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Control de *Phlyctema vagabunda* con levaduras antagonistas

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TABLA DE CONTENIDOS

ÍNDICE DE FIGURAS

Página

CAPITULO II

Endophytic yeasts for the biocontrol of *Phlyctema* vagabunda in apples

ÍNDICE DE TABLAS

Página

RESUMEN

La pudrición ojo de buey, producida por *Phlyctema vagabunda*, es una importante enfermedad de postcosecha en las manzanas. Las medidas actuales para controlar la infección incluyen fungicidas sintéticos. Sin embargo, la creciente preocupación pública por los residuos de fungicidas en los alimentos ha generado interés en el desarrollo de métodos alternativos de control no químicos; siendo el control biológico una de las alternativas más prometedoras. En esta investigación, se aislaron y evaluaron levaduras endófitas nativas para el biocontrol de *P. vagabunda* en manzanas. También se determinaron los mecanismos de acción implicados. Dos aislados, *Vishniacozyma victoriae* EPL4.5 y EPL29.5, mostraron actividad de biocontrol contra *P. vagabunda* a 20°C en manzanas; la incidencia de ojo de buey se redujo en un 39% y 61%, respectivamente, y la severidad de la enfermedad disminuyó en un 67% y 70%, respectivamente, cuando las manzanas fueron inoculadas con estas levaduras 24 h antes de aplicar el patógeno. Los principales mecanismos de acción que podrían estar implicados en la actividad de biocontrol observada fueron la capacidad de formar biopelículas, la producción de compuestos orgánicos volátiles y la inducción de resistencia en la fruta.

ABSTRACT

Bull's-eye rot, produced by *Phlyctema vagabunda*, is an important postharvest disease in apples. Current measures to control infection include synthetic fungicides. However, growing public concern regarding fungicide residues in food has generated interest in the development of non-chemical, alternative control methods; biological control is one of the most promising alternatives. In this research, native endophytic yeasts were isolated and evaluated for the biocontrol of *P. vagabunda* in apples. The mechanisms of action involved were also determined. Two isolates, *Vishniacozyma victoriae* EPL4.5 and EPL29.5, exhibited biocontrol activity against *P. vagabunda* at 20°C in apples, the incidence of bull's-eye rot was reduced by 39% and 61%, respectively, and the severity of the disease was decreased by 67% and 70% respectively, when apples were inoculated with these yeasts 24 h before applying the pathogen. The main mechanisms that could be involved in the observed biocontrol activity were the ability to form biofilms, the production of volatile organic compounds and resistance induction.

CAPITULO I

INTRODUCCIÓN Y OBJETIVOS

El cultivo de manzanos (*Malus domestica*) es uno de los rubros frutícolas más importantes en Chile con 30 mil ha plantadas, concentradas principalmente entre la Región de Valparaíso y del Maule, lo que equivale al 8,8% de la superficie plantada con frutales en el territorio (ODEPA, 2022a), de las cuales 2.650 ha se encuentran certificadas como orgánicas (Eguillor, 2021). Las exportaciones de manzana, principalmente los cultivares 'Royal Gala', 'Granny Smith', 'Richared Delicius' y 'Fuji', han superado las 640 mil toneladas, generando un valor USD FOB de 614 millones durante la temporada 2021, siendo nuestro país el principal productor de esta especie del hemisferio sur; los principales destinos de esta fruta fueron América Latina, Estados Unidos, India y Holanda (ODEPA, 2022b).

Las pérdidas provocadas por las enfermedades de postcosecha en países desarrollados pueden alcanzar entre el 12 y 50% (Usall *et al*., 2016), período en que la fruta es altamente perecible, por su alto contenido de agua y nutrientes, lo que las hace un sustrato ideal para el desarrollo de microorganismos patógenos, como los hongos (Spadoni *et al*., 2015). Los hongos patógenos son los causantes de las mayores pérdidas de fruta durante el almacenamiento (Singh y Sharma, 2018) y a esto se suma la aparición de cepas resistentes a los fungicidas sintéticos actualmente utilizados (Usall *et al*., 2016).

Entre estas enfermedades, actualmente la pudrición denominada "ojo de buey" causada por el hongo *Phlyctema vagabunda* (sin. *Neofabraea vagabunda*) se ha

convertido en una importante enfermedad en postcosecha de manzanas en Chile (Soto-Alvear *et al*., 2013), provocando también grandes pérdidas económicas en Europa (Cameldi *et al*., 2017) y el noreste de Estados Unidos (Henriquez *et al*., 2004), además de ser considerada como cuarentenaria en los lugares de destino de la fruta como China (Wood y Fisher, 2017). En Chile, *P. vagabunda* fue reportada por primera vez el año 2005 (Henriquez, 2005), siendo las variedades más afectadas las de cosecha tardía como 'Cripps Pink', donde se han reportado incidencias que pueden alcanzar un 60%, dependiendo de la estación y la localidad (Soto-Alvear *et al*., 2013). En producción orgánica, las incidencias en cultivares como 'Cripps Pink', pueden llegar a ser del 80-90%, siendo la mayor limitante para la exportación de esta variedad cuando la fruta se mantiene en frío de forma prolongada (Lolas *et al*., 2021).

La infección de la fruta ocurre en el huerto (Cameldi *et al*., 2017), en cualquier momento durante la temporada, pero es más susceptible a medida que se acerca la época de la cosecha (Lolas *et al*., 2021) y es favorecida por periodos largos de lluvias y temperaturas cercanas a los 20°C (Giraud *et al*., 2021). Los síntomas aparecen solo algunos meses después de la cosecha (generalmente 3-4 meses en almacenamiento en frío), cuando numerosas lesiones pueden desarrollarse en una única fruta (Neri *et al*., 2009); estas lesiones se desarrollan a partir de heridas o cavidades lenticelares (Soto-Alvear *et al.*, 2013), donde se ubica el hongo, en la forma de conidia germinada o hifa (Neri *et al*., 2009). Estas lesiones son de 1 a 3 cm de diámetro (Henriquez, 2005), circulares planas a ligeramente hundidas (Cameldi *et al*., 2017) de color marrón con anillos concéntricos, sin embargo,

también se han encontrado lesiones amarillentas sin anillos concéntricos; los tejidos afectados son relativamente firmes y se desprenden fácilmente del tejido sano (Soto-Alvear *et al*., 2013). El acérvulo está frecuentemente presente en lesiones antiguas, bajo condiciones húmedas (Cameldi *et al*., 2017), el cual contiene abundantes macroconidias fusiformes a alantoides, hialinas y aseptadas (Henriquez, 2005). El hongo puede permanecer como saprófito en la corteza de los árboles y en las escamas de las yemas (Giraud *et al*., 2021) y las conidias son dispersadas desde el acérvulo durante todo el año, pero particularmente en otoño (Neri *et al*., 2009). La conidia ingresa a la fruta a través de las lenticelas, y una vez germinada es capaz de penetrar el tejido cortical alrededor de la cavidad subestomática de las lenticelas y permanecer inactivo hasta que la fruta alcanza cierto estado de madurez, cuando puede invadir el resto de la fruta. Él o los mecanismos involucrados en la regulación de la infección latente no están claros, sin embargo, algunos cambios fisiológicos ocurren durante la maduración de la fruta, los que pueden ser responsables de romper la latencia del hongo (Neri *et al*., 2009).

El control de esta enfermedad en Chile incluye tratamientos con fungicidas en pre y postcosecha (Lolas *et al*., 2015). Pero, debido a la creciente preocupación pública acerca de residuos de fungicidas en los alimentos (Spadaro y Droby, 2016), han generado interés en el desarrollo de métodos alternativos no químicos de control, siendo el control biológico la alternativa más promisoria y explorada (Wisniewski *et al*., 2007).

El control biológico consiste en la reducción de la densidad de inóculo o de las actividades productoras de enfermedad de un patógeno o parásito, en un estado activo o dormante, por uno o más organismos, realizado de manera natural o a través de la manipulación del ambiente, del hospedero o de los antagonistas del patógeno (Baker *et al.*, 1974). La utilización de microorganismos antagonistas ha sido reconocido como una de las alternativas promisorias al uso de fungicidas (Spadaro y Droby, 2016), entre estos microorganismos, las levaduras antagonistas aisladas desde la superficie de frutos han mostrado ser efectivas en el control de numerosas enfermedades de postcosecha en manzana (Abdelhai *et al*., 2019; He *et al*., 2020; Mari *et al*., 2012; Türkel *et al*., 2014; Vero *et al*., 2013; Zhu *et al*., 2022). Sin embargo, todavía no se han registrado estudios en *P. vagabunda*.

Las levaduras pueden ser una alternativa viable de control para este patógeno, puesto que poseen algunas propiedades útiles como son la capacidad de colonizar la superficie de frutos por largos períodos de tiempo bajo condiciones de sequía, la producción de polisacáridos extracelulares que mejoran su capacidad de sobrevivencia, utilización rápida de los nutrientes disponibles, son mínimamente afectados por los pesticidas (Wilson y Wisniewski, 1989), no producen esporas alergénicas o micotoxinas (Parafati *et al*., 2015), son genéticamente estables y tienen requerimientos nutricionales simples (Ruiz-Moyano *et al*., 2016).

Los agentes de control biológico son organismos vivos que actúan siguiendo distintas estrategias dependiendo del patógeno, el huésped y el ambiente (Di Francesco *et al*., 2017), conocer sus mecanismos de acción es importante para incrementar su potencial como biocontrolador (Pesce *et al*., 2018), optimizar su

formulación (Lima *et al*., 2013) y su uso en forma segura y efectiva (da Cunha *et al*., 2018).Un buen agente de biocontrol requiere de múltiples mecanismos de acción para antagonizar a un patógeno (Chanchaichaovivat *et al*., 2008), ya que la combinación de los diferentes mecanismos son los que le otorgan a la levadura su capacidad de control (Bautista-Rosales *et al*., 2013) y reduce el riesgo de resistencia por parte del patógeno (Nally *et al*., 2015). Entre los distintos mecanismos de acción descritos en levaduras cabe destacar:

- **Competencia por nutrientes y espacio**. Es considerado el principal mecanismo de acción por el cual actúan las levaduras (Tang *et al*., 2015) y es la consecuencia del requerimiento de los mismos macro y micronutrientes o espacio por dos o más microorganismos (Di Francesco *et al*., 2016). Este mecanismo está relacionado con la habilidad de los agentes de biocontrol para adherirse a sitios específicos, incluyendo los tejidos del huésped y del patógeno, además de la capacidad de proliferación rápida de células en la herida (Droby *et al*., 2009), logrando así la inhibición del crecimiento del patógeno (Lima *et al*., 1997). Se ha descrito que las levaduras *Aureobasidium pullulans*, *Kloeckera apiculata*, *Metschnikowia citriensis*, *Metschnikowia pulcherrima*, *Meyerozyma guilliermondii* y *Pichia galeiformis* reducen eficazmente la pudrición por hongos mediante la competencia por nutrientes y espacio (Chen *et al*., 2020; Di Francesco *et al*., 2017; Huang *et al*., 2021; Liu *et al*., 2013; Wang *et al*., 2020; Yang *et al*., 2021).
- **Resistencia al estrés oxidativo.** La acumulación de especies reactivas al oxigeno (ROS), es una de las primeras señales de reacción de defensa al ataque del patógeno (Liu *et al*., 2013) y cuando las levaduras son aplicadas, los ROS

generados producen estrés oxidativo que puede afectar su viabilidad (Di Francesco *et al*., 2016). Los antagonistas con la capacidad de producir resistencia a este tipo de estrés poseen una ventaja en el control de patógenos de herida (Liu *et al*., 2013), puesto que la tolerancia al estrés oxidativo está relacionado positivamente con la capacidad de colonización de las heridas y la eficacia del biocontrol (Sui *et al*., 2021). Las levaduras donde se ha demostrado dicha capacidad han sido *Cryptococcus laurentii* y *Pichia membranefaciens* (Chan y Tian, 2006; Wang *et al*., 2004).

