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USO DE PROGRAMAS DE MANEJO BASADOS EN PRODUCTOS BIOLÓGICOS PARA EL CONTROL DE ENFERMEDADES EN EL CULTIVO DEL CEREZO

Tesis para optar al grado de Magister en Ciencias Agronómicas

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RESUMEN

El cáncer bacterial y las pudriciones causadas por hongos de pre y post cosecha son enfermedades que afectan al cerezo mundialmente. El uso de productos biológicos es una alternativa de control adecuada para reducir el uso de agroquímicos. Sin embargo, existe poca información sobre la interacción entre estos productos y su efecto sobre las dinámicas poblacionales de los fitopatógenos cuando se incorporan dentro de un programa de manejo integrados de enfermedades. Este trabajo evaluó el impacto fitosanitario de programas basados en productos biológicos, en el manejo de estas enfermedades del cerezo y desarrolló un sistema de qPCR para evaluar presencia de *Pseudomonas syringae* pv. *syringae* (Pss) en tejidos de cerezo. Se establecieron unidades de media hectárea bajo dos tratamientos basados en bioproductos, otra unidad basada sólo en control químico y una unidad bajo el manejo del productor, dentro de un huerto de cerezos 'Sweetheart' ubicado en Chillán, Chile. Se realizaron conteos poblacionales y medidas de incidencia y severidad en estados fenológicos específicos del cultivo. Los resultados indican que los tratamientos basados en bioproductos a base de *Pseudomonas protegens*, *Bacillus* spp. y *Trichoderma* spp. disminuyeron las poblaciones de *Pseudomonas syringae* pv. *syringae*; *Botrytis cinerea* y *Alternaria* spp. durante estados fenológicos específicos, sin ser diferentes respecto a los demás tratamientos. No existieron diferencias significativas en la incidencia y severidad de cancros bacterianos entre los tratamientos, pero si hubo disminuciones en la incidencia de un 87% en pudrición por *Alternaria* spp., y de un 60% en pudrición por *Botrytis cinerea* en fruto maduro en el programa químico con respecto a los basados en productos biológicos, durante la primera y segunda temporada, respectivamente. Estos resultados permiten promover la inclusión de bioproductos en programas comerciales de manejo de enfermedades en cerezo. Se realizaron curvas estándar por qPCR para la cuantificación de Pss en tres estados fenológicos susceptibles, comparando los resultados con los obtenidos por método de conteo en placas Petri. Los resultados indican que el método qPCR es altamente sensitivo, rápido, y efectivo en el monitoreo de la carga de Pss, con límites de detección entre 10^4 y 10^5 UFC mL^{-1}, indicando que es una buena herramienta para la toma de decisiones relacionadas al control del cáncer bacterial.

ABSTRACT

Bacterial canker and pre- and postharvest rots are diseases affecting sweet cherry worldwide. The use of biological products is a suitable alternative to reduce agrochemical applications. However, there is a lack of information on the interaction between these products and their effect on the population dynamics of these pathogens when incorporated into integrated disease management programs. This study evaluated the phytosanitary impact of biological based management programs on sweet cherry disease control. Half hectare units were established under two biological-based programs, one based only on chemical control and the other under the local grower program, in a ´Sweetheart´ sweet cherry orchard in Chillán, Chile. Population counts and incidence and severity measurements were made on cherry at specific phenological stages. Results showed that biological-based programs, which included *Pseudomonas protegens*, *Bacillus* spp. and *Trichoderma* spp., reduced populations of *Pseudomonas syringae* pv. *syringae*; *Botrytis cinerea* and *Alternaria* spp. at specific phenological stages, with no differences regarding chemical and grower programs. There were no significant differences in the incidence and severity of bacterial cankers among all management programs, but there were decreases of 87% for *Alternaria* spp. rot incidence and 60% for *Botrytis cinerea* rot incidence in ripe fruit in the chemical program compared to the biological product-based programs during the first and second seasons, respectively. These results support the inclusion of bioproducts in commercial cherry disease management programs. qPCR standard curves were generated for Pss quantification in three susceptible phenological stages of sweet cherry and the results were compared to those obtained using the bacterial drop count method. The obtained results showed that qPCR is a highly sensitive, fast, and effective method for Pss load monitoring, with detection limits ranging from 10^4 to 10^5 CFU mL^{-1}, indicating that it is a good tool for decision-making related to bacterial canker control.

INTRODUCCION GENERAL

El cerezo (*Prunus avium*) en Chile alcanza un promedio de producción de 321 mil toneladas anuales, en una superficie promedio aproximada de 44 mil hectáreas, entre los años 2018 y 2022, siendo exportadas, en el mismo período, un 85% de esa producción, posicionando a Chile dentro de los principales exportadores del mundo (FAO, 2022a; FAO, 2022b). Para la zona centro sur y sur de Chile, que comprende las regiones del Maule a Los Lagos, la superficie plantada ha aumentado en un 221%, desde el año 2010 al 2020 (ODEPA, 2020). Sumado a lo anterior, la reciente región del Ñuble ha visto un incremento de un 128% de la superficie entre los años 2016 y 2022 (ODEPA, 2022).

Este frutal puede ser afectado por diversas enfermedades que tienen un impacto económico, y cuya incidencia se ve favorecida en condiciones de alta humedad relativa, presencia de agua libre y altas precipitaciones, similares a las que se presentan en la zona sur y centro sur de Chile. Enfermedades bacterianas como la agalla de la corona, causada por *Agrobacterium tumefaciens* Conn; el cáncer bacterial causado por *Pseudomonas syringae* pv. *syringae* van Hall (Pss) y *P. syringae* pv. *morsprunorum* Wormald (Psm) razas 1 y 2; enfermedades fungosas como la pudrición parda, ocasionada por *Monilinia* spp*;* Cloca, causada por *Taphrina cerasi* Fuckel; plateado, causado por *Chondrostereum purpureum* Person; muerte de madera por *Cytospora* spp.; verticilosis ocasionada por *Verticillium dahliae* Klebahn; pudriciones del cuello y raíces por *Phytophthora* spp.; pudriciones diversas por *Alternaria* spp., *Aureobasidium pullulans* de Bary, *Cladosporium* spp., *Rhizopus* spp., entre otras y el moho gris, causado por *Botrytis cinerea* Person, son capaces de afectar severamente la producción del cerezo en Chile (Latorre, 2018).

El cáncer bacterial es considerada la principal enfermedad afectando huertos de cerezo en Chile y es causado principalmente por Pss en Chile; y recientemente se ha detectado infección por Psm en huertos de la Región de Los Lagos (Resolución N° 8498 exenta; García *et al*., 2021). Esta bacteria Gram negativa, infecta a través de aberturas naturales como estomas y lenticelas o a través de heridas naturales (por caída y cicatrices de hojas) y mecánicas (podas y daños por maquinaria agrícola). La bacteria al establecerse dentro del tejido llega a colonizar el tejido cambial y provocar cancros en órganos leñosos, con consecuente gomosis y olor a fermentación característico. Además, durante la fase de crecimiento, parte de la población epífita ingresa a través de las hojas, causando manchas café y negras, las que se evolucionan a perforaciones (Hulin *et al.*, 2018), con futura disminución del área fotosintética. La severidad de los síntomas dependerá de la cepa bacteriana, cultivar, edad de la planta, tejido invadido y condiciones ambientales, llegando a provocar pérdidas en el rendimiento de 10 a 20% en huertos jóvenes e incluso la muerte de las plantas, bajo condiciones ambientales favorables (Khezri y Mohammadi, 2018).

Los hongos *Monilinia fruticola, Monilinia laxa* y *Botrytis cinerea* son los principales agentes causales de las pudriciones de precosecha y postcosecha en el cerezo en Chile. Las conidias de estos hongos son capaces de infectar todos los tejidos de la flor y del fruto, con el mayor riesgo de infección durante el estado fenológico de plena floración, en condiciones de alta humedad relativa y agua libre (Holb 2008). En caso de ocurrir infección durante el estado floral, se puede desarrollar un atizonamiento de la flor, con consecuente muerte floral, o bien terminar en una infección latente en el fruto (Tarbath *et al*., 2014), lo que a futuro podría causar la pudrición en caso de presentarse condiciones favorables. Además, estos patógenos son capaces de invernar en frutas momificadas, de tal forma que, a mayor presencia de estas fuentes de inóculo, mayor será el riesgo de infección (Tarbath *et al.*, 2014). Añadido a estos agentes se encuentran especies del género *Alternaria,* cuyas conidias según Timmer *et al* (2003), en climas mediterráneos, serían capaces dispersarse tras descensos en la humedad relativa; y sobrevivir durante el invierno en suelos, semillas, y tejido perenne (Troncoso-Rojas y Tiznado-Hernández, 2014). En cuanto a las pérdidas ocasionadas por los hongos anteriormente mencionados, la pudrición café por *Monilinia* spp. causa bajas en el rendimiento en postcosecha de hasta un 80% cuando las condiciones climáticas son favorables para el desarrollo de la enfermedad, especialmente en variedades de maduración tardía (Gotor-Vila *et al*., 2017). A su vez, *Botrytis cinerea* es capaz de generar pérdidas por infección latente mayores al 50%, tras la cosecha (Tarbath *et al*., 2014); y para el caso de *Alternaria alternata,* se han detectado incidencias en postcosecha iguales a un 26% (Ahmad *et al*., 2020).

Para el manejo de estas enfermedades se usan programas de manejo integrado, que utilizan bactericidas preventivos basados en el uso de compuestos cúpricos (Husseini and Akköprü, 2020) y antibióticos (Sundin and Wang, 2018). El ión cobre afecta a las bacterias alterando la funcionalidad de enzimas, desplazando co-factores metálicos esenciales, y generando especies reactivas del oxígeno (Husseini and Akköprü, 2020). Además, este ión es capaz de controlar patógenos fúngicos (Mitre *et al*., 2011). Si bien estos productos en base a cobre son eficaces en el control de las bacterias, pueden ocasionar fitotoxicidad y acumularse en el ambiente, en los casos en que se utilizan en altas concentraciones o si se aplican de manera reiterada, lo que conduce a la aparición de cepas resistentes (Aprile, 2021; Beltrán *et al*., 2021) y alteración de la microbiota del suelo (Alengebawy *et al*., 2021). Sumado a lo anterior, existe un alto costo de control debido al número de aplicaciones promedio por temporada en Chile entre las regiones Maule y Araucanía, el que asciende a un valor aproximado de 14,6 millones de dólares (Moya-Elizondo, 2020). En el caso de los antibióticos, en Chile están autorizados ingredientes activos como el Clorhidrato de Kasugamicina hidratado, Sesquisulfato de Estreptomicina y Clorhidrato de Oxitetraciclina, pero su uso inadecuado favorece el rápido desarrollo de resistencia (Sundin and Bender, 1993; Vasebi *et al*., 2019).

El control de hongos se realiza mayoritariamente a través del uso de fungicidas químicos sintéticos. Estos fungicidas tienen diversos modos de acción y ejercen su acción mediante la inhibición de la germinación de esporas e infección de los tejidos del huésped, siendo los preventivos aplicados desde yema hinchada y floración hasta desarrollo del fruto (Tarbath *et al*., 2014). Entre las alternativas más utilizadas en el mercado encontramos fungicidas de contacto tales como Mancozeb; Thiram; Captan; Dicloran; Propiconazol; azufre; y fungicidas sistémicos como Tiofanato-metilo; Miclobutanilo; Benomilo; Fenbuconazol y Tebuconazol. Sin embargo, las opciones son cada vez más limitadas frente a un mercado creciente en el consumo de productos libres de plaguicidas químicos sintéticos, además de nuevas restricciones en el uso de dichos fungicidas químicos, dado el potencial efecto nocivo en la salud humana. Un claro ejemplo de lo anterior se refleja en la reciente prohibición del fungicida Mancozeb por la Unión Europea, del que se ha demostrado su efecto inmunosupresor en ratones (Bano and Mohanty, 2020), y se sugiere como una amenaza para el desarrollo y la reproducción humana (Runkle *et al*., 2017).

Debido a las problemáticas asociadas al uso de bactericidas y fungicidas en la actualidad se están desarrollando alternativas para el manejo de las enfermedades que afectan el cerezo. En Chile está autorizado el uso de productos formulados en base a microorganismos, tales como Mamull®, un fungicida compuesto por cepas de *Bionectria ochroleuca*, *Trichoderma gamsii* y *Hypocrea virens*, usado para el control de plateado; o Nacillus®, bactericida compuesto por cepas de *Bacillus* spp. y *Brevibacillus brevis,* utilizado para el control del cáncer bacterial. Así también están autorizados hongos del género *Trichoderma*, en productos como Trichonativa®, para el control de pudriciones de raíz y cuello, y moho gris; Trichoderma suspensión®, usado para el control de moho gris; 3 TAC-I Beta®, para el control de Pss; y extractos vegetales como EcoSwing®, extracto de *Swinglea glutinosa,* usado para el control de moho gris y moho negro; BC-1000®, extracto de *Citrus x paradisi*, para controlar pudrición parda, moho gris y pudrición ácida; o Bestcure®, extracto de frutos de *Citrus aurantium* L., para control de pudriciones ácidas, tizón de la flor y moho gris; los que sugieren ser eficaces como herramientas en el control preventivo. Sin embargo, a pesar del aumento reciente en la oferta de productos que suponen seguridad para la planta y el medioambiente e inocuidad para la salud humana, resulta imperante el desarrollo de programas de manejo integrado que los involucren y que demuestren eficacia, a fin de disminuir o evitar el uso de agentes tradicionales y sus consecuencias. Frente a ello, existen estudios que han evaluado programas de manejo integrado de agentes de control tradicional y biológico en otras especies de frutales como arándanos y vides (Rotolo *et al*., 2017; Abbey *et al*., 2020), mas no se ha investigado la instauración de estos programas en el cultivo del cerezo.

