 25°C on PDA medium for 25 and 3 days, respectively, until conidia formation. Conidia were harvested from both the pathogen and *T. harzianum* by applying 15 mL of sterile saline solution (0.89%) to the plate to detach the conidia. The resulting suspension was filtered through pre-sterilized gauze to remove residual mycelial fragments and obtain a conidial suspension. The filtered suspension was then adjusted to a concentration of 10^6 conidia mL^{-1}.

The roots of the uninoculated control plants were washed and pruned with scissors, and then kept in sterile distilled water for 30 min. After inoculation, the plants were transferred to 0.25-liter pots containing peat-perlite substrate (3:1). Plants were kept in a growth chamber under a 16 h light/8 h dark photoperiod at 22 °C. They were watered three times per week and fertilized every two weeks with liquid fertilizer containing nitrogen, phosphorus, and potassium (5:3:5).

Three plants from each rootstock were used for each antagonist treatment. The plants were arranged in a completely randomized design with a factorial arrangement, where the interaction between treatments and rootstocks was analyzed.

Three evaluations of the percentage of plant wilting were conducted during the development of the experiment at 15, 30, and 45 dpi. This evaluation was carried out according to the scale proposed by Happstadius et al. (2003), where: 0 = no symptoms, 1 = wilting <25%, 2 = wilting ≥25%, 3 = wilting ≥50%, 4 = wilting ≥90%, and 5 = plant death. The data from the scale were used to determine the disease severity index (DSI) according to the formula described by Chiang et al. (2017):

$$DSI\ (\%) = \frac{\sum(Category\ frequency\ \times\ category\ score)}{(Total\ number\ of\ observations\ \times\ maximum\ disease\ index)} \times 100$$

The severity values obtained from each evaluation were converted to the area under the wilting progress curve (AUWPC) (Molina et al., 2023).

Evaluation of the activity of antagonistic microorganisms, individually and in combination, against *V. nonalfalfae* MLST2 in the Bruno rootstock and its effect on plant physiology

A second experiment was conducted during February and April 2025, using potted plants to determine the efficacy of the individual and combined use of the previously selected antagonistic microorganisms and their effect on the control of *V. nonalfalfae* MLST2 (RGM 3750) and plant physiology. The treatments with bacterial antagonistic microorganisms and their respective combinations with *T. harzianum* (RGM 3510) on *V. nonalfalfae* MLST2 (RGM 3750), evaluated in Bruno rootstock plants (*A. deliciosa*), are described in Table 1.

Bruno rootstock plants were kindly provided by Jingold Chile S.A. They were approximately one year old, with a main stem thickness of 10 mm, and had been grown under nursery conditions in a peat-pine bark substrate in 1.5-L bags. Plants were transferred, with their roots intact, into 4 L pots containing a peat-perlite substrate (3:1, v/v) prior to inoculation. Inoculation of Bruno plants with the respective treatments was carried out using the methodology described by Moya-Elizondo et al. (2016) with modifications. In this case, the treatments were first inoculated with BCAs and, seven days later, with the *V. nonalfalfae* MLST2 RGM 3750. Inoculation was performed using three 15-ml centrifuge tubes, each perforated with eight lateral holes (four holes on each side, spaced10 mm apart). The tubes were inserted into the pot substrate, equidistant from the plant at 5 cm. The substrate was

inoculated by applying 50 ml per pot of a suspension containing 10^7conidia mL^{-1}. Plants treated and inoculated with the pathogen were maintained under a shade structure with a black Rashell mesh cover with 50% light transmission.

Disease severity was evaluated at 30, 45, 60, and 90 dpi. Plants were evaluated using a visual scale based on the percentage of leaf wilting observed in each plant, according to Happstadius et al. (2003). A DSI was estimated as described above (Chiang et al., 2017). The AUWPC was determined using the values from each evaluation to integrate the effects of the treatments over the duration of the trial into a single indicator. A total of 15 plants per treatment were used, with three plants per replicate, arranged in a completely randomized block design with five replicates.

Physiological measurements

All physiological measurements were taken simultaneously at midday (12:00 to 15:00 h) in mature, healthy leaves with no visible symptoms of biotic or abiotic stress. Measurements were conducted in two occasions during the growing season. The initial measurement was taken one week prior to the application of treatments, while the subsequent measurement was taken at the end of the growing season.

Stem water potential (SWP) was measured at midday in one plant per block-treatment combination using a pressure chamber (Model 615, PMS Instruments, Corvallis, USA), following the protocol described by McCutchan and Shackel (1992). Leaf samples were selected from the lower part of the canopy and covered with an opaque airtight bag at least 40 min prior to measurement to allow equilibration of the water potential of leaves and stems.

Stomatal conductance (mmol m^{-2} s^{-1}) was determined using a portable porometer (LI-600, LI-COR Biosciences, Nebraska, USA) on two sun-exposed mature leaves from the upper canopy.

Photochemical Reflectance Index (PRI) was measured using a PRI meter (PlantPen PRI 210, Photon Systems Instruments, Drásov, Czech Republic) on two sun-exposed leaves from the upper canopy.

Maximum photochemical efficiency of Photosystem II (PSII) was estimated as *Fv/Fm* using a chlorophyll fluorescence meter (Pocket PEA, Hansatech Instruments, Norfolk, UK). For

this purpose, two mature leaves per plant were randomly selected from the upper canopy and adapted to darkness for 30 min using opaque clips (Reyes-Díaz et al., 2009). The minimum (*Fo*) and maximum (*Fm*) fluorescence values were recorded, and variable fluorescence (*Fv*) was calculated as the difference between *Fm* and *Fo*.

Statistical analysis

The *in vitro* antagonism and compatibility assays were performed twice, with three replicates arranged in a completely randomized design. The assessment of treatments with antagonistic microorganisms on three different kiwifruit rootstocks, as well as the assessments on the Bruno rootstock, included both the analysis of data from each evaluation and their integration into the AWDPC obtained from the DSI. The data were tested for normality using Shapiro-Wilk test and for homogeneity of variances using Levene test. After verifying these assumptions, an analysis of variance (ANOVA) was performed. When significant differences were detected, the means were separated using Tukey's test ($P < 0.05$) for the data from the *in vitro* assays and the trials under controlled conditions with the three rootstocks, while the study with the Bruno rootstock used Fischer's LSD test ($P < 0.05$). All statistical analyses were performed using InfoStat software, version 2008 (Balzarini et al., 2008).