- **Parasitismo**. Es un importante mecanismo de biocontrol en levaduras (Bautista-Rosales *et al*., 2014), donde ocurre una fuerte adhesión del antagonista a las células del patógeno, debido a un enlace de lectina. La interacción antagonista-patógeno, depende del reconocimiento de proteínas entre levaduras y la hifa del hongo (Spadaro y Gullino, 2004), además, de la habilidad de secretar enzimas hidrolíticas del tipo quitinasas y glucanasas (Tian *et al*., 2018). Estas enzimas degradan las paredes celulares del hongo, ya que hidrolizan glucanos, los cuales son los principales componentes de la pared celular (Gow *et al*., 2017). Sin embargo, varios estudios sugieren que este mecanismo podría estar relacionado con la competencia por nutrientes, en que las levaduras se interponen entre el patógeno y el sustrato, limitando su crecimiento (Bautista-Rosales *et al*., 2014). Este mecanismo de acción ha sido reportado en *Debaryomyces nepalensis*, *Meyerozyma caribbica*, *M. guilliermondii* y *Wickerhamomyces anomalus* frente a *Colletotrichum gloeosporioides*, *M. citriensis*, *Pichia kudriavzevii*, *Pichia membranaefaciens* y

Yarrowia lipolytica frente a *Penicillium digitatum* (Bautista-Rosales *et al*., 2013; Delali *et al*., 2021; Lima *et al*., 2013; Liu *et al*., 2019; Zhou *et al*., 2018).

- **Producción de enzimas hidrolíticas.** La producción de estas enzimas es propuesta como un importante mecanismo de acción de las levaduras antagonistas (Nally *et al*., 2015), puesto que en el caso de la enzima β-1,3 glucanasa actúa sobre los sitios de β-1,3 glucanos en la cadena de polisacáridos de las paredes hifales del patógeno, distorsionando las hifas y filtrando sus componentes citoplasmáticos (Delali *et al*., 2021), causando su lisis y consecuentemente su muerte celular (Banani *et al*., 2015). Se postula que la producción de enzimas β-1,3-glucanasa y quitinasa ocurre en estadíos tempranos de crecimiento de la levadura, por lo que podrían contribuir en la degradación de polímeros complejos y en la firmeza del acoplamiento de las células de levadura a la hifa del hongo (Bar-Shimon *et al*., 2004). La producción de enzimas hidrolíticas como mecanismo de acción frente a patógenos, ha sido sugerido en *A. pullulans*, *Candida stellimalicola*, *Debaryomyces hansenii*, *D. nepalensis*, *Galactomyces geotrichum*, *M. caribbica*, *P. kudriavzevii*, *P. membranaefaciens*, *Saccharomyces cerevisiae*, Schwanniomyces vanrijiae, *W. anomalus* y *Y. lipolytica* (Bautista-Rosales *et al*., 2013; da Cunha *et al*., 2018; Delali *et al*., 2021; Hernández-Montiel *et al*., 2010; Madbouly *et al*., 2020; Parafati *et al*., 2015; Zhang *et al*., 2010; Zhou *et al*., 2016, 2018).
- **Producción de enzimas letales (toxinas killer).** Son proteínas extracelulares o glicoproteínas que degradan la membrana celular de otras especies de levadura, hongos y bacterias susceptibles (Çorbacı y Uçar, 2018), a través de la hidrólisis del mayor componente de la pared celular, los β-1,3-glucanos (Gow *et al*.,

2017), debido a que tienen actividad glucanasa (Çorbacı y Uçar, 2018). Fueron descritas inicialmente en cepas de *S. cerevisiae* y posteriormente se han encontrado en un gran número de especies de levaduras (Santos *et al*., 2004). La estabilidad de estas proteínas está fuertemente influenciada por el pH, puesto que son producidas y activadas bajo condiciones de pH ácido (Chanchaichaovivat *et al*., 2008) y la temperatura, ya que sobre 20°C se pierde rápidamente (Santos *et al*., 2004). La capacidad de producir toxinas killer ha sido reportada en *D. hansenii*, *Pichia guilliermondii*, *P. kudriavzevii*, *Pichia membranifaciens* y *W. anomalus* (Chanchaichaovivat *et al*., 2008; Çorbacı y Uçar, 2018; Grzegorczyk *et al*., 2017; IZGÜ *et al*., 2005; Madbouly *et al*., 2020; Santos y Marquina, 2004).

- **Producción de sideróforos.** Los sideróforos son compuestos orgánicos de bajo peso molecular producidos por las levaduras y otros microorganismos en condiciones de baja disponibilidad de ion férrico (Fe^{+3}) (Ghosh *et al*., 2020), como por ejemplo la herida en la fruta (Di Francesco *et al*., 2016). Su función es facilitar la absorción de Fe^{+3} mediante el enlace y transporte de estos iones al interior de la célula (Baakza *et al*., 2004). El ion férrico es biológicamente importante, porque es constituyente del citocromo (Nally *et al*., 2015) y está involucrado en la patogenicidad de los hongos (Wang *et al*., 2021), ya que es cofactor de enzimas fúngicas. Las levaduras producen sólo sideróforos del tipo hidroxamato (Calvente *et al*., 2001), derivados del aminoácido ornitina y clasificados en cuatro familias estructurales: fusarininas, coprogenos, ferricromos y ácido rodotorúlico (Di Francesco y Baraldi, 2021). Los sideróforos del tipo hidroxamato, se caracterizan por producir un cambio de color desde verde azulado a anaranjado en el método

universal del Cromo Azurol S (CAS) (Pérez-Miranda *et al*., 2007). La capacidad de producir sideróforos ha sido reportada en *A. pullulans*, *Candida sake*, *Leucosporidium scottii*, *M. pulcherrima*, *P. membranifaciens* y *S. cerevisiae* (Di Francesco y Baraldi, 2021; Nally *et al*., 2015; Vero *et al*., 2013; Wang *et al*., 2009; Yang *et al*., 2021).

- **Producción de compuestos volátiles orgánicos.** Los compuestos volátiles orgánicos (COVs) son compuestos de bajo peso molecular, con baja polaridad y alta presión de vapor (Di Francesco *et al*., 2015), son producidos por un amplio rango de microorganismos, incluidos bacterias y levaduras. Algunos COVs tienen actividad antimicrobiana y otros participan en las interacciones microbianas, actuando como compuestos de señalización y de "quorum sensing" (Arrarte *et al*., 2017). Los COVs producidos por las levaduras son principalmente del tipo alcoholes, aldehídos, cetonas y ésteres (Farbo *et al*., 2018; Iñiguez-Moreno *et al*., 2020; Qin *et al*., 2017; Yang *et al*., 2021; Zhou *et al*., 2018), los cuales han mostrado ser efectivos biofumigantes para el control de enfermedades en plantas bajo condiciones herméticas (Huang *et al*., 2011), pudiendo representar un importante mecanismo de control biológico para un amplio rango de patógenos de postcosecha, ya que son biodegradables, no requieren contacto directo con la superficie de la fruta, y no dejan residuos (Parafati *et al*., 2017). La producción de estos volátiles está fuertemente influenciada por su capacidad de asimilar y fermentar hidratos de carbono *(*Fialho *et al*., 2010) y se cree que su actividad antagonista ocurre cuando estos COVs son absorbidos a través de las membranas celulares de los patógenos, lo que incrementa su permeabilidad y difusión de los

iones y metabolitos esenciales con la consecuente pérdida de homeostasis (Medina-Córdova *et al*., 2016), lo que puede repercutir en el crecimiento del hongo. La capacidad de producir COVs con actividad antifúngica sobre hongos patógenos de plantas ha sido descrito en *A. pullulans*, *Candida intermedia*, *C. sake*, *Cryptococcus albidus*, *Cryptococcus victoriae*, *Kluyveromyces marxianus*, *M. pulcherrima*, *P. galeiformis*, *P. kudriavzevii*, *S. cerevisiae*, *W. anomalus* y *Y. lipolytica* (Arrarte *et al*., 2017; Chen *et al*., 2020; Delali *et al*., 2021; Di Francesco *et al*., 2015; Fialho *et al*., 2010; Huang *et al*., 2011; Lutz *et al*., 2013; Parafati *et al*., 2017).

- **Formación de biopelículas.** Las biopelículas son comunidades de microorganismos viables y no viables protegidos por polímeros extracelulares (Bautista-Rosales *et al*., 2014), que crean una barrera mecánica que se interpone entre la herida y la superficie del patógeno (Di Francesco *et al.*, 2016). Se cree que su formación está regulada por “quorum sensing” (Bautista-Rosales *et al*., 2013) y parte con la adhesión de la célula al huésped, la que es conferida por proteínas superficiales denominadas adhesinas (Giobbe *et al*., 2007), luego a través de señales químicas y activación de genes que codifican para la producción de exopolisacáridos, se generan sustancias poliméricas extracelulares desde las microcolonias (Bautista-Rosales *et al*., 2013). Este mecanismo de acción favorece la adhesión, colonización y multiplicación de los antagonistas en la superficie de los frutos, lo que aumenta la capacidad de los microorganismos colonizadores para obtener los nutrientes (Yang *et al*., 2021), permitiendo con ello competir con los patógenos por dichos nutrientes. La capacidad de formar biopelículas ha sido

reportada en *Cryptococcus laurentii*, *C. victoriae*, *D. nepalensis*, *L. scottii*, *M. citriensis*, *M. pulcherrima*, *M. caribbica*, *Pichia fermentans*, *P. membranaefaciens*, *S. cerevisiae* y *W. anomalus* (Bautista-Rosales *et al*., 2013, 2014; Giobbe *et al*., 2007; Liu *et al*., 2019; Lutz *et al*., 2013; Ortu *et al*., 2005; Vero *et al*., 2013; Wang *et al*., 2020; Yang *et al*., 2021; Zhou *et al*., 2018).

- **Inducción de resistencia en el huésped.** Este mecanismo se ha convertido en un enfoque prometedor para manejar el daño en postcosecha de fruta en los últimos años (Sun *et al*., 2018), ya que proporciona resistencia sistémica a largo plazo a un amplio espectro de patógenos (Lai *et al*., 2018). Como elicitor biótico, las células de levadura podrían desencadenar la resistencia en los frutos a través de respuestas bioquímicas de defensa, mediante la acumulación de enzimas, como se ha observado en las levaduras *Hanseniaspora uvarum* la cual induce la actividad de las enzimas superóxido dismutasa (SOD), catalasa (CAT), peroxidasa (POD), polifenol oxidasa (PPO), fenilalanina amonio liasa (PAL) y ascorbato peroxidasa (APX) en frutillas (Cai *et al*., 2015); en *M. guilliermondii* que aumenta la actividad enzimática de PAL, SOD y CAT en manzanas (Zhang *et al*., 2020); *P. membranaefaciens* que estimula la actividad de las enzimas POD, CAT, PAL y PPO en duraznos (Huang *et al*., 2021); *Rhodotorula mucilaginosa* que induce la actividad de las enzimas POD y PPO en manzanas (Li *et al*., 2011) y *Y. lipolytica* que aumenta la actividad de las enzimas PPO, POD y PAL en manzanas (Zhang *et al*., 2017). Además, las levaduras pueden desencadenar la resistencia a través de respuestas moleculares de defensa, activando genes que codifican para proteínas relacionadas con la patogénesis (PR), como lo observado en las levaduras *Candida oleophila* la

cual induce la expresión del gen PR-8 (Quitinasa tipo III) en manzana (Liu *et al*., 2013); *M. guilliermondii* que incrementa los niveles de expresión del gen PR-2 (β-1,3- Glucanasa), PR-9 (Peroxidasa), los factores de transcripción (TFs) relacionados con la defensa WRKY9 y WRKY31 en peras (Yan *et al*., 2018) y la expresión de gen PR-1 y PR-5 (Proteína tipo Taumatina) en manzana (Huang *et al*., 2021); *P. membranaefaciens* incrementa los niveles de expresión de los genes PR-1, PR-4 (Quitinasa) y los TFs ERF115, MYB4, MYC4, WRKY33, WRKY18, WRKY53 en durazno (Zhang *et al*., 2020) y *Y. lipolytica* la cual incrementa los niveles de expresión del gen PR-4, PR-5 (Proteína tipo Taumatina a y b), PR-9 y los TFs Ácido aminociclopropano-1-carboxílico oxidasa (ACO), Isocorismato sintasa (SID) y el MYC3 en manzana (H. Zhang *et al*., 2017).

HIPÓTESIS

En las manzanas existen levaduras endofíticas que controlan *P. vagabunda* agente causal de la pudrición denominada “ojo de buey" y estas levaduras poseen varios mecanismos de acción por el cual antagonizan al patógeno.

OBJETIVO GENERAL

El objetivo general de esta investigación fue aislar y seleccionar levaduras endófitas nativas para el biocontrol de *P. vagabunda* en manzana, además de determinar él o los mecanismos de acción involucrados en dicho control.