HIPÓTESIS

Programas fitosanitarios que incorporan productos biológicos para el manejo integrado son más eficaces en el control de enfermedades en el cultivo del cerezo en la zona centro sur de Chile, en contraste a programas basados en compuestos químicos de control.

OBJETIVO GENERAL

Evaluar la capacidad de control de programas basados en productos biológicos para el manejo integrado de enfermedades de importancia económica en el cultivo del cerezo.

OBJETIVOS ESPECÍFICOS

-Validar una técnica qPCR para la cuantificación de poblaciones de *Pseudomonas syringae* pv. *syringae* en distintos tejidos que afecta en cerezo.

-Comparar programas que incorporen distintos tratamientos biológicos vs programas convencionales basados en plaguicidas químicos en el control de enfermedades de importancia económica en el cultivo del cerezo.

-Evaluar la eficacia de incorporar aplicaciones de cepas de *Pseudomonas protegens* como bioinductores de resistencia y antagonistas dentro de programas fitosanitarios para el control de enfermedades del cerezo.

-Estudiar variaciones poblacionales estacionales de patógenos de importancia económica en programas que incorporen distintos tratamientos biológicos vs programas convencionales.

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Capítulo 1.

A qPCR method for the detection and quantification of *Pseudomonas syringae* pv. *syringae* in susceptible tissues of sweet cherry.

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Abstract

Pseudomonas syringae pv. *syringae* (Pss) is the main causal agent of bacterial canker in sweet cherry worldwide. As bacterial population dynamics is a key factor on infection and disease development, quantification of bacterial load in tissues could help improve disease management strategies. qPCR standard curves were generated for Pss quantification in three susceptible phenological stages of sweet cherry and the results were compared to those obtained using the bacterial drop count method. The obtained results showed that qPCR is a highly sensitive, fast, and effective method for Pss load monitoring, with detection limits ranging from 10^4 to 10^5 CFU mL^{-1}, indicating that it is a good tool for decision-making related to bacterial canker control.

Keywords

Absolute qPCR; Pss; Bacterial canker; *Prunus avium*.

1. Introduction

Bacterial canker is a serious disease of sweet cherry (*Prunus avium* L.) worldwide. It affects young and adult orchards, even causing plant death under favorable environmental conditions, thus resulting in high economic losses (Balestra, 2020; Khezri and Mohammadi, 2018). Two *Pseudomonas syringae* pathovars have been identified as causal agents of the disease, *P*. *syringae* pv. *syringae* (Pss) and *P*. *syringae* pv. *morsprunorum* (Psm) races 1 and 2 (Bultreys and Kaluzna, 2010). In Chile, which is the third largest producer of sweet cherries and the main exporter in the world (FAO, 2021a; FAO, 2021b), this disease is mainly caused by Pss. The presence of Pss was reported in the 1970's (Latorre and Waissbluth, 1979), while Psm has been recently identified in the south of Chile (Garcia *et al*., 2021), being still under official quarantine. Disease symptoms include cankers on twigs, branches, and trunks, with gummosis at infection sites; bud necrosis and blast; shoot dieback; leaf spots and shot holes; blossom blight; and fruit necrotic spots in susceptible cultivars (Balaž *et al*, 2016; Bultreys and Kaluzna, 2010; Scortichini, 2010). Disease cycle of bacterial canker can be divided into winter and summer phases (Crosse, 1966). The summer phase begins early in spring by the colonization and development of large populations of *Pseudomonas syringae* in blossoms, which can reach from 10^4 to 10^6 CFU per blossom, leading to blast symptoms under favorable environmental conditions (Kennelly *et al*., 2007). The primary inoculum originates from overwintering bacteria located in apparently healthy dormant buds, previously colonized during autumn via infection of leaf scars, or in cankers (Kennelly *et al*., 2007; Scortichini, 2010; Sundin *et al*., 1988). Disease control is carried out mainly through calendar-preventive applications of copper-based products or antibiotics, which can develop fast resistance, and cause phytotoxicity and alterations to soil microbiota (Alengebawy *et al*., 2021; Aprile *et al*., 2021; Beltrán *et al*., 2021; Sundin and Bender, 1993; Vasebi *et al*., 2019). Monitoring Pss populations during susceptible phenological stages could help improve disease control efficiency, limiting the use of bactericides in case of high bacterial load situations, which can influence risk of infection (Roy and Kirchner, 2000). Population density of Pss in orchards is currently determined by green-fluorescent bacterial colony count on King B media (Beltrán *et al*., 2021; Heredia-Ponce *et al*., 2020), but this conventional method is unspecific, because it does not allow differentiation between *Pseudomonas* species, which can colonize tissues and also fluoresce on King B media, leading to miscounts. In this sense, molecular methods, particularly quantitative real time polymerase chain reaction (qPCR), could be a more efficient tool that has not yet been validated for Pss detection in cherry orchards. The objective of this study was to develop a qPCR absolute quantification method for monitoring Pss bacterial populations in three susceptible tissues of sweet cherry.

2. Materials and Methods

A qPCR analysis was conducted to quantify the population density of Pss in susceptible tissues of sweet cherry at three phenological stages according to BBCH (Fadón *et al*., 2015): tight cluster bud (stage 55), open cluster bud (stage 56), and fully open flowers (stage 65). Standard curves were prepared for each phenological stage using DNA of the respective tissue and genomic DNA of Pss strain 11116_b1 (Cui *et al*., 2023). DNA extraction was carried out using the protocol described by Doyle and Doyle (1987) with modifications, as follows: 0.5 g of tissue was ground in a sterile mortar with the addition of 9 mL of lysis buffer (20 gL^{-1} cetyltrimethylammonium bromide, 1.4 M NaCl, 0.1 M tris-HCl, and 10 mM EDTA, pH 8.0) and 0.5 g of polyvinylpolypyrrolidone (PVPP). The mixture was then homogenized using an Ultra-Turrax homogenizer (model T25®, IKA Labortechnik, Staufen, Germany). Samples of 250 μL aliquots were taken and placed into sterile microcentrifuge tubes, adding 160 μL of lysis buffer and 90 μL of Pss 11116_b1 King B broth at a known concentration (10^1 – 10^9 CFU mL^{-1}), and then incubated at 60°C for 30 min. Then, a volume of 500 μL of chloroform-octanol (24:1), previuosly pre-chilled at -20°C, was added and centrifuged at 5,000 *g* for 10 min at 4°C. Afterward, the upper aqueous phase was extracted and placed into another sterile microcentrifuge tube, with addition of 400 μL of chloroform-octanol (24:1). After centrifugation at 5,000 *g* for 5 min at 4°C, the upper aqueous phase was placed into another sterile microcentrifuge tube, and 150 μL of 7.5 M ammonium acetate at 4°C along with 220 μL of isopropanol at -20°C were added. After gentle inversion mixing, DNA was allowed to precipitate at -20°C for 60 min and then centrifuged at 13,000 *g* for 20 min at 4°C. The supernatant was removed, and 400 μL of 70% ethanol at -20°C was added to wash the DNA pellet, followed by centrifugation at 11,000 *g* for 5 min at room temperature. Finally, 70% ethanol was removed, and the DNA pellet was allowed to air-dry at room temperature. Ethanol washes were performed in duplicate. The extracted DNA was resuspended in 50 μL of molecular grade water, and after overnight storage at 4°C, quantity (ng μL^{-1}) and quality

(260/280 parameter) were measured using an Epoch™ spectrophotometer (BioTek®). Finally, the samples were stored at -20°C for future use. For quantification, specific primers *syrbaar* and *syrbaaf* were used for the amplification of the *syrB* gene (Petriccione *et al*., 2017). Each qPCR reaction contained a mixture of 5 μL of SYBR® Green (KAPA SYRB® FAST qPCR Kit), 2 μL of DNA (at a concentration of 10 ng μL^{-1}), 1 x ROX (passive reference), and 0.5 μM of each primer, reaching a final volume of 10 μL after the addition of ultrapure sterile water. The qPCR plate was loaded with three technical replicates of each sample. Amplifications were performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) programmed as follows: 20 s at 95°C; and 40 cycles of 3 s at 95°C and 30 s at 60°C.

Tissue samples were collected from a 15-year-old ´Sweetheart´ cherry orchard under different integrated disease management programs. One gram of each tissue was ground in a sterile mortar with the addition of 9 mL of sterile saline solution (0.89% NaCl) and 1 mL aliquot of the first dilution (1:9), and placed into a microcentrifuge tube. The samples were centrifuged at 10.000 *g* for 5 min. Subsequently, the supernatant was removed, and DNA extraction was conducted according to Doyle and Doyle (1987) with modifications as described previously, and considering to add 41.7 mg of PVPP and 750 μL of lysis buffer per sample. DNA quantity, quality, and qPCR of the tissue samples were conducted as described above. To compare the sensitivities of the molecular and traditional methods, drop plate count of 48 tissue samples was conducted by spotting 10 μL of five 10-fold dilution series on King B agar medium (King *et al*., 1954), followed by incubation at 25°C for 48 hours. Green fluorescent bacteria under UV light were then counted and considered positive to *Pseudomonas* genus.

3. Results

Standard curves were constructed using a dilution series of known concentrations of Pss 11116_b1 under a *P*. *avium* DNA background according to each tissue, and by plotting the Ct value versus the logarithm of the bacterial concentration of each dilution (Fig. 1). Efficiency (E) was calculated from the slope (s) of dilution series according to the following formula: $E = (10^{-1/s} - 1) \times 100\%$; efficiency ranged from 1.03 to 1.07. Primers reported by Petriccione *et al*. (2017) were validated by the construction of melting curves on serial dilutions, resulting in specific amplification for the *syrB* gene with a single product-specific melting temperature (84 °C). Standard curves showed a linear relationship with high correlation (R^2 = >0.98) between a 5-log range of bacterial concentration (CFU mL^{-1}) and the threshold cycle values (Ct) for each sampled tissue (Fig.1). Lower detection limits were able to detect 3,6 x 10^4 CFU mL^{-1} in tight cluster buds; 1,8 x 10^4 CFU mL^{-1} in open cluster buds; and 1 x 10^5 CFU mL^{-1} in fully open flowers.

Of the 48 field tissue samples obtained using the bacterial drop plate count method, 82.2% showed fluorescent bacterial population, while only 9 of them were positive to Pss (18.8%) according to the qPCR method. Positive tissue samples for the qPCR and bacterial drop plate count methods can be observed in Table 1. No positive tissue samples were detected in open cluster buds by the qPCR method. For samples at the open cluster bud stage, the bacterial drop plate count method based on green-fluorescent bacterial counts detected positive samples concentrations ranging from 7 x 10^3 CFU mL^{-1} to 7 x 10^6 CFU mL^{-1}.

4. Discussion

For the diagnosis and monitoring of bacterial canker in sweet cherry, Pss is currently quantified by the bacterial plate count method, which can be used alone or in combination with conventional PCR, based on the detection of the *syrB* gene (Sorensen *et al*., 1998). Although this method is cost-effective and confirms the presence of viable bacteria, it can be partly subjective, prone to technical errors, labor intensive and time consuming (Hazan *et al*., 2012). Since changes in phenological stages of sweet cherry occur quickly during the season, the weaknesses of the method are of concern, particularly considering that bactericide applications are necessary to prevent future declines in productivity. Additionally, if not complemented with conventional PCR, the bacterial plate count method could lead to inaccurate counts since some fluorescent colonies can belong to other pathogenic species within the *Pseudomonas* genus in sweet cherry, such as *P. viridiflava* or *P. cerasi* (Beltrán *et al*., 2023; Kaluzna *et al*., 2016), and even including beneficial species such as *P. fluorescens* or *P. protegens* (Panpatte *et al*., 2016; Zhang *et al*., 2020). This becomes particularly relevant in integrated disease management programs that include the use of bacterial antagonists of the last two species, as active ingredients. Similarly, the traditional method becomes even more challenging in situations of high bacterial load as it would require conventional PCR analysis for all bacterial fluorescent isolates, being unsuitable for large-scale or high-throughput experiments. Conversely, qPCR absolute quantification is a highly specific and fast method, which has been widely used to quantify plant pathogenic bacteria (Barrett-Manako *et al*., 2021; Chai *et al*., 2020; Schneider *et al*., 2021). In fact, the method is characterized by its high sensitivity and specificity and low-technical difficulty, requiring limited genome information (Harshitha and Arunraj, 2021). However, qPCR can also detect non-viable bacteria that could overestimate bacterial load (Chai *et al*., 2020), and result in higher costs compared to the conventional method. In this study, qPCR detection limits were 10 times higher than those observed using the bacterial drop plate count method. This decrease in sensitivity could be explained by the presence of PCR inhibitors such as pectin, polyphenols, polysaccharides, or xylan (Schrader *et al*., 2012), which were not removed from the plant tissues by the CTAB modified extraction method. Therefore, other strategies to minimize inhibition should be considered (Demeke and Jenkins, 2010; Mourelos *et al*., 2016). Nevertheless, high risk bacterial load for the analyzed tissues was detected by the qPCR method. In addition, qPCR provided high specificity on Pss detection by discriminating between fluorescent bacterial populations, significantly reducing the margin of error compared to the conventional method from 82.2% of tentatively positive samples to 18.8% confirmed positive samples. These findings indicate that the qPCR detection method is a helpful tool for decision-making related to bacterial canker control.