Results

Selection of antagonists

The identification of *Bacillus* sp. isolates BcB1, BcB8, BcB9, BcB11, BcB12, and BcB13, based on partial amplification of the 16S rRNA, *glpF, ilvD, ptA,* and *tpiA* genes and their phylogenetic analysis, determined that all strains corresponded to *B. velezensis* (Supplementary Material 1). In the evaluation of genes associated with the production of antimicrobial peptides by *Bacillus* isolates determined the presence of the *spoVG*, *fenD*, *bmyB*, *ituC*, *srFAA*, and *bacA* genes in all isolates, and the absence of the *spoVG* gene in all of them (Supplementary Material 2). Based on the BIOLOG analysis, strains BcB1 and BcB8 exhibited the highest utilization of carbon sources (21 sources), while BcB1 was the only strain that showed tolerance to NaCl.

***In vitro* antagonism assays**

In vitro antagonism assays showed that all six isolates of *P. protegens* and all six isolates of *B. velezensis* inhibited *V. nonalfalfae* MLST2 (RGM 3750), although significant differences were only observed between the *P. protegens* strains ($P < 0.05$; Figure 1). On average, *B. velezensis* had greater inhibition on *V. nonalfalfae* (46.6%) compared to *P. protegens* strains, which inhibited mycelial growth by 35.9%. The highest level of inhibition by *P. protegens* was strain C025 (PpC025); while for *B. velezensis* it was strain BcB1, with inhibition percentages of 40.2% and 46.2%, respectively (Figure 1D). In contrast, *T. harzianum* (Th3510) grew across the entire plate in the presence of *V. nonalfalfae* MLST2 (RGM 3750) and reached a value of 1 on the Bell scale after 9 days (Figure 1G).

Compatibility assays

In vitro compatibility assays between bacterial isolates showed that between PpC025 cultured on BcB1 lawn, inhibition halos of 1 mm were observed. In contrast, no growth of *B. velezensis* BcB1 was observed on King's B culture medium (Figure 2 A and B) or on LB culture medium (Figure D and E) onto a PpC025 lawn, indicating a certain degree of incompatibility between the two antagonistic microorganisms. There was no antagonism between the two bacterial strains and the antagonistic fungus 3 dpi (Figure 2 C). Nine days after co-culture, the mycelium of Th3510 reached the edge of the plates, surpassing the lines of bacterial growth without showing signs of inhibition, indicating compatibility among the three microorganisms (Figure 2 F).

Evaluation of the activity of antagonistic microorganisms for the control of *V. nonalfalfae* MLST 2 in three different kiwifruit rootstocks

The integration of disease severity data through the calculation of AUWPC and subsequent analysis showed significant differences between rootstocks ($P < 0.001$) and applied treatments ($P = 0.013$), as well as an interaction between both factors ($P = 0.032$). Of the rootstocks used, Hayward had the highest average AWDPC value (1167), followed by Bounty (763) and Z5Z6 (330), with differences between them ($P < 0.05$), suggesting differences in susceptibility to the pathogen among rootstock genotypes.

The treatments with antagonistic microorganism resulted in significant differences in disease severity among treatments ($P < 0.05$; Table 2). BcB1 showed the highest AUWPC value

(1012) and was statistically similar to the inoculated control (CI), which had an AUWPC value of 854; the treatments with PpC025 and Th3510 had intermediate values of 710 and 671, respectively, while CNI had the lowest AUWPC value of 520.

Considering that there was interaction between both factors, treatments with antagonistic microorganisms were compared within each rootstock. On the Bounty rootstock, no significant differences were observed among treatments ($P < 0.05$; Table 2), indicating that none of the biological treatments evaluated generated a response in this rootstock. On the Hayward rootstock, CI, CNI, PpC025, and Th3510 did not differ from each other, while BcB1 recorded the highest disease severity value (AUWPC =1439), which may indicate a promotion of wilting associated with this treatment on this rootstock. On the Z5Z6 rootstock, significant differences were observed among treatments ($P < 0.05$). Similar to the Hayward rootstock, BcB1 showed the highest AUWPC value (1011) on Z5Z6 rootstock and differed from the rest of the treatments, indicating an increase in disease symptoms. In contrast, the other treatments did not differ from each other, even though CNI and PpC025 reduced wilting in the plants by an average of 85.3% and 87.6%, respectively.

When comparing the effect of microorganisms within each rootstock treatment, differences were observed in the CI and CNI treatments, with the Z5Z6 rootstock differing from Bounty and Hayward. PpC025 showed differences among the three rootstocks, with the greatest reduction in disease severity observed in the Z5Z6 treatment, followed by Bounty. In contrast, BcB1 increased disease expression in the Z5Z6 and Hayward rootstock compared to the severity values observed in Bounty. The use of Th3510 resulted in a significant reduction in wilt severity in Z5Z6, by 87% compared to Hayward, and 81% compared to Bounty; the latter did not differ from Hayward in the level of damage observed. Considering the interaction between the two factors analyzed, these results confirm that the response to the treatments depended on the rootstock. The Z5Z6 rootstock consistently presented the lowest AUWPC values in most treatments, while PpC025 and Th3510 reduced disease severity when applied to Z5Z6. In contrast, BcB1 resulted in an increase of Verticillium wilt severity, particularly in Z5Z6 and Hayward (Table 2).

Evaluation of the activity of antagonistic microorganisms, applied individually and in combination, against *V. nonalfalfae* MLST2 (RGM 3750) in the Bruno rootstock and its effect on plant physiology

The results of the four wilting evaluations performed on the Bruno rootstock are shown in Table 3. In the evaluation performed 30 dpi, significant differences were observed among treatments ($P = 0.0001$; C.V. = 17.1%). The uninoculated control (CNI) exhibited the lowest wilting value, being 60% different from the inoculated control (CI). In contrast, the BCA treatments, applied individually or in combination, showed no effect on disease reduction compared to CI and, on average, differed by 60% from CNI. At 60 dpi, no significant differences were observed among treatments, and no reduction in wilting associated with the BCA treatments was detected. Similarly, at 90 dpi, wilting did not differ significantly among treatments. However, during the final evaluation at 120 dpi, statistically significant differences were observed among treatments ($P = 0.008$; C.V. = 8.75%). The greatest reductions in wilting were observed with PpC025 and Th3510 applied individually to the roots, with reductions of 20% and 22%, respectively. In contrast, the uninoculated control showed 10% less wilting than the inoculated control (Table 3).