OBJETIVOS ESPECIFICOS

Los objetivos específicos fueron: (1) Seleccionar levaduras endófitas nativas para el biocontrol de *P. vagabunda* en manzana, (2) Identificar las especies de levaduras con actividad antagonista frente a *P. vagabunda*, (3) Evaluar la actividad de biocontrol *in vivo* de levaduras frente a *P. vagabunda* en manzanas 'Cripps Pink' (4) Determinar él o los mecanismos de acción utilizados por estas levaduras en el biocontrol de *P. vagabunda*.

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CAPITULO II

Title: Endophytic yeasts for the biocontrol of *Phlyctema vagabunda* in apples

(Horticulturae 2022, 8, 535. DOI:10.3390/horticulturae8060535)

Abstract: Bull's-eye rot, produced by *Phlyctema vagabunda*, is an important postharvest disease in apples. Current measures to control infection include synthetic fungicides, in addition to the application of copper hydroxide and potassium phosphite. However, growing public concern regarding fungicide residues in food has generated interest in developing non-chemical alternative control methods; biological control is one of the most promising alternatives. In this research, native endophytic yeasts were isolated and evaluated for the biocontrol of *P. vagabunda* in apples. The mechanisms of action involved were also determined. Our research found 2 isolates, *Vishniacozyma victoriae* EPL4.5 and EPL29.5, which exhibited biocontrol activity against *P. vagabunda* at 20 °C in apples, the incidence of bull's-eye rot was reduced by 39% and 61%, respectively, and the severity of the disease was decreased by 67% and 70%, respectively, when apples were inoculated with these yeasts 24 h before applying the pathogen. The main mechanisms that could be involved in the observed biocontrol activity are the ability to form biofilms and the production of volatile organic compounds.

Keywords: fruit; *Malus domestica*; mode of action; preharvest

1. Introduction

Phlyctema vagabunda (Guthrie) Verkley (syn. *Neofabraea vagabunda* (E.J. Guthrie) Verkley), the causal agent of bull's-eye rot, has become an important and frequent postharvest disease in apples (Soto-Alvear *et al*., 2013). It has caused great economic losses in Europe (Cameldi *et al*., 2017), the Pacific Northwest of the United States (Henriquez *et al*., 2004), and Chile (Soto-Alvear *et al*., 2013), and is considered a quarantine disease in some fruit destinations, such as China (Wood & Fisher, 2017). In Chile, *P. vagabunda* was first reported in 2005 (Henriquez, 2005). Late-harvest cultivars like 'Cripps Pink' are the most affected varieties, with disease incidences reaching 60%, depending on the season and locality (Soto-Alvear *et al*., 2013). In organic production, incidences in these cultivars can be as high as 80–90%. Fruit infection occurs in the orchard (Cameldi *et al*., 2017) throughout the season (Henriquez *et al*., 2004) and is favored by abundant rainfall (Den Breeyen *et al*., 2020). Infection can become established between petal fall and harvest, increasing susceptibility gradually during fruit development (Pešicová *et al*., 2017). However, symptoms appear only a few months after harvest (usually 3–4 months in cold storage), when numerous lesions can develop on a single fruit (Neri *et al*., 2009). This is, therefore, a major limitation for the late harvest variety 'Cripps Pink' when apples are held under prolonged cold storage (Lolas *et al*., 2021).

Current management practices to control *P. vagabunda* in Chile include pre- and postharvest treatments with fungicides (Lolas *et al*., 2016). However, increasing concerns regarding chemical residues in food (Spadaro & Droby, 2016) have generated interest in developing non-chemical control methods, with biological

control being one of the most promising and explored alternatives (Wisniewski *et al*., 2007).

The use of antagonistic microorganisms has been recognized as one of the most promising alternatives to fungicides (Spadaro & Droby, 2016). Among these microorganisms, antagonistic yeasts have proven to effectively control numerous postharvest diseases in apples (Abdelhai *et al*., 2019; He *et al*., 2020; Li *et al*., 2011; Manso & Nunes, 2011; Mari *et al*., 2012; Navarta *et al*., 2014; Ruiz-Moyano *et al*., 2016; Türkel *et al*., 2014; D. Zhang *et al*., 2010, 2011; Zhang *et al*., 2009). Nonetheless, no studies have yet been carried out on *P. vagabunda*. Yeasts may be a viable control alternative for this pathogen because they have the ability to colonize the surface of fruits for long periods of time under dry conditions, produce extracellular polysaccharides that enhance their ability to survive, and rapidly use available nutrients. Yeasts are also minimally affected by pesticides (Wilson & Wisniewski, 1989), do not produce allergenic spores or mycotoxins (Ocampo-Suarez *et al*., 2017; Parafati *et al*., 2015), are genetically stable (Ruiz-Moyano *et al*., 2016), and have simple nutritional requirements (Parafati *et al*., 2015; Ruiz-Moyano *et al*., 2016). In addition, endophytic yeasts grow in fruit tissues under the same growth conditions as the pathogen *P. vagabunda* and, therefore, are already colonizing these tissues, which gives them an advantage over the pathogen (Madbouly *et al*., 2020).

An effective biocontrol agent requires multiple modes of action to antagonize a pathogen (Chanchaichaovivat *et al*., 2008); a combination of different mechanisms thus provides yeast with its antagonistic capacity (Bautista-Rosales *et al*., 2013) and

reduces the risk of pathogen resistance (Nally *et al*., 2015). The reported modes of action in yeasts include the ability to compete for nutrients and space, oxidative stress tolerance, parasitism, secretion of hydrolytic enzymes, and the ability to produce siderophores, volatile organic compounds, and biofilms, in addition to the induction of resistance (Chan & Tian, 2006; Iñiguez-Moreno *et al*., 2020; Liu *et al*., 2019; Madbouly *et al*., 2020; Tian *et al*., 2018; Yang *et al*., 2021; Zhang *et al*., 2019; Zhou *et al*., 2018).

In this research, we selected a native endophytic yeast for the biocontrol of *P. vagabunda* in apples. The mechanisms of action of the yeast strains involved in the biocontrol activity against the pathogen were also determined. This research could generate beneficial knowledge regarding the application of yeasts for food production and security.

2. Materials and Methods

2.1. Endophyte Isolation and Preparation of Inocula

Endophytic yeasts from ‘Cripps Pink’ apples from organic orchards in the Ñuble and Bío Bío Regions of central Chile were isolated according to the methodology described by Glushakova and Kachalkin (2017), with some modifications. The fruits were treated according to the following scheme: 70% ethanol for 30 min; 2% sodium hypochlorite for 30 min; 70% ethanol for 30 s, followed by sterile water for 10 min. The exocarp was then removed and macerated in 5 mL of saline solution (0.9% NaCl); 100 µL of the suspension obtained was spread on Yeast Peptone Dextrose Agar (YPD) containing 0.05 g L^{-1} of streptomycin (Sigma-Aldrich, St. Louis, MO,

USA) and chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA). Petri dishes were then incubated at 4 °C to observe the development of the colonies (between 30 and 45 days).

Inocula of the antagonists for all of the experiments were prepared in a flask containing 20 mL of yeast dextrose broth with a loop of yeast inoculum. The liquid culture was incubated on a rotary shaker (150 rpm) for 72 h at 25 °C. Antagonist cells were then collected by centrifugation at 1914× *g* for 10 min, washed, and re-suspended in sterile distilled water. The concentration of the suspensions was adjusted to 1×10^9 cells mL^{-1} by means of a Neubauer's chamber.

2.2. Fruit

'Cripps Pink' apples with no visible wounds were harvested from organic orchards in the Maule Region of central Chile. Apples were superficially disinfected with 0.5% sodium hypochlorite for 5 min, rinsed 3 times with distilled water, and air-dried.

2.3. Pathogen Inoculum

P. vagabunda was obtained from apples affected by bull's-eye rot and identified by sequencing the β-tubulin gene (GenBank ID: OL450471) as described by Cao *et al*. (2013). Conidia suspensions were attained according to the methodology described by Cameldi *et al*. (2017). Briefly, a mycelial plug was transferred to Petri dishes with Tomato Agar and incubated in darkness at 5 °C; after 14 days of incubation, pathogen conidia suspensions were prepared by scraping and suspending conidia in sterile distilled water and adjusted to a concentration of 5×10^5 conidia mL^{-1}, using a Neubauer's chamber.

2.4. Selection and Identification of Yeasts as a Potential Biocontrol of *P. vagabunda*

Nine isolates of the most frequently isolated yeast were evaluated as biocontrol agents against *P. vagabunda*. Apples were wounded in the equatorial axis (3 mm diameter and 3 mm deep) using a sterile pipette tip and inoculated with 20 µL of a yeast suspension (1×10^9 cells mL^{-1}). After 24 h, 20 µL of a *P. vagabunda* suspension (5×10^5 conidia mL^{-1}) was inoculated (Neri *et al*., 2009). In the control treatment, the yeast cell suspension was substituted for sterile distilled water. After 20 days at 20 °C, rot incidence and severity were recorded according to Vero *et al*. (2002).

Yeast strains were identified by phylogenetic analyses of the D1/D2 domain of the 26S LSU of rRNA using the primers NL1 (5′-GCA TAT CAA TAA GCG GAGGAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAGACG G-3′) in MEGA version 11. DNA sequences were aligned, together with sequences of homologous regions of closely related species retrieved from the GenBank. Evolutionary distances were computed using the Jukes–Cantor method, and phylogenetic trees were obtained by neighbor-joining. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. The stability of clades was assessed with 1000 bootstrap replications.

Strain identification was confirmed by sequencing the 5.8S-ITS rDNA region using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) in Macrogen Inc. (Seoul, Korea). These

sequences were then compared with those published in the GenBank database with the BLAST program (Ruiz-Moyano *et al*., 2016).

2.5. Antagonistic Activity of Yeast on the Fruit

The isolates that inhibited the fungus during the selection (n = 2) were evaluated for biocontrol activity, apples were wounded as described above, and 20 µL of a yeast suspension (1×10^9 cells mL^{-1}) was inoculated into each wound. After 24 h, the wounds were inoculated with 20 µL of a *P. vagabunda* suspension (5×10^5 conidia mL^{-1}) (Neri *et al*., 2009). In the control treatment, the yeast cell suspension was substituted for sterile distilled water. After 20 days at 20 °C, rot incidence and severity were recorded according to Vero *et al*. (2002).

Three replicates were established for each treatment in a completely randomized design in which each replicate was made up of eight apples. The entire experiment was also repeated twice.

2.6. Biofilm Formation by Yeast

The crystal violet (CV) methodology (Růžička *et al*., 2007) was used to quantify the biofilm formation by yeast. Polystyrene tissue culture multi dishes (Nunclon) with 1800 µL of sterile apple juice were inoculated with a yeast suspension (1×10^9 cells mL^{-1}). The yeast suspension in the control treatment was substituted with distilled sterile water. After 2 days of incubation at 25 °C, the wells were emptied and washed with 2 mL of distilled water using a pipette. This step was carried out 3 times. The biofilm layer on the wall of the wells was fixed by air-drying and stained with 2 mL of 1% crystal violet for 20 min; the cells were then washed and dried again, after which

2 mL of ethanol was added. The absorbance of the eluate was determined at 620 nm with a spectrophotometer (Epoch™ Microplate Spectrophotometer, BioTek, Winooski, VT, USA). Biofilm formation was considered to be positive when absorbance was equal to or higher than that of the control, plus three times the standard deviation (Růžička *et al*., 2007). Four replicates were performed, and the experiment was repeated twice.

2.7. Production of Volatile Antifungal Compounds

The antifungal effect of the volatile organic compounds (VOCs) produced by the yeast strains was assayed by the double Petri dish assay according to Di Francesco *et al*. (2020). For this purpose, plates with YPD or an apple juice agar medium (AJA) were inoculated with 100 µL of a yeast suspension (1×10^8 cells mL^{-1}). Then, 48 h later, 100 µL of a *P. vagabunda* suspension (1×10^4 conidia mL^{-1}) was inoculated in plates with Potato Dextrose Agar (PDA). Subsequently, the plates with the pathogen were individually covered mouth-to-mouth with the plates containing the yeasts, sealed with parafilm, and incubated at 20 °C. The control corresponded to plates containing YPD or AJA without yeast. The inhibition of the colony-forming unit (CFU) and radial growth of the pathogen was calculated after 10 days of incubation using the equation:

$$\text{CFU inhibition } (\%) = \frac{d1-d2}{d1} \times 100 \qquad (1)$$

where d1 is the number of CFU in the control and d2 is the number of CFU in the treated.

$$\text{Micelial growth inhibition } (\%) = \frac{d1-d2}{d1} \times 100 \qquad (2)$$

where d1 is the radial growth (mm of the colony diameter) in the control and d2 is the radial growth (mm of the colony diameter) in the treated.

