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TESIS DE DOCTORADO

Hidrocarburos epicuticulares de insectos plaga de soja y
eucaliptus, composición e interacción con hongos
entomopatógenos

Lic. MSc. Lucía Sessa Jusid

Orientador:
Dr. Nicolas Pedrini

Co-Orientador:
Dr. Ing. Agr. Eduardo Abreo

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Dr. Andrés González - Dra. Almudena Ortiz-Urquiza - Dra. Silvia Batista

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Resumen

La chinche del eucalipto *Thaumastocoris peregrinus* y la chinche de la soja *Piezodorus guildinii* son dos hemípteros plaga que ocasionan daños importantes en estos cultivos en Uruguay, Argentina, Brasil y Estados Unidos. Ambos presentan diferentes susceptibilidades tanto al control con insecticidas químicos como a la infección por hongos entomopatógenos (HEP) como *Beauveria bassiana* y, se presume que esta diferencia podría estar debida en parte a la composición de los hidrocarburos epicuticulares (CHC) de su cutícula, ya que ésta es la primera barrera de defensa. En este trabajo se dilucidó la composición de los hidrocarburos epicuticulares (CHC) de *P. guildinii*, que constó de 72 compuestos de los cuales los más abundantes fueron cadenas lineales saturadas de *n*-C27 y *n*-C29, y las cadenas de monometilo y dimetilo de C31 y C30, además de un grupo de hidrocarburos volátiles con *n*-C13 y *n*-C15 como compuestos predominantes. Por otra parte, se encontraron diferencias en el patrón de CHC entre ninfas y adultos, así como dimorfismo sexual y también se encontró una mayor cantidad total de CHC de los insectos colectados en cultivos de soja tratados con insecticida. Se evaluó el efecto de la nutrición en el hidrocarburo sintético *n*-pentadecano (*n*-C15) sobre el crecimiento y la virulencia de cinco cepas de *B. bassiana* frente a ambos insectos plaga, observándose en algunos casos un aumento en la virulencia (efecto "priming"). En dos de estas cepas (ILB299 hipovirulenta e ILB308 hipervirulenta) además se evaluó el efecto a nivel de la expresión de genes relacionados con la virulencia, observándose en ambas la inducción de algunos genes relacionados con la asimilación de hidrocarburos, como los genes del citocromo P450 monooxigenasa (CYP), junto con genes de adhesión a la cutícula y de tolerancia al estrés, lo cual podría explicar el efecto "priming" observado. Finalmente, ambas cepas fueron evaluadas en cuanto a su respuesta a la nutrición en dos extractos de cutícula de *P. guildinii* y *n*-pentadecano mediante la tecnología RNAseq, encontrándose un perfil de expresión de los genes relacionados con la virulencia diferente entre ambas, así como los términos de ontología génica (GO) y las vías KEGG, lo que podría explicar las diferencias en su virulencia intrínseca pero también del efecto "priming" observado previamente. La cepa hipovirulenta mostró sobreexpresión predominantemente de genes de penetración de la cutícula, como quitinasas, proteasas y CYP, así como los términos GO 'unión de hemo' y 'actividad de monooxigenasa', y la ruta metabólica del 'peroxisoma' enriquecida, mientras que la cepa hipervirulenta mostró sobreexpresión de genes de remodelación de la pared celular y del ciclo celular, y también adhesión de la cutícula y CYP, junto con el término GO de 'actividad del factor de transcripción de unión al ADN' y las vías KEGG de 'Meiosis-levadura' y 'Ciclo celular' enriquecidas. Estos resultados revelaron que ambas cepas se encontraron en diferentes etapas metabólicas en lo que respecta a su nutrición sobre diferentes extractos cuticulares de *P. guildinii* y *n*-pentadecano, evidenciando un "efecto retardado" en la cepa hipovirulenta ILB299 en comparación con la hipervirulenta ILB308, efecto que se correlaciona con la menor virulencia de la primera cepa.