The integration of disease severity levels assessed in each evaluation of the damage caused by *V. nonalfalfae* MLST2 (RGM 3750), calculated as the AUWPC, indicated no significant differences among the individual treatments or the combinations of antagonists under evaluation ($P = 0.11$; C.V. = 4.72; Figure 3). However, the non-inoculated control (CNI) had the lowest AUWPC value (1368). PpC025 showed a similar AUWPC value of 1370, suggesting a suppressive effect on symptom development. In contrast, treatments with BcB1, Th3510, and their combinations showed AUWPC values close to the inoculated control with *V. nonalfalfae* MLST2 (RGM 3750).

Physiological measurements

The B-spline line shows a clear, non-linear, and predominantly negative relationship between DSI and SWP (Figure 4). As disease severity increases, the SWP of kiwifruit vines generally becomes more negative. SWP values ranged from -0.4 to -0.27 MPa, while wilting ranged from 25% to 55%.

Stomatal conductance values were similar for all treatments on the first measurement date. However, the BcB1 treatment showed a 70% higher stomatal conductance than the control on the last measurement date (Figure 5). At the end of the experimental season, stomatal conductance values ranged from 60 to 100 mmol/m²s.

No effect of treatment on the photochemical reflectance index (PRI) was observed on the first measurement date. On the other hand, the PpC025 + BcB1 treatment showed a PRI 67% higher than the control (Figure 6).

Discussion

Controlling vascular pathogens is difficult because they are transmitted from the soil to the plant, produce survival structures that remain in the soil for long periods of time, and in the absence of host plants can infect a wide range of other host plants (Yadeta, and Thomma, 2013). Few studies have addressed on *Verticillium nonalfalfae* MLST2, the causal agent of Verticillium wilt in yellow-fleshed kiwifruit, which has been reported only in Chile (Auger et al., 2009; Auger et al., 2011) and, to our knowledge, this research is the first attempt to evaluate cultural and biological alternatives for its control in kiwifruit.

The use of microorganisms for managing diseases caused by *Verticillium* species has been reported mainly in herbaceous plant species such as cotton, potato, strawberry, and tomato (Snelders et al., 2020; Wei et al., 2019; Cao et al., 2016; Nallanchakravarthula et al., 2014), with few studies focusing on woody hosts such as olive trees (Gómez-Lama Cabanás et al., 2018; Aranda et al., 2011; Mercado-Blanco et al., 2004) or pistachio (Antón–Domínguez et al., 2024).

The first step in achieving effective control of verticillium wilt using microorganisms is to identify antagonists with diverse functional characteristics capable of exerting biocontrol effects (Varo et al., 2016; Chen et al., 2013; Mercado-Blanco and Bakker, 2007). The *Bacillus* sp. isolates used in this study were identified as *B. velezensis*, as six isolates were obtained from walnut fruits; according to the criteria indicated by Fan et al. (2017), plant-associated isolates are classified as *B. velezensis*, while soil-derived isolates are classified as *B. amyloliquefaciens*. These isolates had the presence of genes associated with the production of antimicrobial peptides, including surfactin, bacylisin, fengycina, bacyllomycin, subtilin, and iturina (Mora et al., 2015; Mora et al., 2011), while *P. protegens* C025 strains have genes

associated with the production of antimicrobial compounds such as 2,4-diacetylphloroglucinol (DAPG), pioluteorin, and pyrrolnitrin (Dobrzyński and Kulkova, 2025; Jakubowska et al., 2025; Ajijah et al., 2025; Ramatte et al., 2011, Weller et al., 2007). Notably, *Pseudomonas* species have been reported to inhibit *V. dahliae* in olive trees (Gomez-Lama et al., 2018) and cotton (Bai et al., 2022) under both *in vitro* and *in planta* conditions.

In vitro antagonism assays confirmed the effectiveness of all selected isolates against *V. nonalfalfae* MLST2 RGM 3750 strain. However, PpC025 and BcB1 were selected for plant studies, as they achieved the highest percentage of mycelial growth inhibition. Additionally, biochemical characterization using BIOLOG showed that *B. velezensis* BcB1 could use 21 different carbon sources and exhibited lower chemical sensitivity to NaCl. According to Costa et al. (2002), the ability to use different carbon sources and exhibit lower chemical sensitivity provides valuable insights for developing of products based on antagonistic microorganisms, as competition for carbon sources is considered a key factor influencing biocontrol effectiveness.

In olive production, the use of rootstocks resistant to *V. dahliae* has been studied as a promising strategy for managing this pathogen (Díaz-Rueda et al., 2022; Bubici et al., 2012). However, there are no reports on the use of rootstocks in Chilean kiwifruit production as a preventive measure against Verticillium wilt caused by *V. nonalfalfae* MLST2 on yellow-fleshed kiwifruit. In the present study, Z5Z6 rootstock exhibited the lowest disease severity values, expressed as AUWPC, across most treatments, including the inoculated control, suggesting a higher basal tolerance to *V. nonalfalfae* MLST2. In contrast, Bounty (*A. macrosperma*) and Hayward (*A. chinensis* var. *deliciosa*) rootstocks showed greater disease severity. The Z5Z6 genotype is an improved green kiwifruit variety developed by the Jingold company from pre-existing varieties, with high productive potential and vigor. This vigor may be responsible for its greater tolerance to pathogens compared to the other rootstocks evaluated, consistent with reports in other plant species linking increased vigor to lower pathogen infection (Pagán and Garcia, 2020).

There are few reports on the use of rootstocks to control soilborne diseases in kiwifruit. In Brazil, Oliveira et al. (2021) evaluated the pathogenicity of *Ceratocystis fimbriata* in different cultivars and identified seven potential clones suitable as rootstocks for disease

prevention. In Italy, Mian et al. (2022) evaluated different rootstocks for the control of "Moria" or kiwifruit vine decline syndrome (KVDS), which is associated with soilborne pathogens that, together with waterlogging conditions, leads to the deterioration of roots and canopy. Similar to Verticillium wilt of yellow-fleshed kiwifruit caused by *V. nonalfalfae* MLST2 in Chile, current knowledge of KVDS remains limited, and thus techniques to control this syndrome are ineffective. The use of tolerant rootstocks is among the most promising strategies for managing soilborne diseases (Mian et al., 2022). The Hayward cultivar, like Z5Z6, is a variety grown commercially for its green fruit and belongs to the species *A. chinensis* var. *deliciosa*, but this is also used as a rootstock. However, Hayward has been described susceptible to Moria or KVDS, which could explain the unfavorable behavior of this rootstock in our trial, where it presented the highest disease severity values regardless of the treatment applied. In contrast, the Bounty rootstock, corresponding to *A. macrosperma*, has been reported as a promising alternative for KVSD management. A notable characteristic of this rootstock is its abundant root development compared to other *Actinidia* species, which may enhance the plant's tolerance to biotic or abiotic stresses (Mininni et al., 2024; Mian et al., 2022). However, our results suggest that this rootstock behaves differently when faced with vascular pathogens such as *V. nonalfalfae* MLST2.