Three replicates were used for each treatment, and the experiment was repeated twice.

2.8. Chemical Characterization of Volatile Organic Compounds

The analysis of the composition of VOCs produced by yeast was carried out as reported by Zhou *et al*. (2018). Volatile compounds were collected from yeast samples using Headspace Solid Phase Micro Extraction (HS-SPME), which was identified by gas chromatography–mass spectroscopy (GC–MS; QP2010 Ultra, Shimadzu, Kyoto, Japan). HS-SPME was performed with a 2 cm fiber coated with 50/30 µm DVB/CAR/PDMS. Briefly, we cultured the yeast in a 50 mL Erlenmeyer flask sealed with parafilm and sampled the volatile yeast by inserting the SPME fiber into the head-space of the culture in an Erlenmeyer flask for 5 min at 30 °C. The fiber was injected into a gas chromatograph (GC–MS; QP2010 Ultra, Shimadzu, Kyoto, Japan) containing a 30 m × 0.25 mm fused silica Rxi-5ms column. The chromatographic conditions used were inlet 280 °C; column 40 °C for 2 min followed by ramping at 5 °C min^{-1} to 280 °C. Mass spectral analyses were carried out with gas chromatography–mass spectroscopy (GC–MS; QP2010 Ultra, Shimadzu, Kyoto, Japan). The scan mass range extended from *m/z* 35 to 500. Mass spectra of VOCs were compared with those obtained from the NIST05 library, and comparison

qualities higher than 90% were considered (Standard Reference Data, NIST, Gaithersburg, MD, USA).

2.9. Statistical Analysis

Statistical analyses of incidence and severity of bull's-eye rot were subjected to one-way analysis of variance (ANOVA), and comparison of means was performed by Tukey's with a probability level of 5%. Mycelial growth inhibition (%) was subjected to Student's t-test mean comparison method with a probability level of 5%. All the data were analyzed by statistical software InfoStat (InfoStat® 2011).

3. Results

A total of 29 endophytic yeasts were obtained from 'Cripps Pink' apples, and then 9 isolates of the most frequent yeasts, according to their morphological and phenotypical characteristics, were evaluated against bull's-eye rot in apples at 20 °C in a preliminary assay. Two native yeasts had biocontrol activity against *P. vagabunda* and were identified as *Vishniacozyma victoriae* (EPL4.5 GenBank ID: OL453201, EPL29.5 GenBank ID: OL453202) based on the sequencing of the 5.8S-ITS rDNA region and D1/D2 domain of the 26S LSU of rRNA (Figure 1).

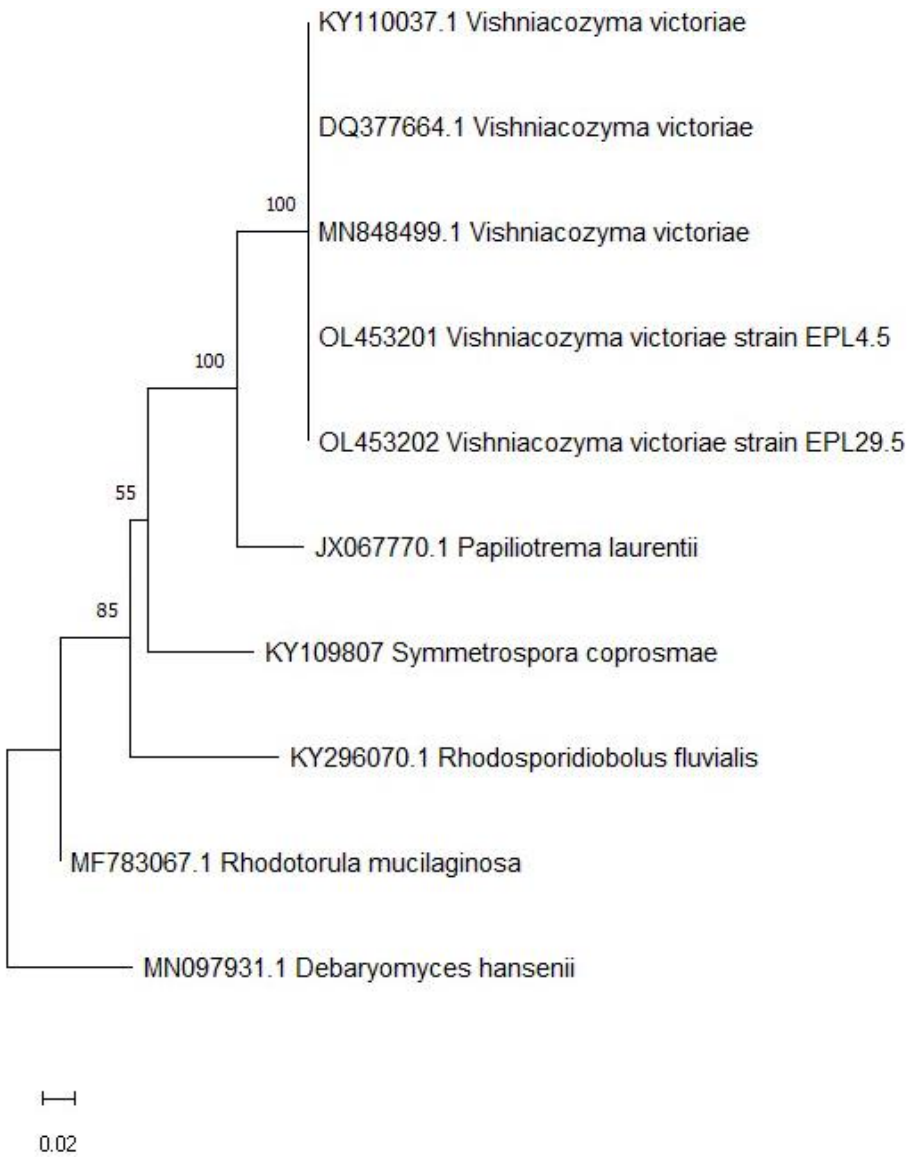


Figure 1. Phylogenetic analysis of yeast *Vishniacozyma victoriae* strains EPL4.5 and *Vishniacozyma victoriae* EPL29.5 with large subunit ribosomal gene nucleotide sequences. This analysis was inferred using the neighbor-joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The scale bar (0.02) indicates 0.2% divergence. This analysis involved 26 nucleotide sequences.

3.1. Antagonistic Activity of Yeast on Fruit

Yeast strains of *V. victoriae* (EPL4.5 and EPL29.5) inhibited bull's-eye rot on apples at 20 °C after apple wounds were inoculated with a yeast suspension (1 × 10^9 cells mL^{-1}), 24 h before inoculation with a *P. vagabunda* suspension (5 × 10^5 conidia mL^{-1}) (Figure 2). Yeast strain EPL4.5 reduced the incidence of bull's-eye disease by 58.3% and the severity by 67.4%, whereas yeast strain EPL29.5 decreased the disease incidence by 37.5% and the severity by 70.3% compared to the control.

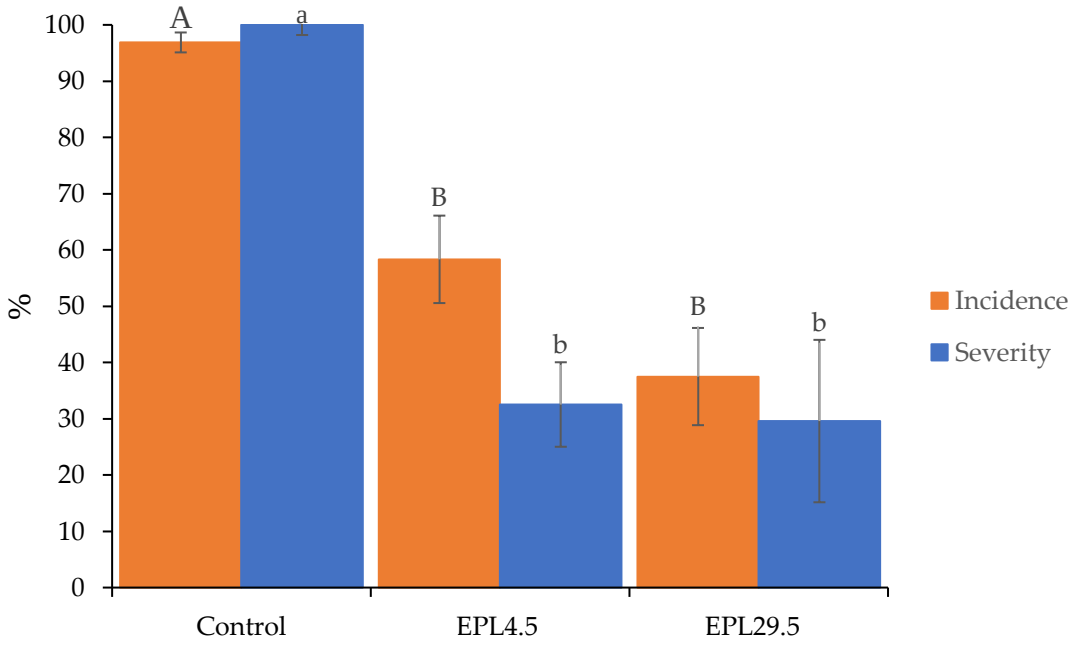


Figure 2. Incidence and severity of bull's-eye rot in 'Cripps Pink' apples treated with endophytic yeasts. Fruits were wounded and treated with a yeast suspension (1 × 10^9 cells mL^{-1}). After 24 h, a spore suspension of pathogen fungus (5 × 10^5 spores mL^{-1}) was applied, and the apples were then stored at 20 °C for 20 days. Mean values of incidence or severity linked by the same letter (upper or lower case, respectively) are not significantly different according to the Tukey's test (Incidence dF = 2; F= 10.16; p = 0.0119; Severity dF = 2; F = 32.25; p = 0.0006).

3.2. Biofilm Formation

The ability to produce biofilm was evaluated in tissue culture multi dishes (Table 1). One of the two evaluated yeast strains (EPL29.5) was able to produce biofilm, and the optical density value (A620) was found to be higher than the cut-off criterion (0.04).

Table 1. Biofilm formation in polystyrene tissue culture multi dishes by *Vishniacozyma victoriae* yeast strains EPL4.5 and EPL29.5 at 20 °C. Biofilm formation was considered to be positive when absorbance was equal to or higher than that of the control, plus three times the standard deviation.

Yeast Strain	Biofilm Formation	
	Absorbance (A_{620})	
EPL4.5	0.023 ± 0.003	-
EPL29.5	0.061 ± 0.006	+

+ Presence of biofilm. - Absence of biofilm. Cut-off value of biofilm formation = 0.04. Data on biofilm formation is expressed as mean ± standard error.

3.3. Production of Volatile Antifungal Compounds

In the double Petri dish assay system (Figure 3), the VOCs produced by yeast strains EPL4.5 and EPL29.5 on the YPD medium inhibited pathogen colony-forming unit (CFU) by 54.8% and 50.6%, respectively, and mycelial growth was reduced around 69.1% and 66.1%, compared to the control. When the yeast isolates were grown on the AJA medium, strain EPL29.5 reduced CFU by 19.4% and inhibited mycelial growth by a maximum of 43.6%. Strain EPL4.5 inhibited CFU by 10.4% and mycelial growth by 42% (Table 2). These results indicate that the production of volatile organic compounds with biological activity was influenced by the culture medium.