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Table 1
Comparison of *Pseudomonas syringae* pv. *syringae* populations in susceptible tissues of sweet cherry between qPCR absolute quantification and the bacterial drop plate count method on Petri dishes (Conventional method).

Tissue	qPCR		Petri dishes counting	
	[Bacterial] (CFU ml-1)	Log[Bacterial]	[Bacterial] (CFU ml-1)	Log[Bacterial]
Tight cluster buds	7,E+05	5,85	1,E+06	6,00
Tight cluster buds	5,E+04	4,71	7,E+04	4,82
Fully open flowers	5,E+06	6,70	1,E+06	6,00
Fully open flowers	4,E+06	6,54	1,E+06	6,00
Fully open flowers	5,E+06	6,74	4,E+06	6,56
Fully open flowers	2,E+05	5,35	1,E+06	6,12
Fully open flowers	2,E+05	5,24	1,E+06	6,12
Fully open flowers	2,E+06	6,29	7,E+05	5,87
Fully open flowers	4,E+06	6,60	7,E+05	5,82

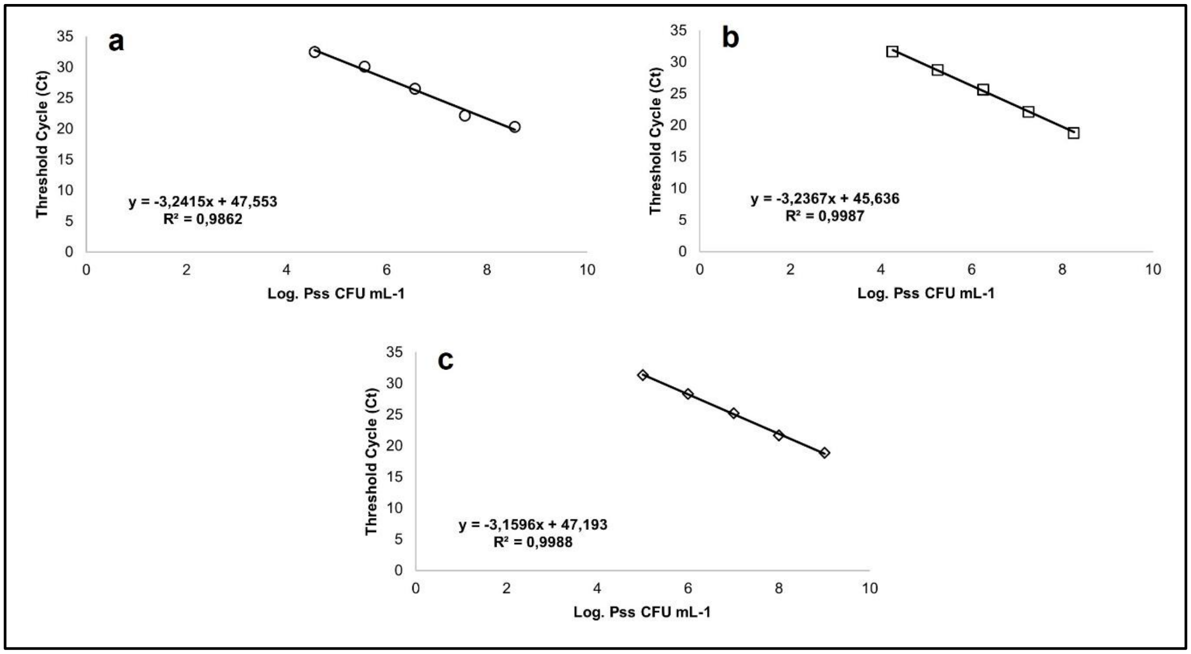

Fig.1. Real-time PCR standard curves for the detection of *Pseudomonas syringae* pv. *syringae* 11116_b1 in tight cluster buds (a), open cluster buds (b), and fully open flowers (c), obtained by plotting the Log. Pss concentration versus the cycle number required to elevate the fluorescence signal above the threshold.

Capítulo 2. Use of biological products-based management programs for the control of sweet cherry diseases.

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Abstract

Bacteria and pre- and postharvest fungal pathogen cause diseases affecting sweet cherry worldwide. The use of biological products is a suitable alternative to decrease agrochemical applications to reduce these problems. However, there is a lack of information on the interaction between these products and their effect on the population dynamics of these pathogens when incorporated into integrated disease management programs. This study evaluated the phytosanitary impact of biological based management programs on sweet cherry disease control. Half hectare units were established under two biological-based programs, one based only on chemical control and the other under the local grower program strategy, in a ´Sweetheart´ sweet cherry orchard in Chillán, Chile. Population counts and incidence and severity measurements were made on cherry at specific phenological stages. Results showed that biological-based programs, which included *Pseudomonas protegens*, *Bacillus* spp. and *Trichoderma* spp., reduced populations of *Pseudomonas syringae* pv. *syringae*; *Botrytis cinerea* and *Alternaria* spp. at specific phenological stages, with no differences regarding chemical and grower programs. There were no significant differences in the incidence and severity of bacterial cankers among all management programs, but there were decreases of 87% for *Alternaria* spp. rot incidence and 60% for *Botrytis cinerea* rot incidence in ripe fruit in the chemical program compared to the biological product-based programs during the first and second seasons, respectively. These results support the inclusion of bioproducts in commercial cherry disease management programs.

Keywords

Bacterial canker; Postharvest diseases; Biological control; *Prunus avium*.

Introduction

Between 2018 and 2022, Chilean sweet cherry orchards reached an annual average production of 321 thousand of tons, distributed on approximately 44 thousand hectares. At the same time, 85% of the production was exported, positioning Chile on world's top exporters (FAO, 2022a; FAO, 2022b). For center south and south zones of Chile, which include Maule region to Los Lagos region, the planted area grew in 221 percent from 2010 year to 2020 (ODEPA, 2020). Additionally, the Ñuble Region sweet cherry orchard area increased in 128%t between 2016 and 2022 years (ODEPA, 2022).

This fruit tree can be affected by several diseases that have an economic impact, and whose incidence is favored by high relative humidity, free water, and frequent rainfall conditions, like those occurring on south and center south zones of Chile. Bacterial diseases like crown gall disease, caused by *Agrobacterium tumefaciens* Conn; bacterial canker caused by *Pseudomonas syringae* pv. *syringae* van Hall (Pss) and *P*. *syringae* pv. *morsprunorum* Wormald (Psm) races 1 and 2; fungal diseases like brown rot, caused by *Monilinia* spp.; black rot caused by *Alternaria* spp.; "witches' broom" a hypertrophic disease caused by *Taphrina cerasi* Fuckel; silver leaf disease, caused by *Chondrostereum purpureum* Person; *Cytospora* spp canker and dieback; verticillium wilt caused by *Verticillium dahlia* Klebahn; crown and root rots by *Phytophthora* spp.; and diverse fruit rots caused by *Aureobasidium pullulans* de Bary, *Cladosporium* spp., *Penicillium* spp., *Rhizopus* spp., among others, and the grey mold, caused by *Botrytis cinerea* Person, are capable of severely affecting sweet cherry production in Chile (Latorre, 2018).

Bacterial canker is considered the main disease affecting sweet cherry orchards in Chile and it is caused mostly by Pss, although recently Psm has been detected in orchards from Los Lagos Region (García *et al.*, 2021). This Gram-negative bacterium infects through natural openings (as stomata and lenticels) or through natural (leaf scars caused by leaf fall) and mechanical (pruning and agricultural machinery) wounds. Once the bacterium has been established inside plant tissue, it can reach cambial tissue and generate cankers on woody organs, with consequential gummosis and characteristic fermented smell. Further, during bacterial growth phase, some epiphytic population penetrates across leaves, causing black and brown spots, which evolve to perforations (Hulin *et al.*, 2018), leading to a future decrease of photosynthetic area. Symptoms severity depends on bacterial strain, cultivar, plant age, invaded tissue, and environmental conditions, leading to yield losses from 10 to 20% in young orchards, and, inclusive, plant death, on favorable environmental conditions (Khezri and Mohammadi, 2018). *Monilinia fruticola*, *M. laxa,* and *B. cinerea* are main causal agents of preharvest and postharvest rots of sweet cherries in Chile. Their conidia are capable of infecting flowers and fruits tissue, with the mayor risk of infection during full bloom growth stage, under high relative humidity and free water conditions (Holb, 2008). In case of flowering stage, blossom blast can be developed, with consequent flowers death, or end in a latent infection (Tarbath *et al.*, 2014), which could cause fruit rot under favorable conditions. Also, these pathogens are capable of overwinter inside mummy fruit, thus, in a mayor presence of inoculum sources, higher will be the risk of infection (Tarbath *et al.*, 2014). Other fruit rot pathogens are *Alternaria* species, whose conidia according Timmer *et al* (2003), in Mediterranean climates, could be capable of spreading after rains and survive during winter on soils, seeds, and perennial tissue (Troncoso-Rojas and Tiznado-Hernández, 2014). Those fruit rot pathogens can cause severe losses. So, brown rot disease caused by *Monilinia* spp. arises yield losses up to 80% when climate conditions are favorable to disease development, especially on late ripening cultivars (Gotor-Vila *et al.*, 2017), whereas *B.s cinerea* generates losses by latent infection over 50% after harvest (Tarbath *et al.*, 2014) and fungus like *Alternaria alternata* has reported 26% postharvest incidence (Ahmad *et al.*, 2020).

Integrated manage programs are used to deal with these diseases in sweet cherries, which use preventive bactericides based on copper compounds (Husseini and Akköprü, 2020) and/or antibiotics (Sundin and Wang, 2018). Copper ion affects bacterium disturbing his enzymes functionality, displacing essential metallic co-factors, and generating oxygen reactive species (Husseini and Akköprü, 2020). Furthermore, copper ion can control fungal pathogens (Mitre *et al*., 2011). Although these copper-based products are effective on bacterial control, it could produce phytotoxicity and being accumulated in the environment, especially if they are used in high concentrations or sprayed repeatedly, which also favor selection of bacterial resistant strains (Aprile, 2021; Beltrán *et al*., 2021), and soil microbiota disturbances (Alengebawy *et al*., 2021). Additionally, there is a high cost of control considering that in Chile, between Maule and Araucanía Regions, has been estimated a cost of 14.6 million dollars per year to management the bacterial canker disease (Moya-Elizondo, 2020). In case of antibiotics, authorized active ingredients in Chile are kasugamycin, streptomycin and oxytetracycline, but their inadequate use favors fast resistant development (Sundin and Bender, 1993; Vasebi *et al*., 2019). Fruit rots caused by fungi are controlled mostly by synthetic chemical fungicides, which have diverse modes of action. Fungicides exert their action by inhibiting spore germination and host tissue infection, being sprayed from swollen bud and full bloom to fruit development to prevent fungal infections (Tarbath *et al*., 2014). In Chile the most used alternatives are contact fungicides such as: mancozeb; captan; dicloran; propiconazol; sulfur; and systemic fungicides like methyl-thiophanate; myclobutanil; benomyl; fenbuconazole and tebuconazole. However, consumers are demanding fruit free of synthetic pesticide, and pesticide options are being more and more reduced, associated with fungicide restrictions by potential hazard on human health. For example, recently UE has banned mancozeb, considering its immunosuppressant effect on mice (Bano and Mohanty, 2020), and possible hazard to human reproduction (Runkle *et al*., 2017).

Problems associated with bactericide and fungicide use have led to the development of new control disease alternatives. In Chile, authorized microorganism-based products are Mamull® (Bioinsumos Nativa SpA., Chile), a fungicide compound by strains of *Bionectria ochroleuca*, *Trichoderma gamsii*, and *Hypocrea virens*; Nacillus® (Bioinsumos Nativa SpA., Chile), a bactericide product based in *Bacillus* spp. and *Brevibacillus brevis* strains and used for bacterial canker control. Product based in fungus of genus *Trichoderma* such as: Trichonativa® (Bioinsumos Nativa SpA., Chile) or 3 TAC-I Beta® (Avance Biotechnology

S.A., Chile) are registered to control crown and root rot, gray mold and Pss. On the other hand, vegetal extracts like EcoSwing® (Gowan Chile S.A.), a product based in extract of *Swinglea glutinosa* are used to control for grey mold and black rot ; BC-1000®, (Chemie S.A., Chile)an extract of *Citrus x paradisi* used for brown rot, grey mold and *Penicillium* rot; or Bestcure®(Bioamerica S.A., Chile), a *Citrus aurantium* L. fruit extract, registered to control blossom blast and grey mold, are recommended in Chile to prevent sweet cherry diseases. However, despite the recently increase of products recognized as safe for the plant and the environment and harmless for human health, the development of integrated management programs that involve their use and that demonstrate their efficacy is required to decrease or avoiding the problems associated with conventional chemical products. In this context, there are studies that have evaluated traditional and biological control agents in integrated disease management programs on blueberry and vine (Rotolo *et al*., 2017; Abbey *et al*., 2020), but the establishment of those phytosanitary programs on sweet cherry orchards have not been deeply investigated.