Regarding the evaluated BCAs, PpC025 effectively reduced infection by *V. nonalfalfae* MLST2, with efficacy dependent on the rootstock, showing a significant control effect on the Z5Z6 rootstock, but not on Bounty or Hayward. This difference between rootstocks may be influenced by host-microorganism compatibility. In this context, root exudates act as chemoattractants to the rhizosphere (Jain et al., 2020; Wang et al., 2017), suggesting greater adaptability of the Z5Z6 rootstock by PpC025. Th3510 did not show significant reductions in AUWPC in most rootstocks compared to controls, although relatively low values were observed in Z5Z6. In contrast, BcB1 consistently showed the highest AUWPC values, particularly on Hayward and Z5Z6 rootstocks, indicating an unfavorable effect on disease control. These findings contrast with reports showing that *B. velezensis* XT1 reduced the severity of *V. dahliae* by 80% in olive trees (Castro et al., 2020).

In the evaluation to determine disease control and physiological responses in the Bruno rootstock, the severity of wilting caused by *V. nonalfalfae* MLST2 had variable responses to the treatments during the experiment. This variability suggests that the effects of the

evaluated antagonistic microorganisms was influenced both by the time of year in which the experiment was conducted and by the host's capacity to respond to infection.

Analysis of disease severity data using AUWPC revealed no significant differences between the microorganisms evaluated, either applied individually or in combination, compared to the inoculated control. This indicates that none of the treatments substantially altered the cumulative progression of the disease. However, the lower AUWPC value recorded in the uninoculated control suggests that other factors may have influenced the observed wilting, indicating the absence of infection in this control. Similarly, PpC025 had an AUWPC value very close to that of the non-inoculated control (CNI), suggesting a potential suppressive effect on wilting development, as observed on the Z5Z6 rootstock, although no statistical differences were observed. Factors such as inoculum density in the substrate may have influenced symptom development. In our experiment, plants were inoculated with 50 mL of a conidia suspension of 10^6 per mL applied in a volume of 4 L of peat-perlite substrate [3:1]. This inoculum level may have been low considering that studies in woody crops, such as olive trees, have reported no symptoms attributable to *V. dahliae* when 20 L pots with naturally infested soil were used, which had a pathogen concentration of 5.5 CFU g^{-1} of soil (Mulero-Aparicio et al. 2020).

The density of pathogen inoculum should also be considered an important factor when establishing trials or commercial plantations. López-Escudero et al. (2007) determined that both high and low densities of *V. dahliae* inoculum affected olive plants similarly over a three-year period. These findings suggest that inoculum volume used in our study may or may not have been sufficient to induce disease development in the Bruno rootstock. In this sense, Varo et al. (2016), despite using very high inoculum concentrations (119 CFU $g^{-1}$of soil), reported only a low incidence of Verticillium wilt in olive trees.

Considering that Bruno plants developed wilting and chlorosis with lower intensity, it is important to note that the plants were one year old and had an abundant root volume, which could have favored the lower expression of symptoms, in addition to the relatively low pathogen inoculum used in the experiment. Another point to consider is that the plants were kept in pots under high summer temperatures, conditions under which wilting may also result from water stress. Water stress is caused by a lack of water and its severity increases when vapor pressure deficit (VPD) is high, a parameter dependent on both air temperature and

relative humidity. During the trial period, the average temperature was 17.2°C, with maximum and minimum values ranging between 25 and 9.4°C, respectively. These temperature conditions were accompanied by an average relative humidity of 65.6% throughout the trial period (Supplementary Material 3). At the physiological level, the findings of the present study demonstrated a negative relationship between DSI and SWP (i.e., water stress severity) in kiwifruit plants, indicating that an increase in disease severity corresponds to an increase in water stress. These results suggest that a more severe *V. nonalfalfae* MLST2 infection may have blocked the plant's vascular system, thus restricting its ability to transport water and causing a reduction in SWP, even under relatively low levels of water stress. In this context, as plants in the study were not subjected to water restrictions and were grown under 50% shade netting throughout the whole growing season, SWP values were expected to fall within the upper range of the plant's water status (between -0.5 and -0.25 MPa). This indicates that the physiological state of the experimental plants was close to the optimal water status for kiwifruit (Calderón-Orellana et al., 2021).

No significant differences in SWP values were observed among treatments on any of the evaluation dates. This finding suggests that the application of the treatments did not alter the severity of water stress, despite the evident correlation between disease severity and water stress. These results may indicate that the treatment had an inconsistent impact on xylem blockage, which may not have induced differences in SWP among treatments. However, as SWP values at the end of the season remained consistently close to or above the optimal physiological water status for the plant, it is plausible that possible obstructions of the vascular system caused by *Verticillium* infection did not result in plant dehydration under conditions of high plant water status and low atmospheric evaporative demand.

As the second ecophysiological measurement was taken near the onset of leaf senescence, stomatal conductance values were low in Bruno kiwifruit plants. Maximum stomatal conductance in mid-season usually exceeds 500 mmol/m² (Calderón-Orellana et al., 2021). The combination of PpC025 + BcB1 showed significantly higher stomatal conductance than the control, by approximately 70% (Figure 5). An increase in stomatal conductance enhances the concentration of CO_2 in the leaf (Ci), which in turn increases the net photosynthetic rate (An). Since the treatments did not affect SWP, it is unlikely that the improvement in stomatal conductance observed in PpC025 + BcB1 treatment was due to an improvement in plant

water status resulting from reduced colonization of the xylem vessels. In olive trees (*Olea europaea* L.), Cardoni et al. (2022) reported significant reductions in stomatal conductance and photosynthesis in the leaves of plants infected with *Verticillium*. It has been determined that toxins and phytohormones secreted by pathogens, such as ethylene and abscisic acid produced by *V. dahliae*, can induce stomatal closure or directly damage the leaf photosynthetic apparatus (Saeed et al., 1992). In addition, the combination PpC025 + BcB1 also showed a significantly higher PRI than the control. More negative PRI values generally indicate greater activation of the xanthophyll cycle as a photoprotective mechanism against some types of stress. Therefore, these results suggest that the leaves of the plants treated with PpC025 + BcB1exhibited lower photoprotective energy dissipation and, consequently, lower stress levels compared to the control. The difference of 0.047 between the PpC025 + BcB1 treatment and the inoculated control is large enough to distinguish between a healthy plant and one experiencing moderate to severe stress, such as that caused by disease. Consequently, these results showed that the combination PpC025 + BcB1 produced the most consistent physiological responses associated with a reduced stress impact, even though no significant differences in AUWPC were detected at the end of the growing season in young Bruno rootstock plants.