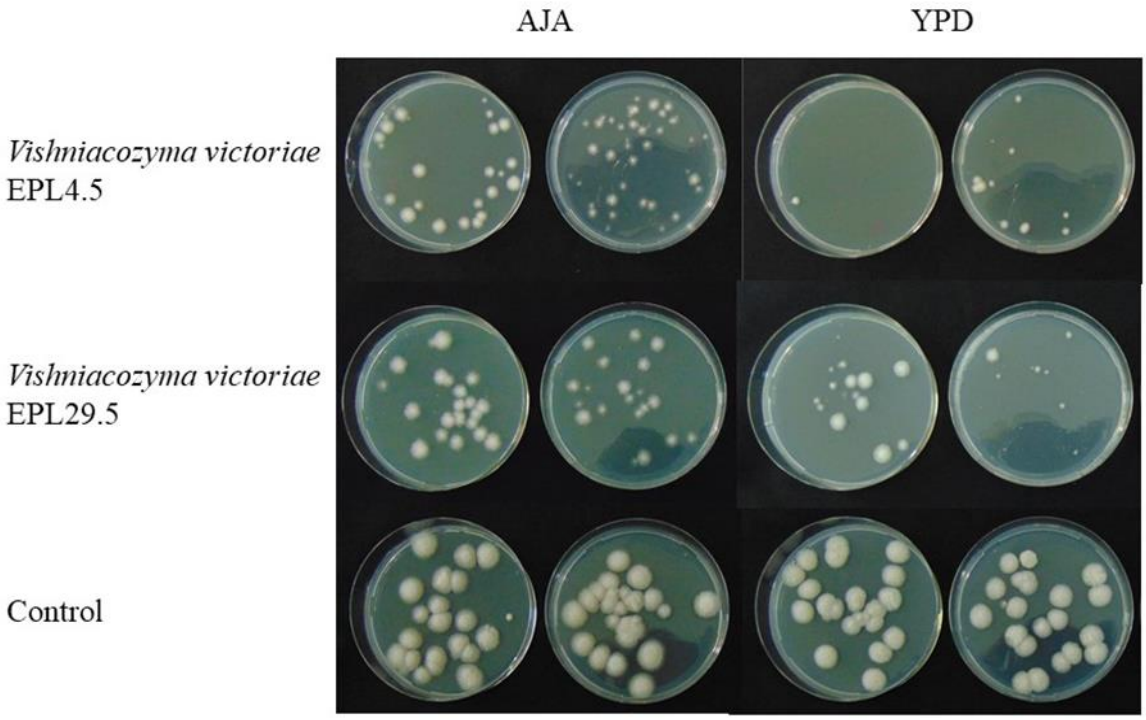


Figure 3. Inhibition of *Phlyctema vagabunda* growth by *Vishniacozyma victoriae* strains EPL4.5 and EPL29.5 antifungal volatile compounds activity on double Petri dish. Pathogen colony-forming unit and mycelial growth were inhibited in the presence of the yeast compared to the control.

Table 2. Effect of VOCs produced by *Vishniacozyma victoriae* yeast strains EPL4.5 and EPL29.5 in Yeast Peptone Dextrose Agar (YPD) and Apple juice agar (AJA) mediums on colony-forming unit and mycelial growth of *Phlyctema vagabunda* at 20 °C.

Yeast Strain	**Colony Forming Unit Inhibition (%)**		**Mycelial Growth Inhibition (%)**	
	YPD	**AJA**	**YPD**	**AJA**
EPL4.5	54.84 ± 27.4	10.38 ± 21.9	69.14 ± 3.6 *	42.1 ± 7.4
EPL29.5	50.63 ± 7.6	19.44 ± 7.3	66.10 ± 12.1	43.62 ± 2.4

Data expressed as mean ± standard error. Asterisks denote a significant difference among treatments for the same yeast strain (EPL4.5 dF = 3, p = 0.046; EPL29.5 dF = 3, p = 0.0997), according to Student's t-test.

3.4. Chemical Characterization of Volatile Organic Compounds

The VOCs produced by yeast strains EPL4.5 and EPL29.5 on the mediums AJA and YPD were analyzed by GC-MS (Figure 4). The VOCs present in just AJA or YPD medium without any yeast were not considered to be produced by *V. victoriae* EPL4.5 and EPL29.5. A total of 10 compounds were detected, including 5 alcohols, 2 ketones, and 3 hydrocar-bons.

On the YPD medium, the most abundant compound was 1-Butanol; the abundance relative area (RA) of this compound was 46.34% and 38.7% for strains EPL4.5 and EPL29.5, respectively. The rest of the components produced by the strain EPL4.5 on YPD medium mainly corresponded to 1-Butanol, 3-methyl and Disulfide, dimethyl representing an RA of 8.18% and 2.47%. The EPL29.5 strain on YPD medium produced mainly corresponded to 1-Butanol, 3-methyl; 1-Butanol, 2-methyl-, (S)- and 1-Propanol, 2-methyl with an RA between 17.63% and 1.97% (Table 3).

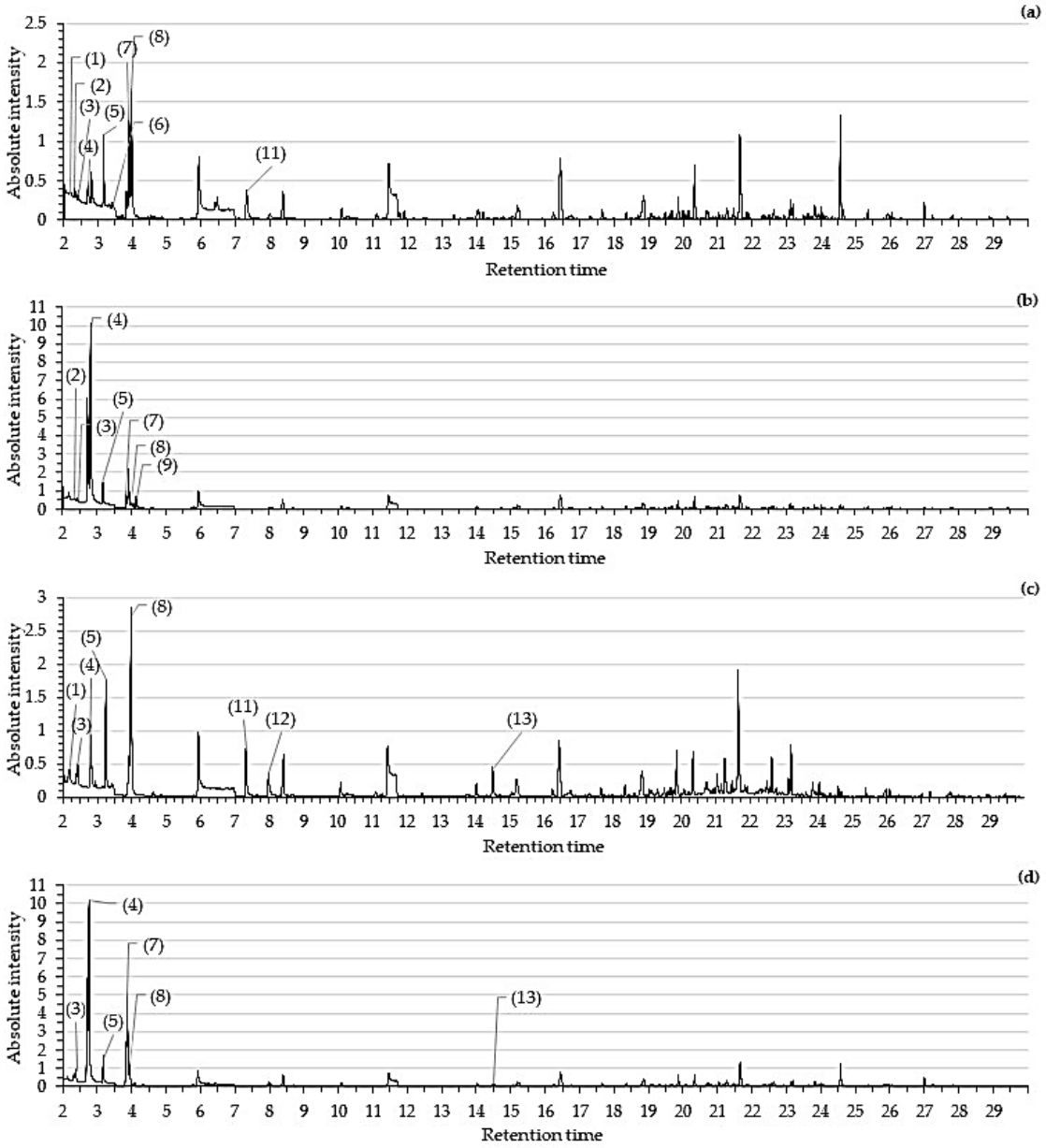


Figure 4. Gas Chromatography–Mass Spectrometry traces (total ion chromatograms) of VOCs collected in SPME. (a) *Vishniacozyma victoriae* EPL.4.5 on AJA medium, (b) *Vishniacozyma victoriae* EPL.4.5 on YPD medium, (c*)* *Vishniacozyma victoriae* EPL.29.5 on AJA medium, (d) *Vishniacozyma victoriae* EPL.29.5 on YPD medium. Peak top numbers refer to compounds listed in Table 3.

Table 3. Chemical characterization and relative abundance (%) of volatile fraction of *Vishniacozyma victoriae* yeast strains EPL4.5 and EPL29.5 in YPD and AJA media.

Peak Number	RT (min)	Possible Compound	Molecular Formula	*m/z*	Relative Abundance (%)				Reference
					EPL4.5		EPL29.5		
					AJA	YPD	AJA	YPD	
1	2.14	Hexane #	C_6H_{14}	41	0.49	N.D.	2.15	N.D.	
2	2.32	Isobutyl chloride	C_4H_9Cl	43	0.65	1.83	N.D.	N.D.	
3	2.41	1-Propanol, 2-methyl	$C_4H_{10}O$	43	0.46	0.53	2.19	1.97	(Di Francesco *et al*., 2015; Jaibangyang *et al*., 2020)
4	2.77	1-Butanol	$C_4H_{10}O$	56	3.98	46.34	5.25	38.7	
5	3.18	Silanediol, dimethy l#	$C_2H_8O_2Si$	77	3.97	2.35	7.82	2.69	
6	3.43	2,5-Dimethylfuran	C_6H_8O	45	1.08	N.D.	N.D.	N.D.	
7	3.87	1-Butanol, 3-methyl	$C_5H_{12}O$	56	1.94	8.18	N.D.	17.63	(Iñiguez-Moreno *et al*., 2020; Yang *et al*., 2021; Di Francesco *et al*., 2015)
8	3.98	1-Butanol, 2-methyl-, (S)-	$C_5H_{12}O$	41	19.06	1.33	13.27	2.97	(Iñiguez-Moreno *et al*., 2020; Di Francesco *et al*., 2015; Jaibangyang *et al*., 2020)
9	4.13	Disulfide, dimethyl	$C_2H_6S_2$	94	N.D.	2.47	N.D.	N.D.	(Farbo *et al*., 2018)
10	5.95	Cyclotrisiloxane, hexamethyl- #	$C_6H_{18}O_3Si_3$	207	5.55	3.25	N.D.	N.D.	
11	7.32	1-Hexanol	$C_6H_{14}O$	56	3.39	N.D.	3.27	N.D.	(Qin *et al*., 2017; Yang *et al*., 2021)
12	7.99	2-Heptanone	$C_7H_{14}O$	43	N.D.	N.D.	1.65	N.D.	(Farbo *et al*., 2018)
13	14.51	2-Nonanone	$C_9H_{18}O$	43	N.D.	N.D.	2.04	0.49	(Yang et al., 2021)
14	16.45	Cyclopentasiloxane, decamethyl #	$C_{10}H_{30}O_5Si_5$	73	N.D.	N.D.	3.99	1.34	
15	20.35	Cyclohexasiloxane, dodecamethyl #	$C_{12}H_{36}O_6Si_6$	88	N.D.	N.D.	2.94	N.D.	

Not detected is referred to as N.D.; # Putative compounds from the column and fiber.

The most abundant compound produced by both yeast strains on the AJA medium was 1-Butanol, 2-methyl-, (S)-; the abundance relative area (RA) of this compound was 19.6% and 13.27% for strains EPL4.5 and EPL29.5, respectively. The rest of the components produced by the strain EPL4.5 on AJA medium mainly corresponded to 1-Butanol; 1-Hexanol and 1-Butanol, 3-methyl with a RA between 3.98% and 1.94%. The EPL29.5 strain on AJA medium produced were 1-Butanol; 1-Hexanol; 1-Propanol, 2-methyl; 2-Nonanone, and 2-Heptanone with a RA between 5.25% and 1.65% (Table 3).