The use of *P. protegens* as a biocontrol agent in fruit trees has been studied in apple for the control of fire blight, caused by *Erwinia amylovora*, and apple ring rot caused by *Botryosphaeria dothidea* (Mikiciński *et al*., 2019; Huang *et al*., 2022), while in lemon leaves *P. protegens* has shown significant reduction of canker lesions caused by *Xanthomonas citri* subsp. *citri* (Michavila *et al*., 2017); and antifungal activity against *B. cinerea* on grapevine leaves (Andreolli *et al*., 2019), suggesting a broad spectrum activity against bacterial and fungal pathogens, but, to the knowledge of the authors, has not been tested on sweet cherry.

This research was conducted with the objective to determine antibiosis of *P. protegens* strains on bacterial and fungal sweet cherry pathogens; to assess the ability to induce resistance genes in sweet cherry plant of these bacterial strains and the effect of integrated programs based on the use of these *P. protegens* strains in reducing plant pathogens affecting sweet cherry under field conditions.

Materials and methods

1. Assessment of antibiosis and ability to induce resistance in sweet cherry plants by *Pseudomonas protegens* strains used in biological-based products.

a) *In vitro* antagonism assay

To dilucidate the antagonist action of *Pseudomonas protegens* strains of the commercial products Taniri® (Bioinsumios native spA., Chile), based in strain Ca2 and ChC7, and Maxgrowth® (Bioprotegens Innovations SpA., Chile), based in strains Ca6 on Pss and fruit rot fungi, such as: *Monilinia fructicola*, *Botrytis cinerea*, and *Alternaria alternata*) an *in vitro* antagonism assay was conducted. *Pseudomonas protegens* strains and Pss strain 11116_b1 (Cui *et al*., 2023) were grown in King B broth for 24 hours at 25°C, in an orbital shaker at 150 rpm. To obtain pure bacterium cells, the following protocol was conducted: 5 min centrifugation at 5000 g, supernatant elimination, and suspension with the addition of 1 mL 1% NaCl sterile saline solution. Bacterial concentration was standardized to 10^7 ufc mL^{-1} by measure optical density (0.1) at 600 nm (Sezonov *et al*., 2007). Additionally, standardized bacterial suspensions were corroborated in its CFU mL^{-1} concentration by plating three drops of 10 uL of each serial dilution on King B agar (KBA). Consecutively, a Pss strain 11116_b1 (Cui *et al*., 2023) lawn was performed on KBA using 500 µL of the bacteria. Once the lawn dried, 10 µL drops of each antagonistic bacterium were equidistant added, and a 10 µL drop of commercial antibiotic solution, based in streptomycin sesquisulfate (0.15 gL^{-1}) plus oxytetracycline hydrochloride (1.92 mgL^{-1}) (Strepto Plus®, Anasac Chile S.A. Chile), was used as a positive control.

The antagonism assay conducted with fruit rot fungus used a *Botrytis cinerea* and an *Alternaria alternata* isolates obtained from symptomatic sweet cherry fruit from the same orchard, while *Monilinia fructicola* was isolated from symptomatic peaches. All fungal isolates were morphologically identified according to macro and microscopic characters in literature. The three fungal species were grown for 5 days in PDA at 25°C in dark conditions, then 6 mm diameter disk of mycelium in active growth were placed in the center of a Petri dish containing PDA with K_2HPO_4 and $MgSO_4$ x $7H_2O$ (PDA+KB agar medium). After this, a 10 µL drop of each *P. protegens* strain and a 10 µL drop of sterile distilled water (SDW), were put equidistant from the mycelial disk and between them on the mentioned medium. Sterile distilled water was used as a negative control.

To assess the antagonistic activity of the bacteria against Pss, after 48 hours of incubation at 25°C, the inhibition area (IA; mm^2) formed by the droplets was measured with a ruler using the following formula: IA = r1 * r2 * π (being r1 and r2 mayor and minor axis of the elliptical area), considering the entire area indicating an antagonistic effect (areas with opaque and clear tones). Regarding the antagonism of *P. protegens* strains against *B. cinerea*, *M. fructicola*, and *A. alternata*, after 7 days of incubation at 25°C, the percentage of radial growth inhibition (PRGI) was evaluated using the equation PRGI = (R1-R2) x R1-1 x 100 (Ezziyyani *et al*., 2004), where R1 and R2 correspond to the radii of fungal growth against negative control and against bacterial antagonist, respectively. Three replicates of the pathogenic Pss bacterium and per each fungal isolate assessed was used and the experiment was repeated twice.

b) Systemic resistance induction Assay

The induction capacity of resistance genes from *P. protegens* strains, which are the active ingredients of Taniri® and Maxgrowth® products was assessed in 2-year-old cv. Santina cherry plants grafted onto ‘Colt’ rootstock. Four plants were used in four treatments (Table 2) in a completely randomized design, with four replicates per treatment. Foliar applications of 100 mL of each treatment were applied using a hand sprayer.

Leaf tissue samples were collected 1 and 7 days after the treatment application. Cherry leaves were immediately stored in liquid nitrogen upon sampling and then kept at -80°C for later analyses.

b.1) RNA Extraction, cDNA synthesis and RT-PCR analysis

Two hundred milligrams of sweet cherry leaf tissue from each treatment repetition were extracted using the Spectrum^{TM} Plant Total RNA Extraction Kit (Sigma-Aldrich), following the manufacturer's instructions. Liquid nitrogen and ceramic mortars and pestles were used to facilitate tissue grinding. The integrity of the RNA was visualized on a 1% agarose gel using the GeneRuler^{TM} 1kb Plus DNA Ladder molecular weight marker (Fermentas). Concentration and purity of the samples (260/280 ratio) were measured using an Epoch® Spectrophotometer (BioTek®). Subsequently, the samples were stored at -80°C. Then, a RT-PCR was conducted to synthesize cDNA from the previously extracted total RNA. Before the reaction, using 1 μg of total RNA in 7 μL of nuclease-free water, a DNA digestion was performed with DNase I RNase-free (Thermo Scientific). Later, cDNA was synthesized using the High-Capacity cDNA kit (Applied Biosystems) with an RNase inhibitor, following the manufacturer's instructions.

b.2) qPCR and relative gene expression quantification analysis

A qPCR analysis on previous obtained cDNA was realized to study proteins NPR3, PR5, PAL and CHS2 gene expression. Primers used in this analysis are described in Table 3, and they were formerly validated on qPCR, showing an efficiency percentage between 95% and 100% in DNA of *P. avium* (data not shown). For the gene expression analysis, a real time thermocycler StepOnePlus Real-Time PCR System (Applied Biosystems^{TM}) was used, with β-*act* (β-actine) as housekeeping gene. Relative gene expression quantification was performed on diluted cDNA, using ΔΔCt method. Every reaction had a mixture of 5 μL SYBR® Green (KAPA SYBR® FAST qPCR), 2 μL cDNA (10 ng μL^{-1}), 0.5μM of each primer and nuclease free water, for a 10 μL volume per reaction. qPCR plate was charged with four biological replicas of each treatment, in two technical replicas of each sample. qPCR amplifications were programmed as follows: 20 s at 95°C; and 40 cycles of 3 s at 95°C and 30 s at 60°C. Collected data was then normalized with respect to β-*act.*

Assessment of effectiveness of integrated disease management programs based in products with P. protegens compared to farmer and chemical programs under field conditions

2. Study site and experimental design

The study site was located on "Los Tilos" orchard, Chillán, Puente Ñuble (GPS: 36°32'16'' S; 72°05'33'' O), during 2021/2022 and 2022/2023 seasons. Biological study models were sweet cherry trees 'Sweetheart', on 'Colt' rootstock, planted in 2007, and conducted on a central axis training system. Orchard planting system was 5 m x 2.5 m (800 trees hectare^{-1}). For experimental design, four sectors of ~0.5 hectares each were considered. A disease management program was conducted for each sector (Table 1). Main differences between management programs are the inclusion on farmer's habitual program (FP) of phytosanitary products based on microelements fertilizer and microbial exudates (Wert® y Ph4®, Tavan

Chile S.A.), a single application of cuprous oxide (Cuprodul® WG, Quimetal Industrial S.A.), organic acids extracts (Carboxigram®, Fitological Chile S.A.), pyraclostrobin (Comet®, BASF Chile S.A.), and tebuconazole and fenhexamide (Tie Break® 416,7 SC, Bayer S.A); Chemical program (CP) inclusion of copper hydroxide (Champ® DP, Quimetal Industrial S.A), two applications of cuprous oxide (Cuprodul® WG, Quimetal Industrial S.A), pyraclostrobin (Comet®, BASF Chile S.A), and tebuconazole and fenhexamide (Tie Break® 416,7 SC, Bayer S.A); and differences on the two biocontrol based programs: first one (BBP1), which includes applications of *Pseudomonas protegens* strain Ca6 (Maxgrowth®, Bioprotegens Innovations SpA), *Trichoderma harzianum* strain T22 (Harzstop®, Biogram S.A); and the second one (BBP2), composed by *Pseudomonas protegens* strains Ca2 and ChC7 (Taniri® WP, Bio Insumos Nativa SpA), *Bacillus* spp. and *Brevibacillus brevis* (Nacillus®, Bio Insumos Nativa SpA), *Trichoderma harzianum* strain Queule, *Trichoderma virens* strain Sherwood and *Trichoderma parceramosum* strain Trailes (Trichonativa®, Bio Insumos Nativa SpA). Each application considered a spraying volume of 750 L of water, except BBP2 which was diluted on 500 L, according to commercial dose. Spraying was conducted with an orchard airblast sprayer of 1500 L (Nebulizador Parada, Pulverizadores Agrícolas Parada SpA., Chile) on leaf fall (BBCH 93-96 stages); after frost events during winter dormancy; on open cluster; full bloom; petal fall; calyx fall; fruit about 80% final size; and fruit ripe for harvesting, during 2021/2022 and 2022/2023 seasons. Growth stages were based on BBCH scale, proposed by Fadón *et al.*, (2015). The experimental design had around 500 sweet cherry trees per sector, selecting 64 of them (16 per sector), and choosing 4 random trees in every evaluation, with at least two border trees on peripheral rows, so application drift effect was avoided. For Pss control efficacy and copper resistant assay, 4 subplots were established per sector, of 24 trees each, leaving 2 trees on each sector peripheral rows. 4 trees were selected in each subplot at every evaluation time. All products were obtained as commercial presentations in the market.

Sampling. For each tree, plant tissue was observed or sampled, depending on the pathogen of interest, by dividing the tree into four opposing quadrants. For Pss, twigs were observed; while for *Monilinia* spp., *Botrytis cinerea*, and *Alternaria* spp., flowers, hypanthium, fruits about 80% final size, and ripe fruits were sampled, then incubated in a humid chamber for 7 days at room temperature (~23°C). Tissues affected by fungi were sampled after product spraying, excepting on stage 65, and stage 72 during the first season, when plant tissues were sampled before spraying.

Assessments. Incidence and severity of diseases caused by sweet cherry pathogens was conducted as indicated below:

i) *Pseudomonas syringae* pv. *syringae*. Incidence was calculated as a percentage based on the total number of branches with bacterial canker symptoms out of 100 observed branches. For severity, the length of cankers on twigs was measured with a caliper. In both measurements, during the 2020/2021 season evaluations, vegetative growth from that season was considered, while during the 2021/2022 season evaluations, growth from previous season (2020/2021) was also included. Likewise, in both cases, an average of measurements per tree was taken, which were then treated as independent data.

ii) Fungal rot pathogens (*Monilinia spp*., *Botrytis cinerea* y *Alternaria* spp.). During various growth stages, incidence and severity of sweet cherry rot fungus were observed and registered. The corresponding BBCH growth stages were full bloom (65), calyx fall (72), fruit about 80% final size (78), and fruit ripe for harvesting (89) (Fadón *et al*., 2015). Incidence was calculated as a percentage based on the total number of flowers at full bloom (BBCH's 65 stage), hypanthium (BBCH's 72 stage), and fruits (BBCH's 78 and 89 stages) showing mycelium of each pathogen compared to the total collected samples. Severity per each fungal pathogen was calculated based on the percentage of tissue surface covered by mycelium, according to the visual scale modified from Romanazzi *et al*., 2001, where: 0 = no infected fruit; 1 = up to 25% superficial mycelium; 2 = 26-50% superficial mycelium; 3 = 51-75% superficial mycelium; 4 = more than 75% superficial mycelium. Finally, with the scale values was determined a severity index by using the following formula:

$$Severity\ index = \frac{\sum(d \times n)}{T}$$

With *d* as scale value score; *n* as number of tissues observed in this category; and *T* as total number of observed tissues. Evaluated data, in both cases, were the average measurements per tree.