The integration of BCAs with suitable rootstocks represents a promising, sustainable, and effective strategy to mitigate the impact of Verticillium wilt on yellow-fleshed kiwifruit, promoting both physiological stability and plant resilience. However, environmental variability and the complexity of microbial interactions under field conditions require larger-scale studies, the development of stable microbial formulations, and analyses of the associated microbiome. These studies should include different seasons throughout the year and irrigation strategies that mimic variations in water availability and demand associated with climate change. Such advances will help consolidate the use of beneficial microorganisms as key components of integrated vascular disease management programs in fruit trees.

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Tables and figures

Table 1. Treatments used to evaluate the efficacy of microorganisms applied individually or in combination for the control of *Verticillium nonalfalfae* MLST2 and their effects on the physiology of 'Bruno' kiwifruit rootstocks.

Treatment code	Treatments
CI	Inoculated control
CNI	Non-inoculated control
PpC025	*Pseudomonas protegens* C025
BcB1	*Bacillus velezensis* BcB1
Th3510	*Trichoderma harzianum* RGM3510
PpC025 + BcB1	*P. protegens* C025 + *B. velezensis* B1
Th3510 + BcB1	*T. harzianum* + *B. velezensis* B1
Th3510 + PpC025	*T. harzianum* + *P. protegens* C025
PpC025 + BcB1 + Th3510	3 BCA*

*3 BCA corresponds to a consortium with strains of *P. protegens*, *B. velezensis*, and *T. harzianum.*

Table 2. Results of the area under wilting progress curve (AUWPC) values associated with root treatments with antagonistic microorganisms applied individually on three different kiwifruit rootstocks, inoculated or not with *Verticillium nonalfalfae* MLST2.

	Rootstocks			
Treatments	**Bounty**	**Hayward**	**Z5Z6**	**Average**
CI	1010 a A	1128 ab A	422 b B	**854 ab**
CNI	666 a A	856 b A	39 b B	**520 b**
PpC025	794 a B	1303 ab A	33 b C	**710 ab**
BcB1	586 a B	1439 a A	1011 a AB	**1012 a**
Th3510	759 a A	1108 ab A	144 b B	**671 ab**
Average	**763 B**	**1167 C**	**330 A**	

Treatment (Trt) *P* = 0.013*

Rootstock (Rst) *P* = 0.001*

Trt x Rst *P* = 0.0317**

Different lowercase and uppercase letters indicate statistically significant differences among treatments within each rootstock and among rootstocks within each treatment, respectively.

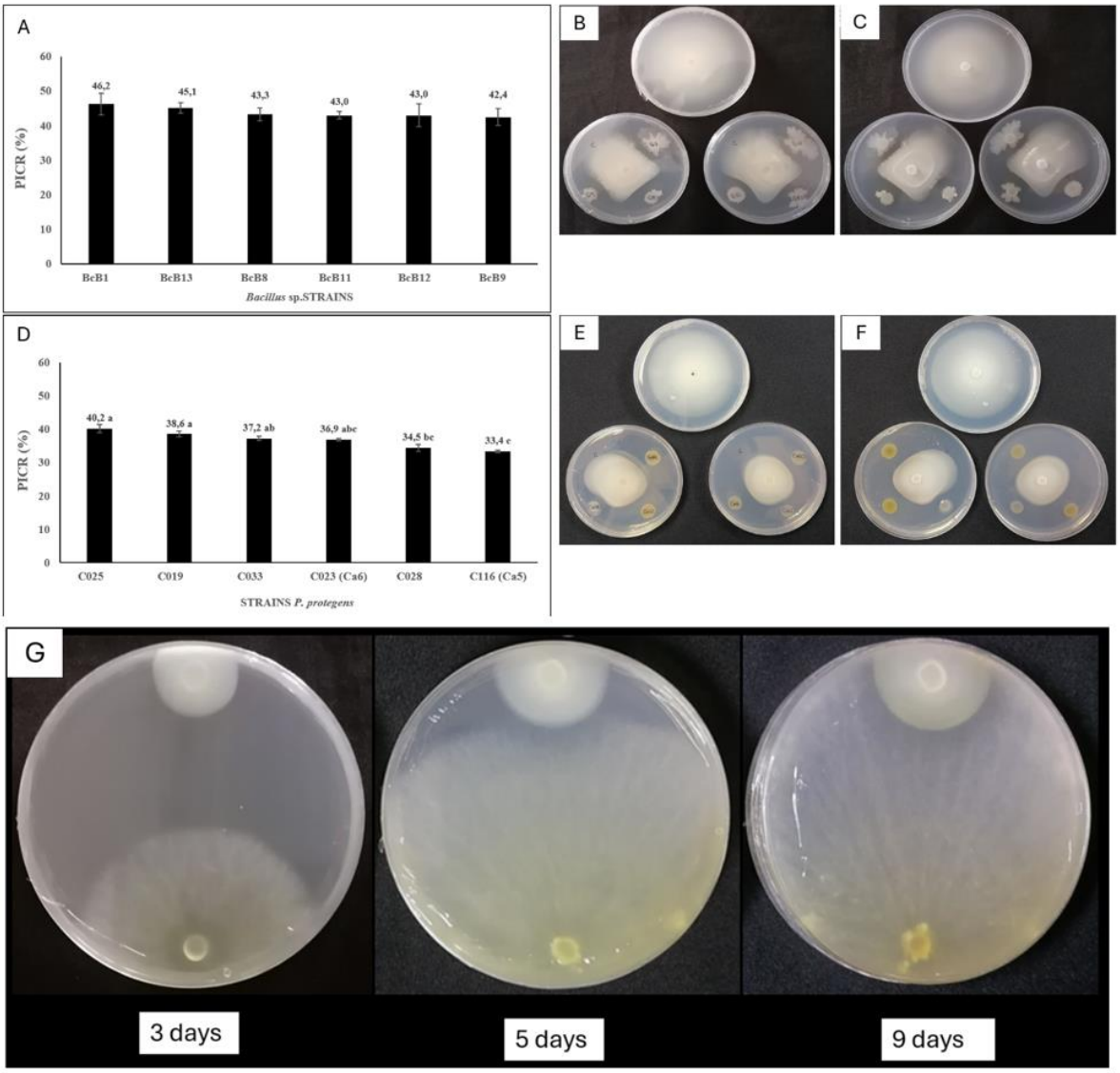
* *P* value for treatments and rootstocks are indicated. ** Interaction *P* value 0.0317, according to Tukey's multiple range test, with a *P* value < 0.05. Bold italic uppercase letters indicate mean differences among rootstocks, while bold italic lowercase letters indicate differences among antagonistic microorganism treatments. Antagonistic microorganism treatments are described in Table 1.