4. Discussion

In this study, we screened endophytic yeasts isolated from organic 'Cripps Pink' apples for the control of *P. vagabunda*. The most effective yeast that significantly decreased the incidence of bull's-eye rot in apples was the EPL29.5 strain which was identified as *Vishniacozyma victoriae*. Previously, the yeast *V. victoriae* had been reported to be an effective biocontrol agent for *Botrytis cinerea* and *Penicillium expansum* on pears (Lutz *et al*., 2020) and *Penicillium crustosum* and *Mucor piriformis* on cherries (Villalba *et al*., 2016). To the best of our knowledge, no study on *P. vagabunda* has yet been recorded, making this the first report of a yeast controlling bull's-eye rot in apples.

P. vagabunda fruit infection occurs in the orchard (Cameldi *et al*., 2017), so the presence of the antagonist prior to or during the initial phases of the disease cycle is of crucial importance, considering the difficulties in controlling previously established infections (Villalba *et al*., 2016). We tested that the application of *V.*

victoriae EPL29.5 24 h before the pathogen reduces the incidence of *P. vagabunda* by 58.3%. The pre-harvest application of antagonistic yeasts could protect fruits against pathogen infection in the orchard, but for the biological control to be successful, the yeast needs to possess effective mechanisms to cope with the abiotic stresses to which they are exposed (Sui *et al*., 2015). In this sense, endophytic yeasts could be a very promising new source of biological control agents because they can grow and develop inside plants, thus avoiding the negative influences of environmental factors such as solar radiation and desiccation (Glushakova & Kachalkin, 2017).

It has been suggested that biological control agents utilize different strategies depending on the pathogen, host, and environment (Di Francesco *et al*., 2017). Understanding these diverse mechanisms is essential to determine how a combination of different yeasts affects pathogen control in order to take advantage of their multiple means of action (Liu *et al*., 2017). This is also an important topic in the development process of bio fungicide formulations because it permits an increase in the performance of biocontrol agents (Pesce *et al*., 2018).

In this study, we evaluated different mechanisms of action of *V. victoriae* (EPL4.5 and EPL29.5), including antibiosis, pathogen hyphal adhesion, and siderophore production (Figure S1–S3 Supplementary Material), biofilm-forming capacity and production of volatile organic compounds.

Only *V. victoriae* EPL29.5 formed biofilms in sterile apple juice at 20 °C. This mechanism has been demonstrated by Lutz *et al*. (2013) at 0 ± 1 °C in pear juice with glucose peptone yeasts extract as a culture medium. Biofilms are a network of

cells and extracellular polysaccharides that form a gel that holds microorganisms together (Klein & Kupper, 2018), creating a mechanical barrier between the wound and the pathogen surface (Di Francesco *et al*., 2016), thereby preventing the onset of the infection process.

Several studies have shown that the production of VOCs by yeasts has a significant role in their antagonistic activities (Arrarte *et al*., 2017; Konsue *et al*., 2020; Medina-Córdova *et al*., 2016). In this study, VOCs emitted by the *V. victoriae* strains EPL4.5 and EPL29.5 reduced colony-forming unit (CFU) and mycelial growth of *P. vagabunda*. Strain EPL4.5 reduced CFU by 54.8% and mycelial growth by 69.1% when grown in a YPD medium, while strain EPL29.5 reduced CFU by 50.63% and mycelial growth by 66.1%.

The analyses of the VOC profiles produced by the evaluated yeast strains were conducted with HS-SMPE coupled with GC-MS, which indicated that the VOCs were mainly alcohols, with the main components being 1-Butanol, 2-methyl-, (S)- in the AJA medium and 1-Butanol in the YPD medium (Table 3). These compounds have previously been observed in the yeasts *Aureobasidium pullulans* and *Meyerozyma caribbica* when grown on a Nutrient Broth, Yeast Extract, Dextrose Agar, and PDA (Di Francesco *et al*., 2015; Iñiguez-Moreno *et al*., 2020). Arrate *et al*. (2017) found that the main VOCs produced by *C. sake* in an AJA medium were 3-Methylbutyl hexanoate, 3-Methylbutyl pentanoate, and 2-Methylpropyl hexanoate; however, none of the compounds found in this research were detected in the current study. This difference may be due to the fact that the production of such volatiles is strongly

influenced by the ability of yeast to assimilate and ferment carbohydrates (Fialho *et al*., 2010).

The production of VOCs characterized as effective biofumigants for disease control in plants (Huang *et al*., 2011) may represent an important biological control mechanism for a wide range of postharvest pathogens. In this study, the main components produced by the yeast strains in the media evaluated were alcohols, which damage the plasma membrane and rapidly denature proteins, producing a subsequent interference with metabolism and cell lysis (Iñiguez-Moreno *et al*., 2020) and could explain the observed decreases in colony-forming unit and mycelial growth of *P vagabunda*.

Our results suggest that strains EPL4.5 and EPL29.5 of the endophytic yeast *V. victoriae* are potential biocontrol agents of *P. vagabunda* in apples and could be used in preharvest applications. Due to the fact that these yeasts are endophytes, they could colonize fruit tissues, including wounds and lenticels, through rapid cell proliferation, thus allowing them to compete with the pathogen. Further research is needed to demonstrate the biocontrol activity of these isolates against bull's eye rot in orchards.

Supplementary Material

Figure S1. No production of diffusible antifungal compounds of (a) *Vishniacozyma victoriae* EPL4.5 and (b) *Vishniacozyma victoriae* EPL29.5 on Potato Dextrose Agar (PDA) medium at 20°C. Lawns on plate *Phlyctema vagabunda*.

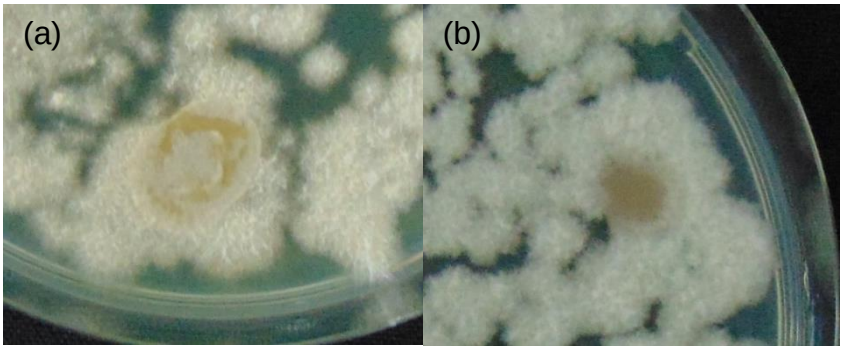


Figure S2. Lack of attachment of (a) *Vishniacozyma victoriae* EPL4.5 and (b) *Vishniacozyma victoriae* EPL29.5 to the hyphae of *Phlyctema vagabunda*, after 24 h incubation at 20 °C on Potato Dextrose Agar (PDA) medium.

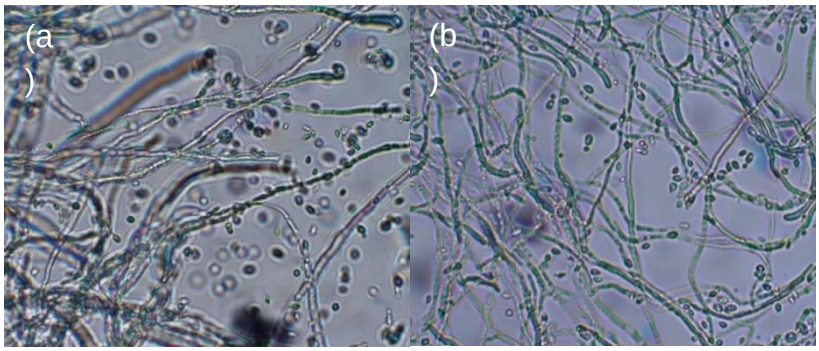
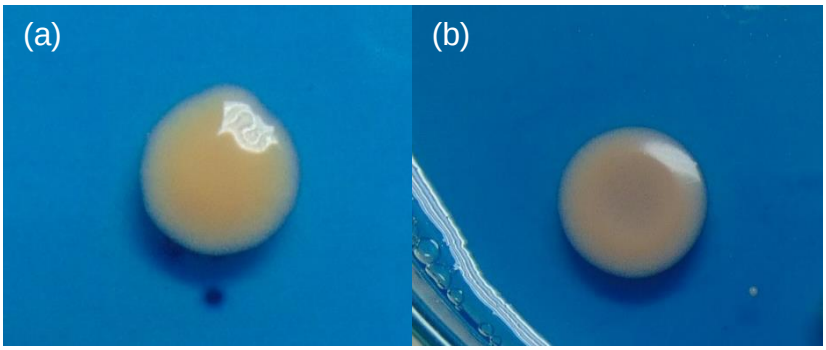


Figure S3. No detection of siderophore production by (a) *Vishniacozyma victoriae* EPL4.5 and (b) *Vishniacozyma victoriae* EPL29.5 after 15 days of incubation at 20°C on Chrome Azurol S (CAS) agar medium.



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CAPITULO II

Title: Effects of *Vishniacozyma victoriae* on defense response in ‘Cripps Pink’ apples

(Enviado, Biological Control)

Abstract: Apple is one of the most economically important fruit crops in the world, but it is susceptible to fungal diseases that cause significant losses. Due to increasing concern about chemical residues in food, alternative non-chemical control methods such as biological control have been explored, where yeast antagonists have been shown to be effective in controlling postharvest pathogens in apples. Yeasts use different strategies to control the pathogen, with resistance induction being the most promising, as it provides long-term systemic resistance to a broad spectrum of pathogens. In this study, we used qPCR analysis to explore the relative expression of several defense-related genes in ‘Cripps Pink’ apples treated with the yeast *Vishniacozyma victoriae* EPL29.5. We demonstrating that, the presence of yeast up-regulates the relative expression compared to control, of defense-related genes PAL, POD, CAT, PR8, PR3-CH4, TLP1b, PRPL, WKYR31, SID and MYC3, suggesting that yeast *V. victoriae* EPL29.5 is capable of inducing resistance in ‘Cripps Pink’ apples.

Keywords: Fruit, gene expression, host-defense, *Malus domestica*

1. Introduction

Apple is one of the economically most important fruit crops in the world, with an estimated production 81.8 million tons in 2021/2022 season (USDA, 2021). In Chile, the production of this fruit in the 2021/2022 season reached 1.1 million tons, of which 643,000 tons were exported, taking the fourth place in importance cultivated area of fruit trees (ODEPA, 2022).

In process of storage, transportation and sale after harvesting, apples are susceptible to fungal diseases, which cause significant losses in apple production (Ferrada *et al*., 2021). In Chile a serious post-harvest diseases of apple fruits is bull's eye rot caused by *Phlyctema vagabunda* (Lolas *et al*., 2021).

Current management practices to control of this pathogen in Chile include pre- and postharvest treatments with fungicides (Lolas *et al*., 2016). However, due to growing concerns about chemical residues in the food (Spadaro and Droby, 2016), have generated interest in the development of alternative non-chemical control methods, with the biological control as one of the most promising and explored alternative (Wisniewski *et al*., 2007). The use of yeast antagonists has been proven to effectively control postharvest diseases in apples (Li *et al*., 2011; Mari *et al*., 2012; Vero *et al*., 2013; Zhu *et al*., 2022).

The yeast *Vishniacozyma victoriae* has previously been reported to be an effective biocontrol agent for *Botrytis cinerea* and *Penicillium expansum* on pears (Lutz *et al*., 2020) and *Penicillium crustosum* and *Mucor piriformis* on cherries (Villalba *et al*., 2016) and in previous research conducted by our laboratory we demonstrated this yeast is an effective biocontrol agent for *P. vagabunda* (Sepúlveda *et al*., 2022).

Typically, the mechanisms conferring yeast antagonists with the ability to inhibit postharvest pathogens include competition for nutrients and space (Tian *et al*., 2018), parasitism (Zhou *et al*., 2018), secretion of hydrolytic enzymes (Madbouly *et al*., 2020), the production of organic volatile compounds (Arrarte *et al*., 2017) and induction of host resistance (Zhang *et al*., 2019). Among these, the induction of host resistance to pathogens has become a promising approach to manage postharvest biocontrol disease over recent years (Sun *et al*., 2018), since it provides long-term systemic resistance to a broad spectrum of pathogens (Lai *et al*., 2018).