Assessment of populations variations of sweet cherry pathogens

a) Population variations of *Pseudomonas* spp. and *Pseudomonas syringae* pv. *syringae.* Sampling was carried out from late leaf fall, covering the phenological stages of winter bud (BBCH's stage 50); swollen bud (BBCH's stage 53); tight cluster (BBCH's stage 55); open cluster (BBCH's stage 56); and full bloom (BBCH's stage 65). A tree per assessment plot was divided into four opposing quadrants, with one basal, one central, and one apical fruiting center chosen per quadrant. This plant material was used to conduct bacterial counts by using the bacterial drop plate count method on Petri dishes. So, plant tissues to be examined varied according to phenological stage. For winter bud, swollen bud, tight cluster, and open cluster, a total of 20 buds were selected for each phenological stage, per tree. On full bloom, 20 opening flowers per tree were selected. Subsequently, 50% of each tissue were surface sterilized by immersion on 70% ethanol for 45 seconds, followed by two consecutive rinses in SDW for 1 min each. Then, each group of tissues, with and without surface sterilization, were ground using a sterile mortar and pestle and sterile saline solution (0.89 % NaCl). The amount of saline solution to be added on the ground buds and flowers was determined by the average weight of 10 buds or flowers, in triplicate, to standardize the weight of 10 tissue samples to make up the first dilution (1:9). From the resulting dilution, 1 mL aliquot was put in microcentrifuge tubes at 4°C for later use. Then, a total of 5 serial dilutions (1:9) were made in microplates. Aliquots of 10 μL of each dilution were plated in triplicate on Petri dishes with KBA medium for subsequent incubation at 25°C for 48 hours. The remaining volume of the first dilution was stored at -20°C for qPCR absolute quantification assays.

Finally, counts were obtained in colony-forming units (CFU) per bud, and CFU per flower, based on morphological observations and fluorescence under UV light. Population variations curves of *Pseudomonas* spp. were generated according to phenological stages over time for each disease management program. Sampling and counting were conducted over two growing seasons.

b) Population variations of *Monilia* spp, *Botrytis cinerea* y *Alternaria* spp. Sampling was conducted as described above. With those results, bar charts of population variations were generated according to changes in disease incidence for each pathogen in the different phenological stage where samples for fungal analysis were collected from each disease management program.

4. Statistical analysis

For *in vitro* antagonism assay, data were subjected to an analysis of variance and comparison of means using Tukey's test ($\alpha = 0.05$). Prior to ANOVA, the data were analyzed to determine normality of distribution and homogeneity of variance using the Shapiro-Wilks and Levene tests, respectively. Kruskal-Wallis test was realized in case ANOVA assumptions could not be achieved after Arcsen or square root transformations of the original data.

Results of fields assays were analyzed by using descriptive statistic. Media values and their respective standard deviations were calculated. All the statistical analysis were conducted in the software INFOSTAT (Di Rienzo *et al*., 2008).

Results

In vitro antagonism assay

The antagonistic action of *P. protegens* Ca2 and ChB7 (i.a.s of Taniri® product) and strain Ca6 (i.a. of Maxgrowth® product) was observed in all bacterial strains against Pss. However, only strains Ca6 and Ca2 showed inhibition halos, while strain Chc7 formed a bacterial biofilm around the Pss lawn (Figure 1a). Despite the above, the antibiotic product had a significantly larger halo and percentage of inhibition than the other treatments (Table 4; Figure 1a). The fungus *Botrytis cinerea* was inhibited by all the bacterial strains, but strains ChC7 achieved the highest PRGI values (35.8%). *Monilinia fructicola* and *A. alternata* were equally reduced in their mycelial growth by the three strain of *P. protegens* in around 46% and 36%, respectively (Figure 1c and 1d, Table 4). The used suspension of the strains Ca6 and Pss 11116_b1 reached a concentration of 1 x 10^8 CFU mL^{-1}; meanwhile, the strains Ca2 and Chc7 reached concentrations of 1.3 x 10^8 CFU mL^{-1} and 1.5 x 10^8 CFU mL^{-1}, respectively, according to bacterial dilution count in KBA.

Sweet cherry i*n vivo* systemic resistance induction assay

The expression of the four genes analyzed in sweet cherry plants sprayed with *P. protegens* based products increased in comparison to non-inoculated plants ,(Figure 2). The expression of the *npr3* gene increased in all treatments compared to the control at 24 h after spraying, with a high equivalent expression between the chemical inducer (ASM) and the bacterial strain Ca6. Seven days after treatments spraying the *npr3* gene expression was active but lower compared with the observed at 24 h. The *pr5* gene expression was lower for *P. protegens* strains at 24 h and 7 days, but ASM showed a noticeable increase in the gene expression of this gene at 24 h. The *chs2* gene, despite high variability in biological replicates, showed numerical increases in all treatments compared to the control non inoculated, while the relative expression of the *pal1* gene differentially increased only in the treatment with the strain Ca6. In general, after 7 days post-inoculation, all treatments experienced maintenance in the relative expression of the analyzed genes, except for the *npr3* gene which decreased, and the *chs2* gene in the chemical inducer treatment, which increased by 421% compared to the first 24 hours after inoculation.

Incidence and severity of bacterial canker caused by *Pseudomonas syringae* pv. *syringae* under field conditions

Incidence and severity of canker lesions for both seasons can be seen on Figures 3 and 4, respectively. In first season (2021/2022), notorious differences on the canker incidence were observed only between BBP1 (12%; S. E = 1.63) and BBP2 (21%; S. E = 6.19), whereas no differences were founded on the average severity between every disease management program, with canker length values less than or equal to 38.5 mm (S. E = 3.87), having BBP2 the highest numerical value. In second season, BBP1, based in *P. protegens* strain Ca6, showed the lowest incidence value (14%; S. E =3.83), contrasting to CP (25%; S. E = 5.97), FP (35%; S. E = 5), and BBP2 (35%; S. E = 9.98). The canker length during the second season showed no major difference between the programs, being consistent with 2020/2021 season. Cankers incidence was lower in 2020/2021 season (values less than or equal to 21%), compared to 2021/2022 season (values less than or equal to 35%). Also, cankers length decreased on FP and BBP2 during the second season.

Incidence and severity of rots caused by *Monilinia* spp., *Botrytis cinerea* and *Alternaria* spp. on sweet cherry tissues.

On the first season, *B. cinerea* full bloom (BBCH 65) incidence was the highest among all growth stages for all disease management programs, in which BBP1 showed the highest control of grey mold disease reaching a 3.3 incidence percentage, followed by CP with a 5%

(S.E = 0.98). Excluding BBCH 78 stage, where grey mold was not observed, the lowest incidence percentage occurred at BBCH 89, with BBP2 having the highest incidence of grey mold with a 3% (S.E = 0,58). At this growth stage, symptoms and signs of the disease were not observed for FP, CP and BBP1 (Figure 5a). Regarding first season's grey mold severity, the highest and lowest values founded on disease management programs corresponded with incidence evaluations, having BBP2 the highest severity index at BBCH's 65 (0.44, S.E = 0.09) and 89 (0.08, S. E. = 0.01) stages, in contrast of BBP1 who had the lowest index value (0.07, S.E = 0,02) at full bloom and all others disease programs at BBCH's 89 stage as grey mold was not observed (Figure 6a). Second season's incidence of *Botrytis cinerea* grey mold showed an increase in the programs based in *P. protegens* strains during full bloom (BBCH 65), reaching a 16.7% disease incidence at BBP1, and in all disease management programs at calyx fall (BBCH 72), with a CP incidence media statistically equal to BBP2 media at both growth stages (Figure 5b). For BBCH 89 stage, CP was the only treatment with no grey mold symptoms, and the rest of them were below of 5% incidence. Also in this season, grey mold severity was higher during calyx fall for all management programs, with BBP1 having the lowest severity index compared to BBP2, FP, and CP. In case of BBCH 89 stage, the second lowest severity index was obtained in BBP1 (0.02, S. E = 0.01), and the highest value in the management programs used by the grower (FP, 0.11, S.E = 0.03) (Figure 6b), contrasting first season's disease severity results.

Alternaria incidence during first season BBCH's 65 stage was over 75% for all treatments, with BBP2 having the lowest percentage (Figure 5c). BBP2 media (75.0%, S.E = 3.96) showed difference compared to CP media (89.2%, S.E = 2.49). The highest incidence was observed during calyx fall, when all management programs had over 90 percent of *Alternaria* colonization. As occurred in grey mold at BBCH 78, no symptoms or signs of the disease were observed. Additionally, the incidence observed in CP at BBCH 89 stage was the lowest between all treatments, with a media value of 1.5% (S.E = 4.0), eight times lower than BBP2 and BBP1. In case of *Alternaria* severity, FP had a severity index equal to 0.32 at full bloom (BBCH 65), and CP a 0,03 index at fruit ripe (BBCH 89) for harvesting, being the two lowest values for their respectively growth stages (Figure 6c). Referring to biological-based management programs at BBCH 89 stage, both had no difference, with means values among 0.23 and 0.28 of severity index. On the second season, at BBCH 65 stage *Alternaria* incidence was kept at high values on flowers, being FP the highest (95.9, S.E = 2.1) and BBP2 the lowest (60, S.E = 7.7). No infection by *Alternaria* were observed at BBCH 72 and 78 stages. At BBCH 89 stage BBP1 and BBP2 held lower numerical values compared to FP and CP programs, with media values of 16.5 and 18.3, respectively (Figure 5d).

For second season's *Alternaria* severity at full bloom, FP increased in a 250% and BBP2 decreased in a 57%. Also, in contrast of first season, *Alternaria* was not observed on chalices. Instead, calyx tissue was colonized by *Cladosporium* spp. at level of 100% of incidence (data not shown). Additionally, at BBCH 89 stage, BBP1 showed the lowest severity index (0.13, S.E = 0.04), being different compared to CP (0.24, S.E = 0.05) and FP (0.31, S.E = 0.06) (Figure 6d).

Finally, *Monilinia* brown rot was rarely seen in both seasons, only affecting very few fruits (data not shown).

Bacterial counts using Petri dish plates

Bacterial counts were conducted at the phenological stages 50, 53, 55, 56, and 65 (Fadón *et al*., 2015). Both seasons were characterized by high variability in bacterial populations in each disease management program and among them. Similarly, the trends shown by disease management programs differed between seasons, whether in the absence or performance of previous sample disinfection. Additionally, tissue samples with surface disinfection using 70% ethanol exhibited lower peaks of bacterial populations. Thus, throughout the first season, CP exhibited higher fluorescent bacterial populations than BBP1 at 55 stage, and higher than BBP2 at 50 and 56 stages, both for samples without disinfection and samples prior disinfection (Figures 7a and 7b). Also, at stage 53, CP showed the highest bacterial fluorescent bacterial load among all management programs in samples without disinfection. In contrast, during the second season, bacterial populations obtained by CP were the lowest at 55 stage in samples with prior disinfection, and equivalent to all others management programs in samples without disinfection. Also, at stage 56 in prior disinfected samples, the lower values were exhibited on CP (2.45, S.E = 1.42) and BBP2 (2.41, S.E = 1.44) (Figure 7c). In addition, in both seasons and sample processing, no differences were observed on fluorescent bacterial populations during stage 65 between management programs, excepting on BBP1 on first season (4.1, S.E = 1.4). Also, there was generally a trend toward higher bacterial concentration in winter bud and fully open flowers tissues for all management programs, except for BBP2 during the first season at stage 50.

Discussion

Pseudomonas protegens is a species characterized by its ability to produce antimicrobial metabolites such as 2,4-diacetylphloroglucinol (2,4-DAPG), pyrrolnitrin, and pyoluteorin (Castro *et al*., 2020; Zhang *et al*., 2020); and whose antagonistic potential against fungal pathogens has been widely studied (Andreolli *et al*., 2019; Castro *et al*., 2020; Cesa-Luna *et al*., 2020; Zhang *et al*., 2020). The *in vitro* antagonistic activity of strains Ca6, Ca2, and ChC7 showed significant differences in inhibiting *B. cinerea* and Pss 11116_b1, with ChC7 exhibiting antifungal capacity equivalent or superior to the other strains but with a lower control potential against Pss under *in vitro* conditions. While all strains exhibited an inhibition capacity over Pss significantly lower than a mix of antibiotic (Table 4), mechanisms of action other than growth inhibition by antimicrobial metabolites, such as competition for niche and nutrients or induction of resistance, may take place at the field level, explaining the resulting bacterial canker control. On the other hand, the method of concentration adjustment by OD only provides an approximation to the actual bacterial concentration observed after plating, so establishing bacterial growth dynamics over time by every antagonist isolate should be necessary for a more accurate evaluation in terms of determining the effective bacterial concentrations to control Pss.