Table 3. Wilting severity (%) observed at different evaluation dates in 'Bruno' kiwifruit rootstocks treated with antagonistic microorganisms applied individually or in combination for the control of *Verticillium nonalfalfae* MLST2.

Treatments	Evaluation dates			
	1st evaluation 30 dpi	2nd evaluation 45 dpi	3rd evaluation 60 dpi	4th evaluation 90 dpi
CI	11.7 a	14.0	27.7	42.0 a
CNI	4.7 b	16.3	29.0	38.0 ab
PpC025	11.3 a	16.7	29.0	33.7 b
BcB1	11.7 a	15.7	29.0	39.7 ab
Th3510	12.3 a	16.3	29.3	32.7 b
PpC025 + BcB1	12.7 a	15.3	29.0	40.3 ab
Th3510 + BcB1	12.0 a	16.7	29.3	37.3 ab
Th3510 + PpC025	12.0 a	17.7	29.0	40.7 ab
PpC025 + BcB1 + Th3510	11.7 a	17.0	28.0	40.0 ab
C.V	17.1	13.41	7.27	8.75
***P*-value**	0.0001	0.3228	0.9213	0.0008

Values correspond to mean wilting values across four different evaluation dates. Dates with different letters indicate statistically significant differences among the treatments applied to the 'Bruno' plants, *P* < 0.05, according to Tukey's multiple range test. Description of the antagonistic microorganism treatments is available in Table 1.

Figure 1. Results of percentage of radial growth inhibition (PRGI) and view of *in vitro* antagonism assays obtained with six isolates of *Bacillus* sp. (BcB1, BcB8, BcB9, BcB11, BcB12, and BcB13) (A, B, and C)), six strains of *Pseudomonas protegens* (C019, C023 (Ca6), C025, C028, C033, C116 (Ca5)) (D, F, and E) after 30 days of co-culture, and with *Trichoderma harzianum* RGM3510 after 3 evaluation dates (G) , against *Verticillium nonalfalfae* MLST2 strain RGM 3750.



Different letters indicate statistically significant differences among strains of *Pseudomonas protegens* (D), $P < 0.05$ according to Tukey's multiple range test.

Figure 2. Compatibility assays among the antagonistic microorganisms *Pseudomonas protegens* C025, *Bacillus velezenzis* BcB1, and *Trichoderma harzianum* RGM 3510. KB medium plate seeded with *B. velezensis* BcB1 lawn and subsequently inoculated with four 10 µL aliquot of *P. protegens* C025 (A) and vice versa (B). LB medium seeded with *B. velezensis* B1 lawn and subsequently inoculated with four 10 µL aliquot of *P. protegens* C025 (D) and vice versa (E), after 3 days of inoculation. PDA plates co-inoculated with three antagonistic microorganisms (C and F), *T. harzianum* RGM 3510 (T) in the center, and two lines of *P. protegens* C025 (*Pp*) and *B. velezenzis* BcB1(*B*) after 5 (C) and 9 days (F) of co-culture.