As a biotic elicitor, yeast cells could trigger resistance in fruits through biochemical defense responses by the accumulation of defense-related enzymes (Zhu *et al*., 2022) and through molecular defense responses, activating defense pathogenesis related (PR) proteins (Lai *et al*., 2018). Several reports have indicated that yeast can induce the expression of defense-related genes in apple fruit, some of the species include *Meyerozyma guilliermondii* up-regulated the relative expressions of genes PR-1, PR-3 (Chitinase), PR-4 (Chitinase), PR-5 (Thaumatin-like), PR-9 (Peroxidase), PPO (Polyphenol oxidase) and CAT (Catalase) (He *et al*., 2020; Huang *et al*., 2021) and *Sporidiobolus pararoseus* up-regulated the relative expressions of genes PR-3, PR-4, PR-5 and PR-9 (Zhao *et al*., 2018). To the best of our knowledge no study on *V. victoriae* has yet been recorded, making this the first report on the stimulation of defense-related genes expression by this yeast in ‘Cripps Pink’ apples. Therefore, in this study we explored the relative expression of several defense-related genes in ‘Cripps Pink’ apples treated with the yeast *V. victoriae*.

2. Materials and methods

2.1 Fruit and pretreatment

‘Cripps Pink’ apples with no visible wounds were harvested from organic orchards in the Maule Region of central Chile. Apples were superficially disinfected with 0.5% sodium hypochlorite for 5 min, rinsed three times with distilled water, and air-dried prior to the experiment.

2.2 Yeast

Yeast *Vishniacozyma victoriae* EPL29.5 was originally isolated from apple fruit. It was first cultured on Yeast Peptone Dextrose Agar, then inoculated to 20 mL of Yeast Dextrose Peptone Broth in 100 mL conical flask and incubated at 25°C for three days on a rotary shaker at 150 rpm. Yeast cells were then collected by centrifugation at 1914× *g* for 10 min, washed and re-suspended in sterile distilled water. The concentration of the suspensions was adjusted to $1x10^9$ cells mL^{-1} by means of a Neubauer's chamber.

2.3 Assay of expression of defense-related genes

Apples were wounded in the equatorial axis (3 mm diameter and 3 mm deep) using a sterile pipette tip and inoculated with 30µL of suspension of *V. victoriae* EPL29.5 or sterile distilled water as the control. After air drying, the apple fruits were stored in closed plastic trays at 20°C. In order to measure the relative expression of defense-related genes, 2 g of tissue surrounding the wound of each apple was collected at 0 (2h after treatment), 1, 2, 3, 4 and 5 days and immediately frozen in liquid nitrogen,

then stored at -80°C for further RNA extraction (Yan *et al*., 2018). Each treatment was repeated 3 times and each group treated samples comped of 12 apples.

2.4 RNA extraction and cDNA synthesis

Total RNA was extracted according to the procedure of Asif *et al*. (2006) and purified using an E.Z.N.A. Total RNA kit I (Omega Bio-Tek, Norcross, GA, USA) according to manufacturer´s instructions. The quantity and quality of the total RNA extracted was determined using a micro Spectrophotometer (Epoch™ Microplate Spectrophotometer, BioTek, Winooski, VT, USA). The extracted RNA was subsequently used for first-strand cDNA synthesis by using the High-capacity cDNA reserve transcription kit (Thermo fisher scientific, NY., USA) according to the description of protocol in the product manual and stored at -20°C before further used.

2.5 Real-Time PCR Quantification (qPCR)

For the expression analysis of defense-related genes, specific primers were used (Table 1). RT-qPCR was performed using an Agilent AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) and the data were analyzed using Agilent

AriaMX 1.5 Software (Agilent Technologies, Santa Clara, CA, USA). The PCR reaction was carried out using 1× of KAPA SYBR FAST Universal 2× qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA) according to manufacturer´s instructions on under the following conditions: an initial denaturation for 60s at 95°C, followed by 40 cycles of 10s at 95°C, 10s at 58°C (or 59°C, or 60°C), and 10s at

72°C. At the end of each RT-qPCR, melting curve analysis was performed over the range from 60 to 95°C to determine the specificity of the amplicons. Relative fold changes in gene expression were calculated after normalization with an endogen gene (β-Actin), as described by Pfaffl (2001).

Table 1. Specific primers used for quantitative Real-Time PCR (qPCR) to analyze the defense-related genes expression in 'Cripps Pink' apples treated with the yeast *Vishniacozyma victoriae* EPL29.5.

Primer name	Primer sequence	Annealing temperature (°C)	References
ACTIN	F: CCCAAAGGCTAATCGGGAGAAA R: ACCACTGGCGTAGAGGGAAAGA	60	(He *et al*., 2020)
CAT2	F: AGACACCTGTCATTGTGCGT R: CATGGATCACGTCCGGGAAT	60	(Abdelhai *et al*., 2019)
GLU	F: CCTTACTTCAGCTACAATGACAAC R: GTACTGAGCGTCCAGGAGAG	60	(Yan *et al*., 2018)
MYC3	F: CCATAAACTCCTCCCATACC R: GCCCACGACAAAGAAACT	56	(Zhang *et al*., 2017)
PR3CH4	F: TGTGGTACTGGATGAACAACG R: CAGGTGAGATTGTCACCAGG	56	(Hassani *et al*., 2016)
PR8	F: GCCACTGCAACCCCGCTAGT R: GCGGGCGCGAATCTGACTGA	60	(Liu *et al*., 2013)
PAL2	F: GGCATTTGGAGGAGAACTTG R: AGAACCTTGAGGGGTGAAGC	60	(He *et al*., 2020)
POD	F: AAGCCTATAGCCCCACCAGA R: CTTGAAGCTACGTGGGTCGT	60	(Abdelhai *et al*., 2019)
PRPL	F: TCCCACCATCGTCCAACTCAAAGA R: GCTACAACCCGTCAGAAGAAACC	58	(Zhang *et al*., 2017)
SID	F: CGCTCGTGGACCAACTAA R: TTCAATGCCCGATACAAAA	60	(Zhang *et al*., 2017)
TLP1a	F: GTCAACAAGGCTAACATCGTA R: ATGGTCTGGTCGCTTCTG	60	(Zhang *et al*., 2017)
TLP1b	F: ATGGTCTGGTCGCTTCTG R: ACAGCCGCAGCCGACAACTA	60	(Zhang *et al*., 2017)
WKYR31	F: ATGGACAAAGGAGGACGGCTAC R: CTGGGAGTGATGATGTTGGTGAT	60	(Yan *et al*., 2018)

2.6 Statistical analysis

Three biological replicates were performed for each treatment and each biological replicate contained three technical replicates. Data are represented as the means ± standard error (SE). The significant differences at each time point were detected by Studen's t-test ($p \leq 0.05$) using InfoStat software 2016e (FCA-UNC, Córdoba, Argentina).

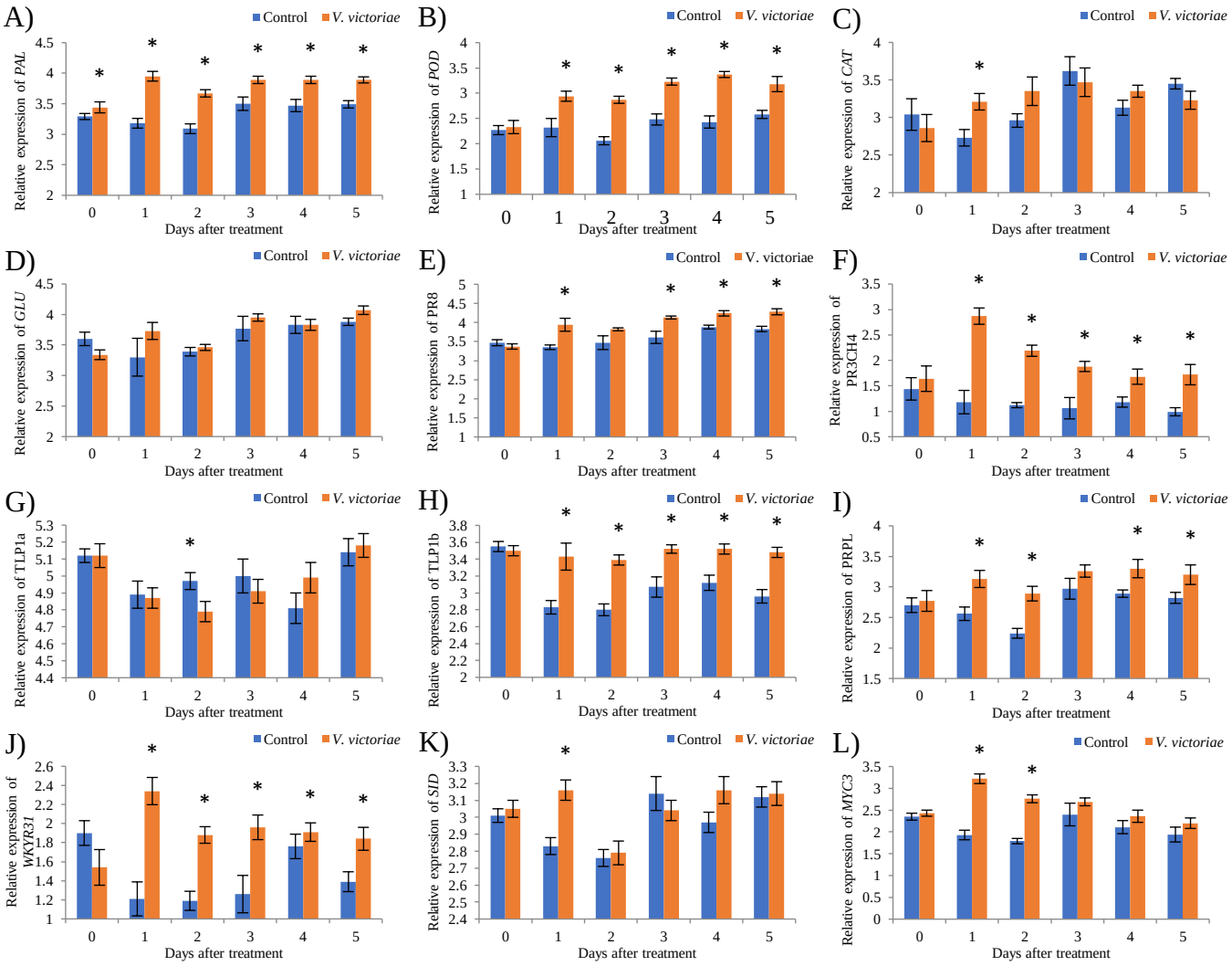
3. Results

V. victoriae applied in 'Cripps Pink' apples wounds, induced the relative expression of gene of defense-related enzymes Catalase (CAT), Peroxidase (POD) and Phenylalanine ammonia lyase (PAL); PR proteins Chitinase (PR3-CH4, PR8), Pathogen-related Protein like (PRPL), Thaumatin-like protein (TLP1b) and Transcription factors (TFs) MYC3, Isochorismate synthase (SID) and WKYR31 (Figure 1 and 2).

The relative level of PAL expression was significantly higher during the whole evaluation period, at 1 d the yeast-treated apples wounds reached the peak, being 24% higher than the control (Figure 1A).

As shown in Figure 1B, the relative expression of the POD gene in the wounds of apples treated with *V. victoriae* EPL29.5 was up-regulated from 1d, remaining significantly higher than the control throughout the evaluation period, with a peak at 2d of 39%. Meanwhile, CAT gene was up-regulated in the presence of yeast only 1d, with a relative expression level 11% higher compared to the control (Figure 1C).

Figure 1. Effects of *Vishniacozyma victoriae* EPL29.5 on defense-relates gene in apple fruit. PAL (A), POD (B), CAT (C), GLU (D), PR8 (E), PR3CH4 (F), TLP1a (G), TLP1b (H), PRPL (I), WKYR31 (J), SID (K), MYC3 (L). The fruit was incubated at 20°C. Each data represents the mean + EE of three biological independent replicates, including three technical repetitions. Asterisks denote a significant difference from control (t test, $p \leq 0.05$).



The GLU gene was not up-regulated in apples wounds treated with *V. victoriae* EPL29.5 (Figure 1D). On the other hand, the PR8 gene was up-regulated with regard to the control on 1d, 3d, 4d and 5d, with a peak on 1d of 17% (Figure 1E). Meanwhile, PR3-CH4 was up-regulated on 1d with a peak of 133% higher than the control, remaining up-regulated during the entire evaluation period (Figure 1F).