Induction of resistance in plants can occur through the activation of the expression of defense genes, which, in turn, code for pathogenesis-related proteins (PR proteins), such as: chitinases (CHT), phenylalanine ammonia-lyases (pal), β-1,3-glucanases (glu), or peroxidases (POD) (Qin *et al*., 2003; Yao and Tian *et al*., 2005). Currently, studies on resistance induction in cherries have mainly focused on postharvest disease control in fruit under storage conditions, including the action of phytohormones such as salicylic acid (SA); microbial antagonists; and other compounds, against fungal pathogens including *B. cinerea*, *A. alternata*, *Penicillium expamsum*, and *M. fructicola* (Chan *et al*., 2006; Liu *et al*., 2020; Liu *et al*., 2021; Pan *et al*., 2022; Wang *et al*., 2015; Xu *et al*., 2008). Within this, the available information on microbial resistance inducers in cherry trees is limited, particularly through the use of *P. protegens*. In this context, the increase in the relative expression of the *pal* gene observed in this study after the first day of foliar applications of acibenzolar-S-methyl (ASM) and the strain Ca6 aligns with results reported in kiwi after the application of ASM for the control of *P. syringae* pv. *actinidae* (Wurms *et al*., 2017); and the application of *P. protegens* strain CHA0 in wheat for the control of *Zymoseptoria tritici* (Ashrafi *et al*., 2020). Concerning the application method, the use of recombinant strains of endophytic *Pseudomonas*, to which the 2,4-DAPG gene cluster was transferred from *P. protegens* strain Pf-5, showed increases in the relative expression of the *pal* gene between 1.31 and 2.74 times compared to untreated plants, after eight days of application (Patel and Archana, 2018). These values were close to those observed in this study after seven days since the application. Thus, more significant increases in the expression of the *pal* gene from 2,4-DAPG-producing strains could occur at earlier stages, as seen in this study, necessitating an early assessment of the behavior of this gene in sweet cherry trees. Chalcone synthase (CHS) is a key enzyme in the flavonoid and phytoalexin synthesis pathway, involved in the SA defense pathway, and plant growth and development (Dao *et al*., 2011; Hou *et al*., 2022). Although there was high variability in the expression for all treatments for *chs2* gene, the increases in the means were considerably higher than for all other analyzed genes, being accentuated on the seventh day after application. This variation could be explained by the role of CHS in multiple cellular stress reactions, also increasing its expression in response to UV exposure, high-intensity light, and low temperatures (Wang *et al*., 2017; Zhang *et al*., 2020). PR-5 has demonstrated antifungal activity in *Prunus domestica* (El-kereamy *et al*., 2011), describing in *P. avium* a β-1,3-glucanase activity capable of degrading the cell wall of fungal pathogens (Kebede and Kebede, 2021). The notable increase in this gene only in the chemical inducer may be due to the bacterial nature of the biological inductors used, unable to trigger the specific antifungal expression of this gene. NPR3 is a receptor of SA that acts as a negative regulator of the defense response in plants linked to SA (Fu *et al*., 2012; Ding *et al*., 2018). In the absence of pathogens, baseline levels of NPR3 repress the expression of SA-dependent defense genes (Ding *et al*. 2018), while, in the face of increased cellular SA levels due to applications or during an infection, NPR3 has increased stability, inhibiting its degradation to control cellular levels of NPR1, a co-activator of the expression of SA-related defense genes (Mou *et al*., 2003; Zhou *et al*., 2023). Under this regulation dynamics, the increase in the relative expression of *npr3* for all treatments should be due to the activation of the plant defense system mediated by SA, confirming resistance induction. However, to follow the model proposed by Zhou *et al*. 2023, it is necessary to monitor the levels of *npr1* and its gene product, which was not performed in this study. Nevertheless, the analysis of the relative expression of the genes included in this assay represents a significant approach to the study of resistance induction in cherry trees, which must be improved with monitoring of related gene products and include the analysis of other defense genes, as well as their evaluation in other plant tissues, including more instances of assessment over time. Also, it is necessary to elucidate the direct control effect of this alternative on bacterial canker caused by Pss and post-harvest rot in cherry, in order to parameterize its disease control potential.

Pss is capable of overwintering inside cankers and buds, making them the main sources of inoculum that define the population development in spring and the progression of the disease into the orchard. Therefore, winter applications included in management programs aim to reduce this inoculum source. Bacterial counts at stage 50 (dormancy) showed no significant variations between disinfected and non-disinfected samples across both seasons (Figures 1a, 1b), which aligns with low epiphytic bacterial activity during the winter stage. In this state, the lowest florescent-bacteria populations were found in BPP2, which suggest that the prior application and during the recess of the consortium of *Bacillus* spp. and *Brevibacillus brevis* had the greatest effect in reducing bacterial population, compared to FP and CP programs, which included one and four applications of copper-based products, respectively (Table 1). This decrease in the effectiveness of copper applications could be associated with an increase in the populations of copper-resistant bacteria, which has been reported in high percentage for Pss in Chilean orchards (Beltrán *et al*., 2021). After stage 56 (open cluster), the two programs based in BCAs (BBP1 and BBP2) tended, in general, to be comparable or lower than the chemical program (CP), suggesting a biocontrol effect of the strains of *P. protegens* Ca6, ChC7, and Ca2. During the second season, counts, in general, reached higher values, probably due to more favorable environmental conditions for Pss development, or a lower persistence of phytosanitary products in the field due to an increase in the quantity and frequency of precipitation (Annex 1). The incidence and length of cankers with symptoms compatible with bacterial canker were studied in both seasons of the trial in late spring. At the beginning of spring, bacterial concentration per untreated flower showed a situations of high disease risk of Pss infection, because bacterial concentrations were between log 4.0 to log7.0 CFU per flower in the programs with BCAs and FP (Kennelly *et al*., 2007). In this context, canker lesions did not experience variations in their length across all management programs, with lengths not exceeding 39 cm, contrary to what was observed in sweet cherry susceptible cultivars without control (Farhadfar *et al*., 2016; Mgbechi-Ezeri *et al*., 2017), suggesting control capacity by the antagonists applied during the period (*Trichoderma* spp. and *P. protegens* strains) . However, Mgbechi-Ezeri *et al*. (2017) report only a moderately high correlation between canker length and bacterial population size, explaining that variations between the populations found are not substantially reflected in the length of the lesion. On the other hand, the increase in the average incidence of cankers in all treatments in the second season could be explained by the significant increase in millimeters of rainfall during May and November 2022 (Annex 1), which facilitates Pss spread into the orchard and within the canopy; as well as bacterial aggregation (Kearns, 2010; Xin *et al*., 2018), favoring infection in buds and abscission zones during May, and wounds on lignified tissue in November. Despite this, this increase was only notorious in FP and CP programs, with BBP1 maintaining the lowest values among programs during both seasons (Figures 3 and 4), suggesting a greater control on canker incidence by *P. protegens* Ca6.

Assessments conducted during stage 65 (full bloom) to stage 89 (ripe fruit), flowers, hypanthia, growing fruits, and ripe fruits allowed to monitor the presence and degree of tissue colonization by fruit rot pathogens such as *Monilinia* spp., *Botrytis cinerea*, and *Alternaria* spp.. Incidence values and severity index of *B. cinerea* in flowers were below 18% and 0.44, respectively, during both seasons in all management programs, with at least one of the biological product-based programs showing equal or better control over the incidence and/or severity of the disease compared to the program based only in chemical fungicides (CP), demonstrating control capacity during bloom, which is a susceptible stage, or avoiding latent infections that could be observed during fruit ripening (Legard *et al*., 2001; Tarbath *et al*., 2014). However, the increase in *B. cinerea* rot incidence in BBP1 in flowering stage during the second season was probably due to the presence of rain two days before sampling, which could favored infection by environmental inoculum (Annex 1) and negatively affected the remaining population and the control of the Ca6 strain. Thus, under environmental conditions that increased disease development pressure, P. protegens Ca6 could diminish its control capacity. Infection. For fully open flowers colonized by *Alternaria* spp., the application of the *P. protegens* ChC7 and Ca2 strains achieved lower incidence of the fungus during both seasons, compared to the other management programs. However, their effects on severity are not entirely clear, showing a decrease between seasons of 57% in the severity index. Similarly, pyraclostrobin applications showed an increase in the index by350% for FP and a decrease of 26% for CP, between the first and second seasons. This variation in severity could be influenced by the previous active ingredient applications along the season that modify their control capacity over overwintering inoculum sources under the environmental conditions observed during the study. *B. cinerea* incidence values in hypanthia were below 7% and 20% depending on the season; while *Alternaria* spp. (first season) and *Cladosporium* spp. (second season; data not shown) reached incidence levels above 90% in all treatments (Figure 5c), Incidence of pathogenic fungi on hypanthia suggests that this tissue would be an important environmental inoculum source, capable of generating infections in future fruit development stages. For example, during the first season, *B. cinerea* incidence levels in the FP, CP, and BBP1 programs were comparable between the 72nd stage and the 89th stage, whereas this trend persisted between both stages during the second season, except for CP. While fenhexamid is described as a fungicide specific to *B. cinerea* (SAG, 2023), its fungicide broad-spectrum action has been observed on different postharvest fungi that affect sweet cherries, being reported as highly effective on *A. alternata* mycelial growth (Feliziani *et al*., 2013). Incidence levels of *Alternaria* spp. observed in CP after stage 89 were equal to or lower than the other management programs, despite the common use of fenhexamid. However, programs that included the application of biological products during stage 78 found incidence values below 12%, and severity indices below 0.28 in *Alternaria* spp.; and below 3% incidence, and severity indices below 0.08 in *B. cinerea*, for both seasons. Therefore, despite the control efficacy demonstrated by CP, the application of *Trichoderma* spp. and *P. protegens* strains during stages 67 and 78 would contribute to the control of both fungi, allowing a reduction in fenhexamid applications and thereby reducing resistance development, already documented in Chile for Bc isolate from grapevines (Esterio *et al*., 2007). Finally, during stage 89 (fruit ripe for harvesting), optimal environmental conditions for *B. cinerea* infection, such as relative humidity above 90%, presence of free water for more than 4 hours, and temperatures between 15 and 20°C (Carisse, 2016), did not occur, then study fruit rot control under conditions more predisposing to the disease may be necessary.

Finally, all these results suggests that biological based products could be incorporated into disease management programs for the control of sweet cherry main diseases, contributing to reduce the negatives impacts derived from the excess application and misuse of chemical fungicides and bactericides.

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Tables and figures

Table 1. Sweet cherry disease management programs and their products used in each different BBCH growth stage (Fadon *et al* , 2015).

BBCH Growth Stage	Disease management programs^z			
	FP	CP	BBP1	BBP2
93	Cu + Mg + Zn + Mn + Biotoxins	Copper hydroxide	*Pseudomonas protegens* strain Ca6	*Pseudomonas protegens* strains Ca2 and Chc7
94-95	Cuprous oxide	Cuprous oxide	*Pseudomonas protegens* strain Ca6	*Bacillus subtilis*. + *Brevibacillus brevis*
96	-	Copper hydroxide	*Pseudomonas protegens* strain Ca6	*Bacillus subtilis*. + *Brevibacillus brevis*
50	Carboxylic acids + Zn	Copper hydroxide	*Pseudomonas protegens* strain Ca6	*Bacillus subtilis*. + *Brevibacillus brevis*
56	Pyraclostrobin	Pyraclostrobin	*Pseudomonas protegens* strain Ca6	*Pseudomonas protegens* strains Ca2 and Chc7
64	*Bacillus subtilis*. + *Brevibacillus brevis*	-	-	*Bacillus subtilis*. + *Brevibacillus brevis*
65	Fluopyram + Tebuconazole	Fluopyram + Tebuconazole	Fluopyram + Tebuconazole	Fluopyram + Tebuconazole
67	Tebuconazole + Fenhexamide	Tebuconazole + Fenhexamide	*Trichoderma* spp. + *Pseudomonas protegens* strain Ca6	*Trichoderma* spp.
72	Captan	Captan	Captan	Captan
78	-	-	*Pseudomonas protegens* strain Ca6	*Pseudomonas protegens* strains Ca2 and Chc7
89	Fenhexamide	Fenhexamide	Fenhexamide	Fenhexamide

[z] Symbol description of each disease management program: FP= Orchard program used by the grower; CP= program based in chemical bactericides and fungicides; BBP1 = Biological-based program 1; BBP2 = Biological-based program 2.

Table 2. Treatments and their concentrations assessed in a resistance induction assay on sweet cherry cv. ‘Santina’.

Treatment (a.i.)	a.i. Concentration	Dose
Sterile distilled water	-	-
P. *protegens* strains Ca2 and ChC7[a]	5 x 10^3 CFU g^{-1}	1 g L^{-1}
P. *protegens* strain Ca6[b]	1 x 10^5 CFU mL^{-1}	1 mL L^{-1}
Acibenzolar-S-methyl[c]	50% w w^{-1}	2 g L^{-1}

[a] Taniri® WP, Bioinsumos Nativa SpA., Chile; [b] Maxgrowth®, Bioprotegens Innovations SpA., Chile; [c] Bion® 50 WG, Syngenta S.A., Chile.

Table 3. List of gene resistance primer sequences used to assess resistance induction on sweet cherry plants.