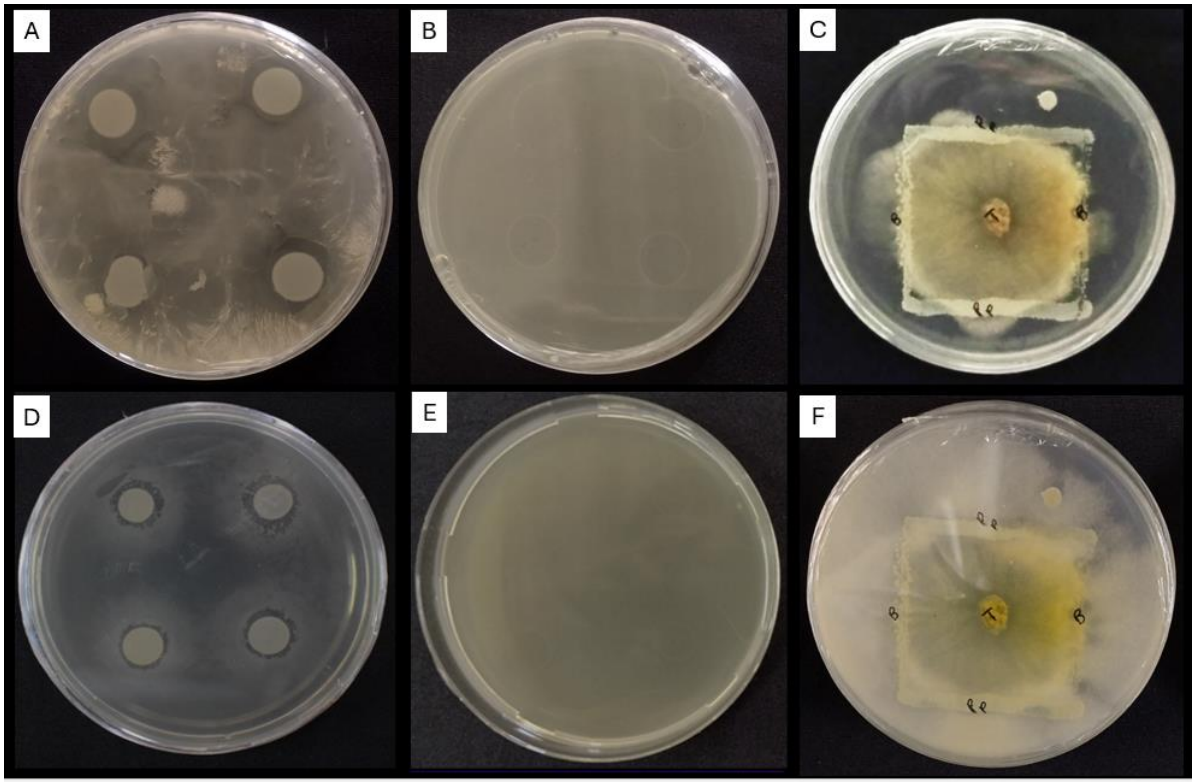
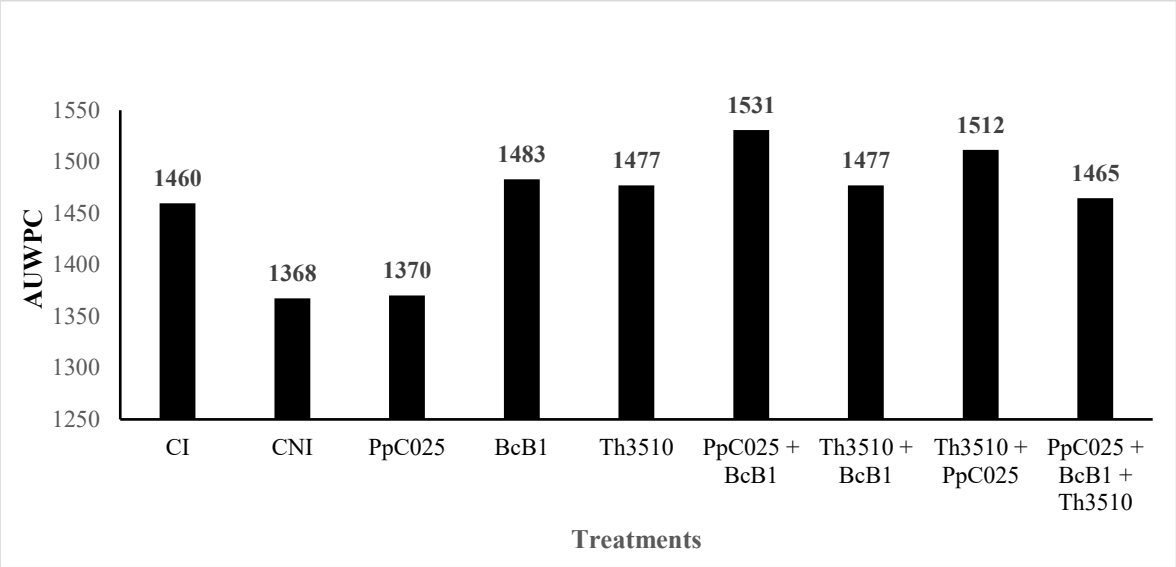


Figure 3. Area under the wilting progress curve (AUWPC) observed in 'Bruno' kiwifruit plants after 120 days of growth in pots, inoculated or not with *Verticillium nonalfalfae* MLST2 under nursery conditions, treated with different microorganism-based treatments. Treatment description is provided in Table 1.



Values correspond to the AUWPC average after four evaluation dates, with no statistically significant differences according to Fisher's LSD test ($P < 0.05$) (p value = 0.118, C.V= 4.72).

Figure 4. Penalized B-Spline for the relationship between the severity index disease and midday stem water potential (12:00-15:30 h) of kiwifruit vines (*Actinidia chinensis* var. *deliciosa*) subjected to several treatments of Verticillium wilt control. Error bars represent ± 1 s.e.

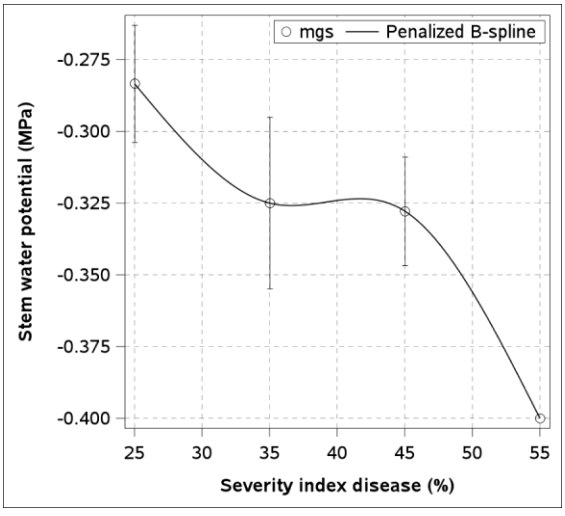
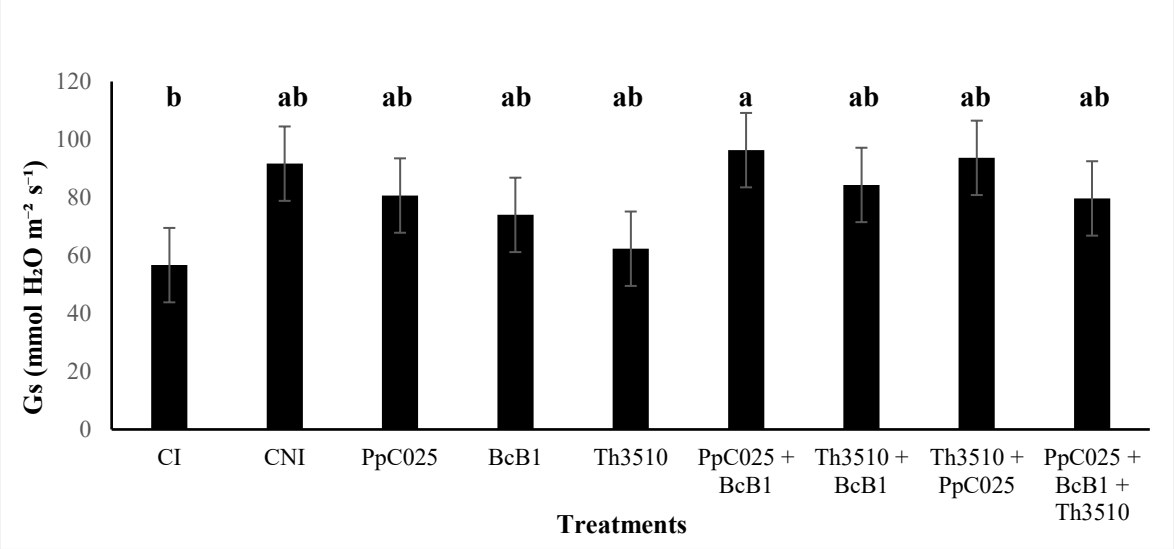
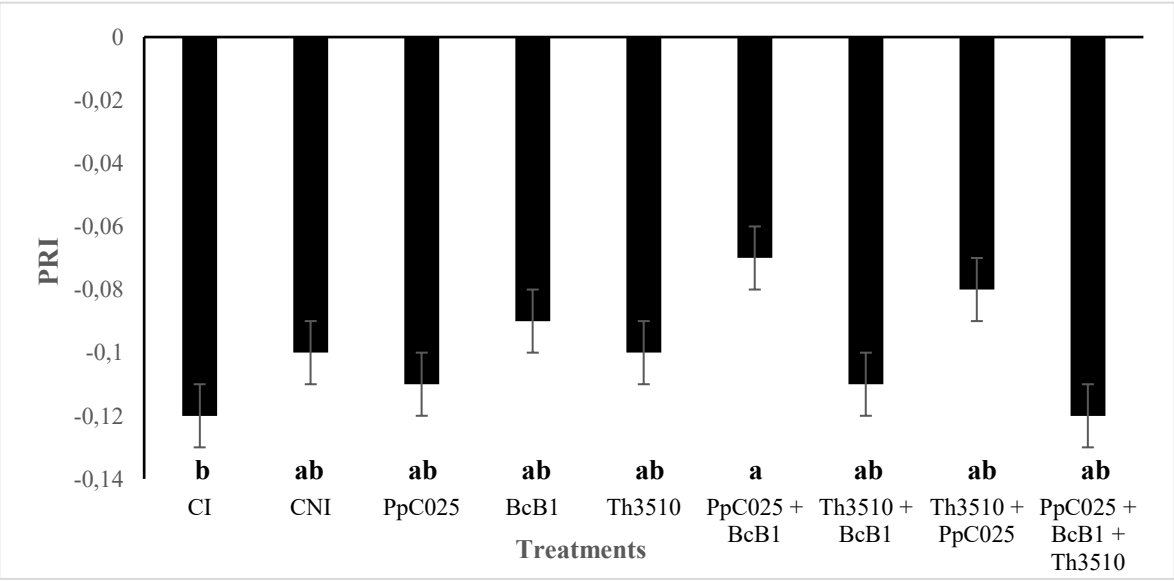