The TPLP1a gene was down-regulated 3% compared to the control at 2d in yeast-treated apples wounds (Figure 1G). On the other hand, The TPLP1b gene, was up-regulated compared to the control from 1d, remaining significantly higher throughout the evaluation period reaching a peak in 2d of 22% relative to the control (Figure 1H). The PRPL gene in the presence of yeast was up-regulated most of the days of evaluation, the peak was on day 2, which was 30% higher than the control (Figure 1I).

As shown in Figure 1J, the WKYR31 gene was up-regulated in yeast-treated apples wounds on 1d with a peak 65% higher compared to the control, then remaining up-regulated throughout the entire evaluation period (Figure 1L). Another gene that was up-regulated at 1d was SID with a relative expression level 14% compared to the control (Figure 1K). Meanwhile, MYC3 gene was up-regulated at 1d and 2d, with the peak on 1d, which was 70% higher than the control (Figure 1L).

4. Discussion

Results of the present study provide evidence that the yeast *V. victoriae* EPL29.5 inoculated in apple wounds was capable of eliciting to up-regulated the expression of defense-related genes PAL, POD, CAT, PR8, PR3-CH4, TLP1b, PRPL, WKYR31,

SID and MYC3 (Figure 1 and 2). Several investigations have shown that yeasts trigger the induction of defense-related genes like PR-1, PR-2 (β-1,3- Glucanase), PR-3 (Chitinase), PR-4 (Chitinase), PR-5 (Thaumatin-like), PR-9 (Peroxidase), PPO, APX (Ascorbate peroxidase) and CAT (Apaliya *et al*., 2017; He *et al*., 2020; Huang *et al*., 2021; Lai *et al*., 2018; Wang *et al*., 2019; Yan *et al*., 2018; Zhang *et al*., 2019; Zhao *et al*., 2018), indicating that induction of host resistance could be a viable strategy for postharvest pathogen control in fruit.

The PAL gene coding for the enzyme Phenylalanine ammonia lyase was significantly expressed compared to the control from 0d and remained up-regulated throughout the evaluation period. The results of this study agree with those obtained He *et al*. (2020) who demonstrated that *M. guilliermondii* increased the relative expression of PAL in apples from 0d, and remained up-regulated compared to the control during the 6 days of evaluation. Other yeast species that also increased the relative level of PAL gene expression, but in grapes, were *Hanseniaspora uvarum* that increased the expression level from 0d, remaining up-regulated during the three days of testing (Apaliya *et al*., 2017); while *Yarrowia lipolytica* increased expression level on days 1, 2, 3 and 6 of evaluation (Wang *et al*., 2019).

V. victoriae EPL29.5 up-regulated relative expression since 1d the POD gene coding for the enzyme Peroxidase. Meanwhile, Zhao *et al*. (2018) reported that inoculating apples with yeast *S. pararoseus* up-regulated POD gene expression, but on only two of the six days evaluated. Other yeast that has been reported to induce the expression of the POD is *M. guilliermondii*, which up-regulated gene expression in pears from day 0 and remained up-regulated throughout the evaluation period (Yan

et al., 2018). While, yeast *Wickerhamomyces anomalus* up-regulated the expression of POD only on four of the six days tested (Zhang *et al*., 2019).

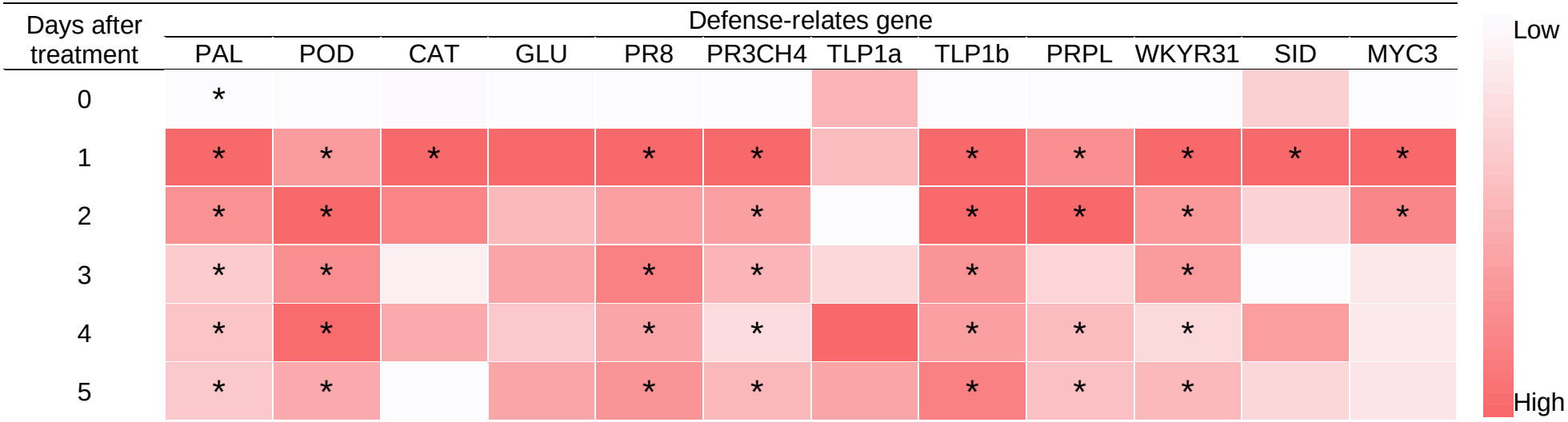
The CAT gene coding for the enzyme Catalase was up-regulated in the presence of *V. victoriae* EPL29.5 only 1d. Different results to the present study were reported by He *et al*. (2020) who indicated that *M guilliermondii* up-regulated CAT in apples from day 0 remaining up-regulated during the 6 days of evaluation. While Abdelhai *et al*. (2019) reported that *S. pararoseus* up -regulated CAT compared with the control among 3d and 6d. Other yeasts that have been reported to induce the expression of the CAT were *H. uvarum* which up-regulated in grapes the expression on all three days of testing (Apaliya *et al*., 2017), *Y. lipolytica* which up-regulated CAT in grapes some days of evaluation, with a peak 2.18-fold higher than the control at the third day (Wang *et al*., 2019), and *W. anomalus* which up-regulated CAT in pears from 3d and peaked at the 5d (Zhang *et al*., 2019).

Several studies have shown that the expression levels of PAL, POD and CAT genes are related to enzymatic activities and biocontrol against pathogens (He *et al*., 2020; Wang *et al*., 2019; Zhang *et al*., 2019), so *V. victoriae* EPL29.5 could be very important to reinforce fruit resistance to pathogen infection. In fact, PAL is a rate-limiting enzyme, that catalyzes the first step of phenylpropanoid metabolism in apples (He *et al*., 2020), and is involved in the biosynthesis of disease resistance-related substances like lignin and phytoalexin and defense hormone salicylic acid, which is associated with systemic acquired resistance in plants (Abdelhai *et al*., 2019; Lai *et al*., 2018). On the other hand, POD and CAT are antioxidants and play an important role in the defense response of plant cells against oxidative stress

caused by pathogen invasion (Lai *et al*., 2018), these enzymes can scavenge reactive oxygen species (ROS) in order to protect cells and tissues from free radical damage, such as O_2^- and H_2O_2 (Wang *et al*., 2019). In addition, POD participates in the last step of lignin biosynthesis, thus improving plant cell wall resistance to pathogen invasion, along with oxidizing phenolic compounds to quinones, which are toxic to pathogens (Lai *et al*., 2018).

The PR8 and PR3-CH4 genes coding for Chitinase type III and IV, respectively, were up-regulated in apples by *V. victoriae* EPL29.5 especially PR3-CH4, which at 1d was 133% higher relative to control. Zhao *et al*. (2018), reported that *S. pararoseus* induced CHI gene coding for the Chitinase type III in apples from day 0, staying up-regulated during the whole testing period. While, Zhang *et al*. (2019) indicated that *W. anomalus* up-regulated CHI gene in pears from 3d to 6 d and peaked at the 4d, which was 8.97 times higher than the control. In most fungal species the inner cell wall consists of a core of covalently attached branched β-(1,3) glucan with 3 to 4% interchain and chitin (Gow *et al*., 2017). Therefore, up-regulation of PR8 and PR3-CH4 in the presence of *V. victoriae* EPL29.5 observed in this study, could play the major role in the defense reaction against the fungal pathogen (Sun *et al*., 2018), since chitinase enzymes may be involved in the degradation of fungal walls (Nally *et al*., 2015).

Figure 2. Heat-map illustrating the intensity of up-regulation of defense-relates gene in apple fruit wounds treated with the yeast *Vishniacozyma victoriae* EPL29.5. Asterisks denote a significant difference from control (t test, p≤0.05).



Another gene that was up-regulated by *V. victoriae* EPL29.5 in apples from day 1d, and remained up-regulated the rest of the evaluation period compared to the control, was TPLP1b. On the other hand, the TPLP1a gene in the presence of yeast was down-regulated on day 3d compared to the control. While, Zhao *et al*. (2018) reported that *S. pararoseus* induced TLP1a in apples from day 2 to 6, with the maximum reached at 4 d, and Zhang *et al*. (2017) who indicated that the yeast Y*. lipolytica* in apples up-regulated TLP1a and TLP1b genes where the expression level of TLP1a was increased more than 1.58-fold, been significantly different compared to the control. This gene is known Thaumatin-like proteins, are involved in plant defense system against various biotic and abiotic stresses (Cao *et al*., 2016), which are believed to be capable of creating transmembrane pores. Several TLPs exhibit a special binding activity to β-1,3-glucans and may act in concert with PR-2 enzyme to disrupt fungal cell wall synthesis and/or prevent proper fungal wall assembly during hyphal extension, because also been reported to display β-1,3-glucanase activity (Cao *et al*., 2016; Liu *et al*., 2010). Then, that yeast activating this gene in apple could prevent pathogen infection, by membrane disruption of the spore or infective hyphae.

In this study we demonstrated that *V. victoriae* EPL29.5 in apples up-regulated of expression of transcription factors (TFs) related to defense WKYR31, SID and MYC3. In particular, the TF WKYR31 was up-regulated from day 1, and remained up-regulated for the remainder of the evaluation period. TFs participate in the control of genes expression, by acting as positive regulators (Yan *et al*., 2018). WRKY31 promotes Salicylic Acid related gene expression and enhanced the content of CAT

and POD by repressing MdHIR4 (Zhao *et al*., 2019), which could explain the up-regulation of POD seen in this study. MYC proteins are a family of Helix-Loop-Helix (bHLH) TFs responsible for activation of most Jasmonates mediated responses, by ethylene, light and abscisic acid (Ortigosa *et al*., 2020) and in particular MYC3, acts additively with MYC2 in the activation of Jasmonates responses (Zhang *et al*., 2017), which are phytohormones involved in abiotic and biotic stress responses (Goossens *et al*., 2015). SID is involved in the biosynthesis of Salicylic Acid from Chorismate, Salicylic Acid protects plants against pathogens by accumulating in infected areas and areas adjacent to the site of infection (Wildermuth *et al*., 2001) and after infection, promotes the generation of antimicrobial compounds, such as PR proteins and the phytoalexins (Zhao *et al*., 2019).

Our results suggest that *V. victoriae* EPL29.5 induces resistance in 'Cripps Pink' apples by up-regulating resistance defense-related genes. Further research is needed to demonstrate whether induction of these genes reduces or inhibits infection of apple pathogenic fungi.

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CONCLUSIONES GENERALES

Los resultados de esta investigación sugieren que las cepas EPL4.5 y EPL29.5 de la levadura endófita *V. victoriae* son potenciales agentes de biocontrol de *P. vagabunda* en manzanas. Los mecanismos de acción utilizados por las levaduras para antagonizar al patógeno incluyen la capacidad de formar biopelículas, la producción de compuestos orgánicos volátiles como 1-Butanol; 1-Butanol, 2-metil-, (S)-; 1-Butanol, 3-metil; 1-Propanol, 2-metil y 1-Hexanol. Además, de la inducción de resistencia mediante la sobrerregulación de los genes relacionados con la defensa PAL, POD, CAT, PR8, PR3-CH4, TLP1b, PRPL, WKYR31, SID and MYC3. Por tanto, estas levaduras podrían utilizarse en aplicaciones de precosecha y al ser endófitas, pueden colonizar los tejidos de la fruta, incluidas las heridas y las lenticelas, mediante una rápida proliferación celular y de esta forma controlar al patógeno.