Gen-Protein	Gen codification	Forward primer sequence (5' - 3')	Reverse primer sequence (5' - 3')	Amplicon size (pb)	Reference
β–actine	β-*act*	TTGTGCTGGACTCTGGTGATG	GCTCAGCAGTGGTGGTGAAC	165	NP
NPR3-like protein	*npr3*	TGGTCTGGGTCTAGCTGATGTTA	GCCCCTGCTCTGTTTTTGAATGA	212	NP
Phenylalanine ammonia lyase-1	*pal1*	CTAACAGGGGAAAAGGTCAGGTCA	GGACACAGAAGTAGTGGAATGGAAT	80	Xu *et al.*, 2020
Pathogenesis-related protein 5	*pr5*	AGTCCTCAGCCTCAGCTTAACCAT	TTGGGATGCTAACTCGAACCCTGT	157	El-Kereamy *et al.*, 2011
Chalcone synthase 2-like	*chs2*	CCCACAAGACACATACTATCTGAGTA	CCCAAACCCAAACAGCACACCCCAA	147	NP

Table 4. Fungal radial growth (cm) and percentage of radial growth inhibition (PRGI) on three pre and post-harvest rot fungi after 7 days of dual culture in PDA-KB medium, and area of inhibition (mm^2) on *Pseudomonas syringae* pv. *syringae* (Pss) strain 11116_b1 for different strains of *P. protegens* after 48 hours under *in vitro* condition.

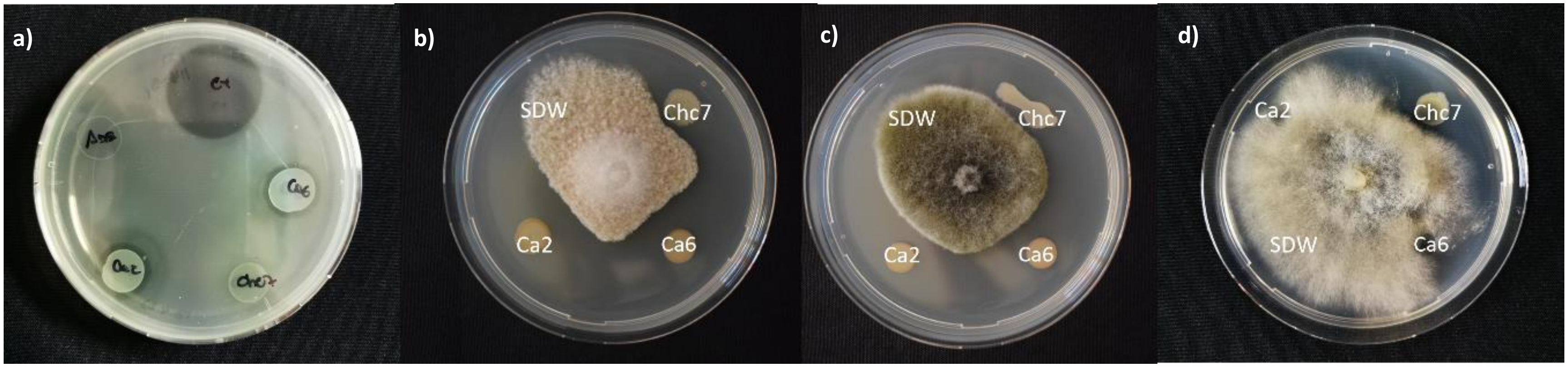
Strain	Fungal radial growth and PRGI (%)			Inhibition area
-	*Botrytis cinerea*	*Monilinia fructicola*	*Alternaria alternata*	Pss 11116_b1
Control				0.0
Ca6	3.80 (5.0%) b[z]	2.07 (49.3%) A[y]	2.30 (37.6%) a	8.9 bc
Ca2	3.40 (15.0%) b	2.10 (48.5%) A	2.33 (38.5%) a	9.9 b
ChC7	2.57 (35.8%) a	2.43 (40.2%) A	2.57 (31.3%) a	6.7 c
C+[x]	-	-	-	21.4 a
Coefficient of variation				
P-value				

[z] Different lowercase letters inside a column indicate significant differences ($p < 0.05$) using one way ANOVA with Tukey's test.

[y] Capital letters inside a column indicate no significant differences ($p < 0.05$) using non-parametric Kruskal-Wallis test.

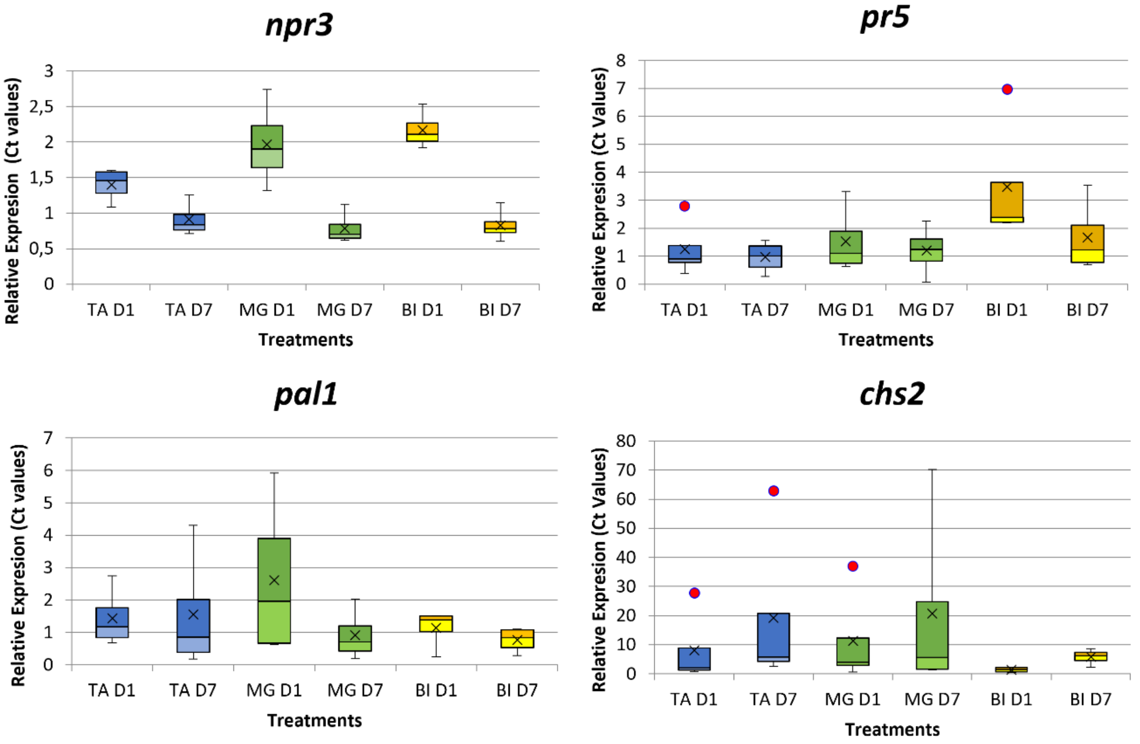
[x] C+ = Positive control based in antibiotics

Figure 1. Inhibition halo and inhibition of mycelial growth caused by *Pseudomonas protegens* strains against Pss 1111-6 B1 (a) and *Monilinia fructicola* (b), *Alternaria alternata* (c), and *Boytritis cinerea* (d), respectively. SDW or ADS in a) correspond to sterile distillated water and C+ in a) corresponds to a positive control based in the antibiotics streptomycin sesquisulfate and oxytetracycline hydrochloride.



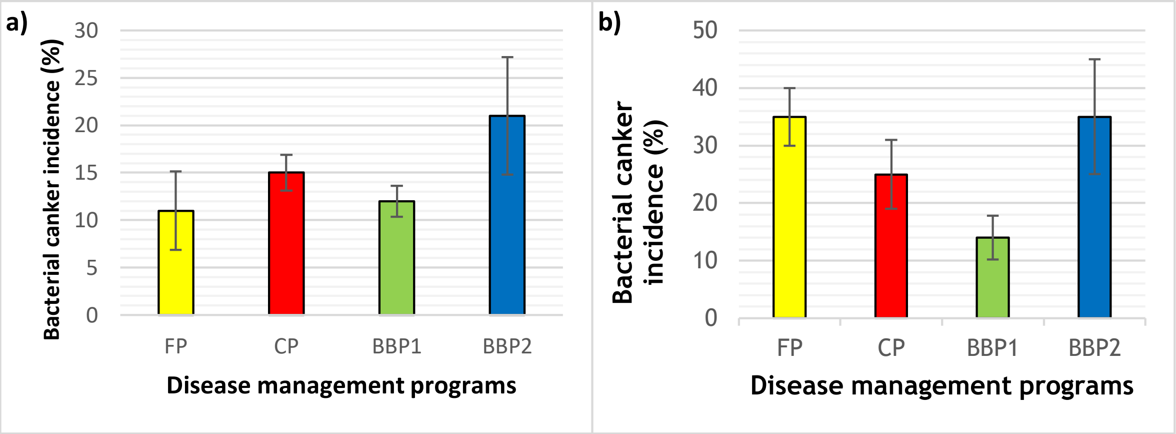
C+ = Positive control (Strepto Plus®); ADE = Sterile distilled water.

Figure 2. Relative expression of four genes involved on induced systemic resistance used in this study. Data normalized with respect to *β-actin* gene expression on control treatment.



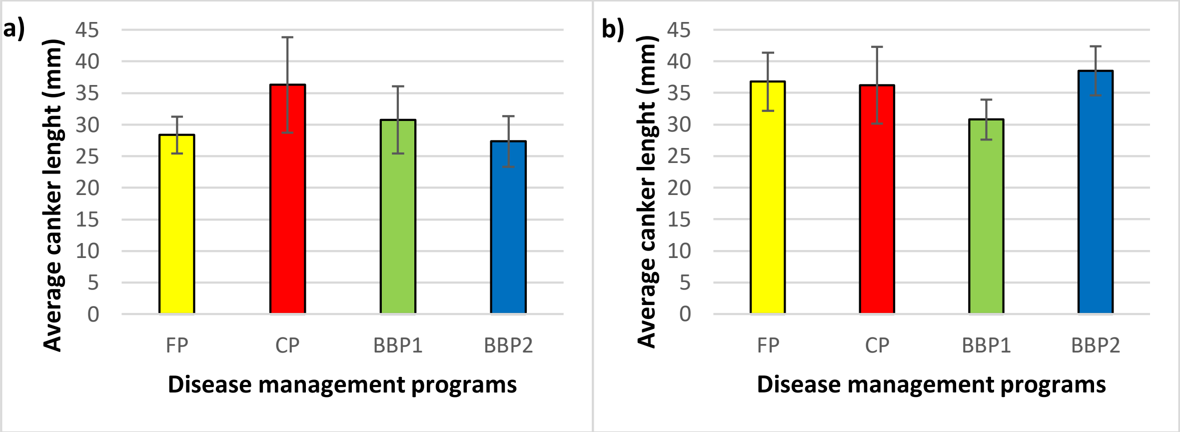
TA: *P. protegens* strains Ca2 and ChC7 (Taniri®); MG: *P. protegens* strain Ca6 (Maxgrowth®); BI: Acibenzolar-S-methyl (Bion® 50 WP). D1 = 1 day after spraying; D7 = 7 days after spraying.

Figure 3. Pss bacterial canker incidence (%) according to disease management program a) 2020/2021 season and b) 2021/2022 season. Error bars are for standard error.