Figure 5. Stomatal conductance at midday (12:00-15:30 h) of leaves from kiwifruit vines (*Actinidia chinensis* var. *deliciosa*) subjected to several treatments of *Verticillium* wilt control at the end of the experimental season. Treatment description is provided in Table 1.



Different letters indicate significant differences (Tukey) at 95% confidence level. Error bars represent ± 1 s.e. N=3.

Figure 6. Photochemical reflectance index (PRI) at midday (12:00-15:30 h) of leaves from 'Bruno' kiwifruit vines (*Actinidia chinensis* var. *deliciosa*) subjected to several treatments of *Verticillium* wilt control with three antagonistic microorganisms at the end of the experimental season. Treatment description is provided in Table 1.



Different letters indicate significant differences (Tukey) at 95% confidence level. Error bars represent ± 1 s.e. N=3.

Conclusiones generales

Pseudomonas protegens C025 (Pp C025) y *Bacillus velezensis* BcB1fueron seleccionados como agentes de control biológico (ACB), ya que mostraron mayor inhibición del crecimiento micelial sobre *Verticillium nonalfalfae* MLST2 en pruebas *in* vitro realizada con 6 aislados de cada género bacteriano, presentando *P. protegens* C025 crecimiento incompatible sobre *B. velezensis* BcB1. Por su parte, *Trichoderma harzianum* RGM 3510 (Th3510) creció sobre *V. nonalfalfae* MLST2 y no presentó incompatibilidad con ambos aislados bacterianos seleccionados.

En base a la evaluación de las dos bacterias seleccionadas (Pp C025 y BcB1) y Th3510 sobre tres portainjertos de kiwi (Hayward, Bounty y Z5Z6) se observó que el genotipo influye significativamente en la severidad de la marchitez causada por *V. nonalfalfae* MLST2 y en la eficacia de control de los ACB sobre este fitopatógeno. El cultivar Z5Z6 (*Actinidia chinensis* var. *deliciosa*) al ser utilizado como portainjerto fue el genotipo que presentó la mayor tolerancia a la enfermedad, seguido de Bounty (*A. macrosperma*) y Hayward (*A. chinensis* var. *deliciosa*). La efectividad de los microorganismos antagonistas estuvo condicionada por la interacción entre el hospedero, el patógeno y el mismo microorganismo benéfico. PpC025 y Th3510 presentaron las mayores reducciones en la severidad de la enfermedad con los tres portainjertos, especialmente con Z5Z6, mientras BcB1 incremento el daño causado por el patógeno.

Los resultados de las cuatro evaluaciones de marchitez realizadas en el portainjerto Bruno indicaron que no hubo mayor efecto en la reducción de la enfermedad de los tratamientos con ACB, aplicados individualmente o en combinación, en las tres primeras evaluaciones, pero en evaluación final a los 90 días post inoculación, se observaron reducciones en el marchitamiento con PpC025 y Th3510 aplicados individualmente a las raíces.

A nivel fisiológico, los hallazgos del presente estudio con el portainjerto Bruno demostraron una relación negativa entre la severidad de la enfermedad y la severidad del estrés hídrico en plantas de kiwi, mientras que no se observaron diferencias en los valores de SWP (Potencial hídrico) entre los tratamientos en base a ACB en ninguna de las dos fechas de evaluación. Por su parte, La combinación de PpC025 + BcB1 mostró una conductancia estomática significativamente mayor y tuvo un PRI (Índice de reflectancia fotoquímica) significativamente más alto que el control no tratado. Estos resultados sugieren que las hojas

de las plantas del portainjerto de kiwi Bruno tratadas con PpC025 + BcB1 exhibieron menor disipación de energía fotoprotectora y, en consecuencia, menores niveles de estrés en comparación con el control.

Finalmente, la integración de ACB con portainjertos adecuados representa una estrategia prometedora, sostenible y eficaz para mitigar el impacto del marchitamiento por Verticillium en el kiwi de pulpa amarilla, promoviendo tanto la estabilidad fisiológica como la resiliencia de la planta a la enfermedad.