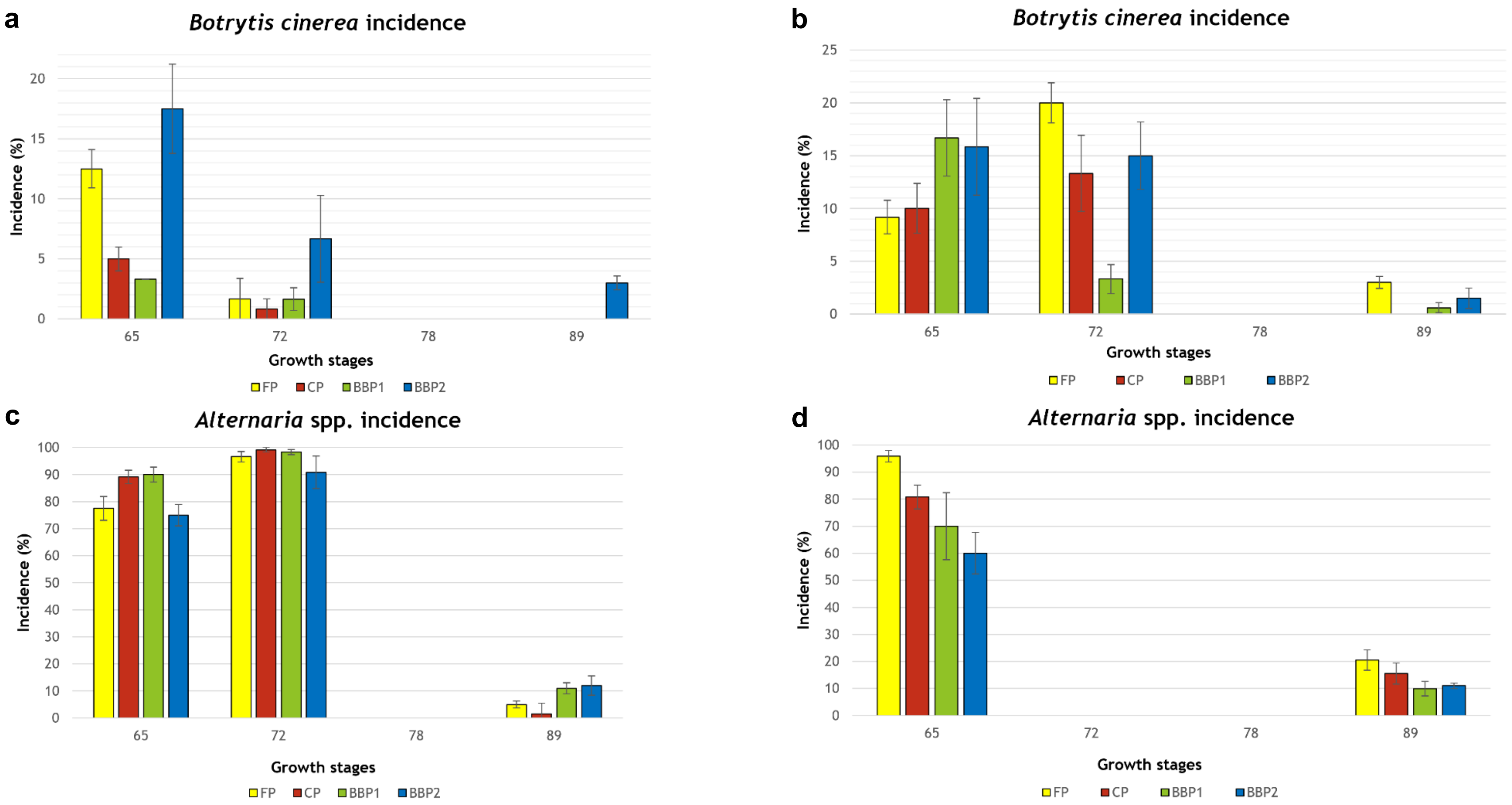
FP = Farmer's habitual program; CP = Program based in chemical product; BBP1 and BBP2 = programs based in biological control agents.

Figure 4. Average canker length associated with *Pseudomonas syringae* pv. *syringae* infection in sweet cherry trees cv. 'Sweetheart' observed by four different disease management programs during 2020/2021 a) and 2021/2022 (b)season. Error bars are for standard error.



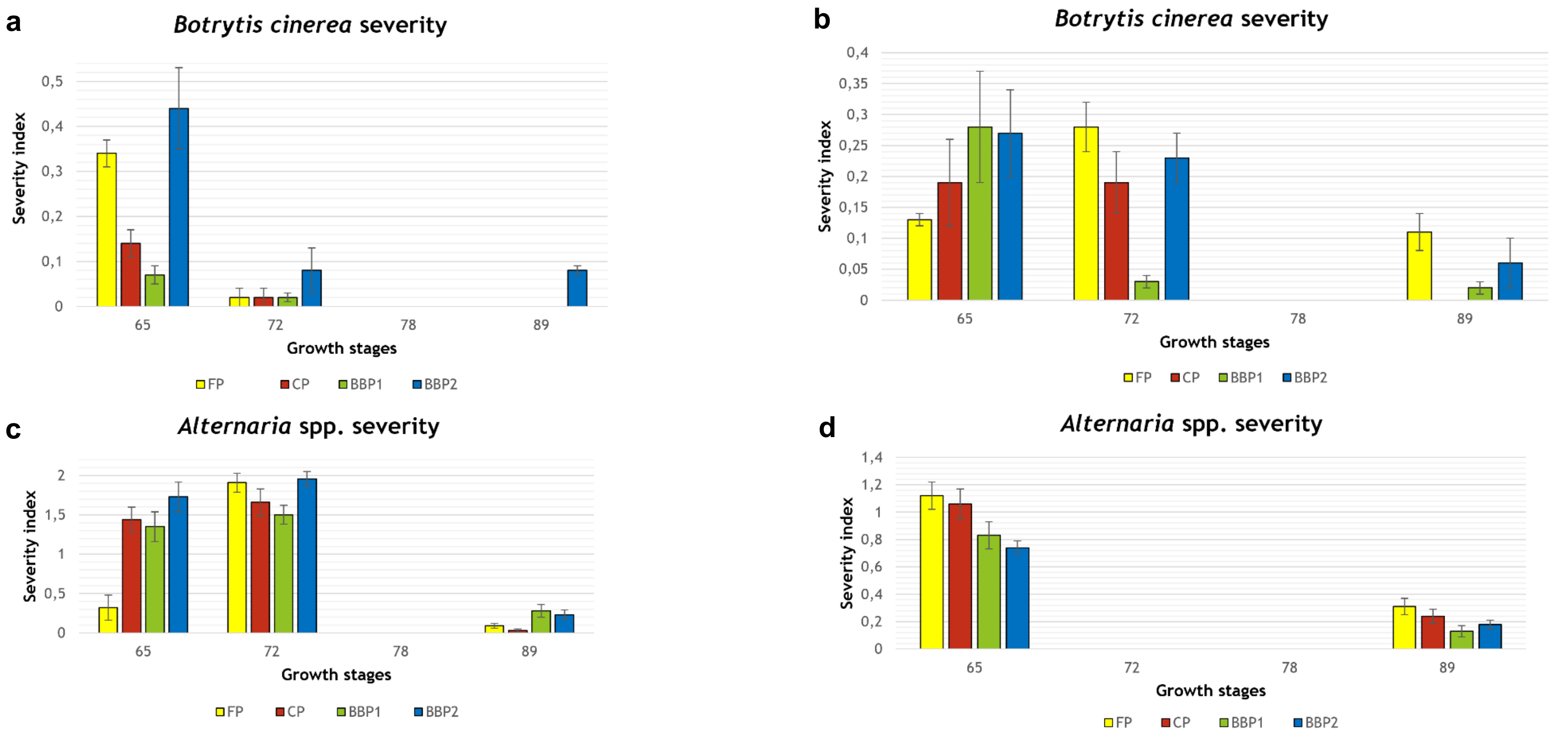
FP = Farmer's habitual program; CP = Program based in chemical product; BBP1 and BBP2 = programs based in biological control agents.

Figure 5. *Botrytis cinerea* incidence during season 2021 (a) and 2022 (b), and *Alternaria* spp. incidence during season 2021 (c) and 2022 (d). Error bars are for standard error.



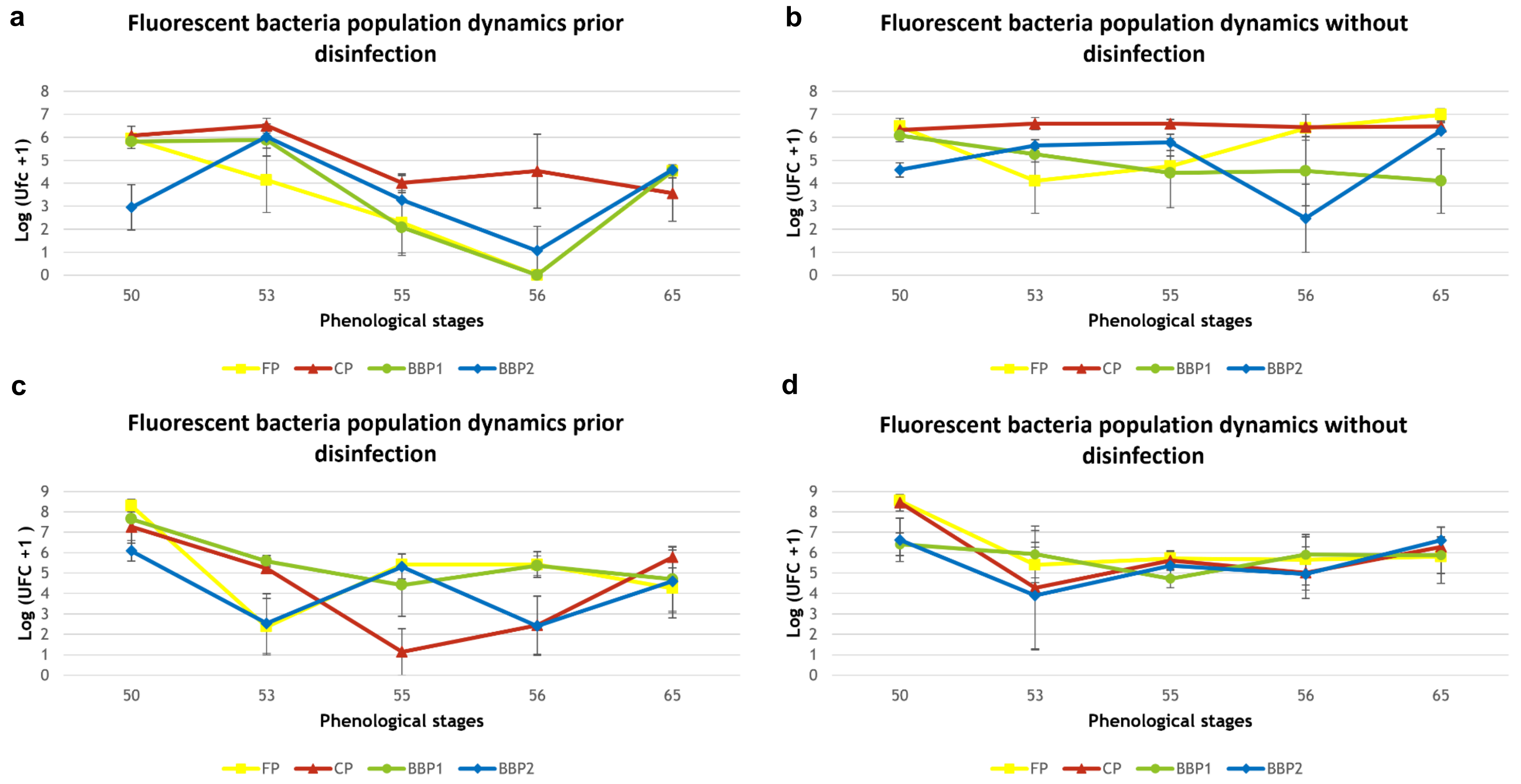
FP = Farmer's habitual program; CP = Program based in chemical product; BBP1 and BBP2 = programs based in biological control agents.

Figure 6. *Botrytis cinerea* severity during season 2021 (a) and 2022 (b), and *Alternaria* spp. severity during season 2021 (c) and 2022 (d). Error bars are for standard error.



FP = Farmer's habitual program; CP = Program based in chemical product; BBP1 and BBP2 = programs based in biological control agents.

Figure 7. Fluorescent bacteria population dynamics with and without disinfection for season 2020/2021 (a) and (b); and season 2021/2022 (c) and (d), respectively, isolated from different phenological stages. Error bars are for standard error.


a Fluorescent bacteria population dynamics prior disinfection

b Fluorescent bacteria population dynamics without disinfection

c Fluorescent bacteria population dynamics prior disinfection

d Fluorescent bacteria population dynamics without disinfection

FP = Farmer’s habitual program; CP = Program based in chemical product; BBP1 and BBP2 = programs based in biological control agents.

CONCLUSIONES GENERALES

La detección y cuantificación de la carga bacteriana de *Pseudomonas syringae* pv. *syringae* mediante qPCR representa un método rápido y preciso para el diagnóstico del riesgo del desarrollo de cáncer bacterial, sin presentar los inconvenientes de la siembra en placa, contribuyendo a la toma de decisiones para el manejo de la enfermedad.

El uso de programas de manejo con inclusión de microorganismos antagonistas resulta en un control eficaz del cáncer bacterial causado por *Pseudomonas syringae* pv. *syringae*, de manera equivalente al control en programas químicos. De igual forma, el uso de antagonistas microbianos en conjunto a fungicidas químicos sintéticos dentro de un programa de manejo permite un control de las pudriciones en pre y post-cosecha causadas por *B. cinerea*, *Alternaria* spp. y *Monilinia* spp., permitiendo un uso racional de fungicidas químicos.

La incorporación de cepas de *P. protegens*, *Trichoderma* spp., y *B. subtilis y Brevibacillus brevis* dentro de programas de manejo fueron capaces de disminuir poblaciones de *Pseudomonas syringae* pv. *syringae* y *Botrytis cinerea* durante botón blanco y plena flor; y plena flor y caída de chaqueta, respectivamente, reduciendo fuentes de inóculo que afectan negativamente el rendimiento potencial. Así mismo, el programa de manejo basado en *P. protegens* fue capaz de reducir pudriciones por *Alternaria* spp. en frutos durante la segunda temporada, homologándose al programa químico.

La expresión de los genes *npr3*, *chs2*, *pal1* y *pr5* relacionados con respuestas de resistencia sistémica en cerezo fue inducida por aplicaciones foliares de cepas de *P. protegens*, sugiriendo una opción de control mediante el uso de estas cepas, que podría explicar la eficacia observada de estos tratamientos usados en programas de manejo de la enfermedad que usaron los aislados Ca6 y la mezcla Ca2 y ChC7.