Abstract

The eucalyptus bug *Thaumastocoris peregrinus* and the soybean stinkbug *Piezodorus guildinii* are two hemipteran pests that cause significant damage to these crops in Uruguay, Argentina, Brazil, and the United States. Both display different susceptibilities towards chemical insecticides and infection by entomopathogenic fungi such as *Beauveria bassiana*, and it is presumed that this difference could be due partly to the composition of the epicuticular hydrocarbon layer (CHC) of their cuticle, because that is the first defense barrier. In this work, the composition of the epicuticular hydrocarbon layer (CHC) of *P. guildinii* was elucidated, which consisted of 72 compounds, being the most abundant saturated *n*-C27 and *n*-C29 linear chains, and monomethyl and dimethyl C31 and C30 chains, as well as a group of volatile *n*-C13 and *n*-C15 hydrocarbons. What is more, differences were found in the CHC pattern between nymphs and adults, as well as sexual dimorphism, and a higher total amount of CHC in insects collected in soybean crops treated with insecticide. The nutrition effect of the synthetic *n*-pentadecane (*n*-C15) on the growth and virulence of five *B. bassiana* strains against both pest insects was evaluated, observing in some cases an increase in virulence ("priming" effect). In two of these strains (hypovirulent ILB299 and hypervirulent ILB308) the effect was also evaluated at the genes expression level, observing in both strains the overexpression of some genes related to the hydrocarbon assimilation, such as cytochrome P450 monooxygenase (CYP) genes, together with cuticle adhesion and stress tolerance genes, which could explain the observed "priming" effect. Finally, both strains were evaluated regarding their response to nutrition in two *P. guildinii* cuticle extracts and *n*-pentadecane using RNAseq technology, finding a different expression profile of virulence genes related between both, as well as gene ontology (GO) terms and KEGG pathways, which could explain the differences in their intrinsic virulence but also the priming effect previously observed. Hypovirulent strain showed predominantly overexpression of cuticle penetrating genes such as chitinases, proteases and CYPs, as well as the GO terms 'heme binding' and 'monooxygenase activity', and the enriched 'peroxisome' metabolic pathway, whereas the hypervirulent strain showed overexpression of cell wall remodeling and cell cycle genes, and also cuticle adhesion and CYP genes, along with the GO term for 'DNA-binding transcription factor activity' and the KEGG 'Meiosis-yeast' and 'Cell cycle' pathways enriched. These results revealed that both strains were in different metabolic stages regarding their nutrition on different cuticular extracts of *P. guildinii* and *n*-pentadecane, evidencing a "delayed effect" in the hypovirulent ILB299 strain compared to the hypervirulent ILB308., an effect that correlates with the lower virulence of the former strain.

Introducción General

Las plantaciones que ocupan grandes extensiones representan un desafío no sólo para el manejo integrado de plagas y enfermedades sino para la sustentabilidad del sistema productivo. La chinche del eucaliptus *Thaumastocoris peregrinus* (Carpintero y Dellapé) (Heteroptera: Thaumastocoridae) y la chinche de la soja *Piezodorus guildinii* (Westwood) (Heteroptera: Pentatomidae) son dos hemípteros plaga de difícil control que provocan daños importantes en estos cultivos en Uruguay y otras regiones productoras. Una diferencia fundamental entre ambos hemípteros es la relación que mantienen con los hongos entomopatógenos: mientras la chinche del eucaliptus suele sufrir epizootias causadas por estos hongos (Simeto et al., 2012), la chinche de la soja es poco sensible al ataque de estos organismos en condiciones de campo. Esta diferencia en la susceptibilidad de los insectos a los hongos biocontroladores puede deberse, entre otras causas, a la composición de la capa más externa de la cutícula del insecto, la epicutícula, compuesta principalmente por hidrocarburos o CHC por su sigla en inglés (de Cuticular HydroCarbons), donde se inicia el proceso de infección a partir de la adhesión de las esporas del hongo.

Es así como la efectividad del control biológico mediante el uso de hongos entomopatógenos depende, entre varios factores, de la capacidad de estos hongos de penetrar al interior del insecto blanco través de la cutícula y colonizarlo (Pedrini et al., 2007). La cutícula se compone de múltiples capas, cada una de las cuales presenta una estructura química y propiedades fisicoquímicas diferentes. La capa más externa de la cutícula, la epicutícula, constituye la primera barrera contra la acción de no sólo bioinsecticidas fúngicos sino también agentes bacterianos e insecticidas químicos, es por sobre todo resistente a la degradación enzimática, restringe la pérdida de agua, controla la absorción de sustancias químicas y actúa en procesos de comunicación química entre individuos (Juárez et al., 1994). Uno de sus componentes principales son los hidrocarburos de cadena carbonada larga, los cuales se ha visto se encuentran presentes en todos los órdenes de insectos, y en muchos casos son el componente prevalente. La composición de los CHC de los insectos influirá en cierta medida en el proceso de infección fúngica, ya que se ha reportado que algunos insectos varían su composición de hidrocarburos no constitutivos durante el proceso de infección (Sosa-Gómez et al., 1997), siendo algunos más fáciles de degradar que otros, e incluso algunos son inhibidores de la germinación de esporas (Pedrini et al., 2007; Pedrini et

al., 2013). El hongo entomopatógeno *Beauveria bassiana* (Bals.) Vuill ha sido usado como agente microbiano de control biológico para el control de varias plagas (García-Estrada et al., 2016). El uso de estos agentes de biocontrol aparece como una estrategia complementaria al uso de insecticidas químicos, menos dañina para la salud humana y animal y más amigable con el medio ambiente, a la vez de no presentar un gran riesgo de generación de resistencia. Aislamientos de esta especie pueden degradar una variedad de hidrocarburos similares o idénticos a los de su insecto blanco, utilizándolos como fuente de energía para su metabolismo, pero también incorporando sus productos de degradación en diferentes lípidos fúngicos (Pedrini et al., 2007). Además, se ha reportado que la previa exposición de hongos entomopatógenos a CHC en medio de cultivo como única fuente de carbono, aumenta su virulencia, probablemente favorecida por el aumento de la afinidad debido a la inducción de genes de la asimilación de hidrocarburos (Pedrini et al., 2009, 2010, 2013; Zhang et al., 2012).

Comprender la interacción entre la composición de CHC de *P. guildinii* y *T. peregrinus* y diferentes cepas de *B. bassiana* puede generar el conocimiento necesario para manipular dicha interacción, con el objetivo final de aumentar la capacidad de infección de *B. bassiana* sobre estos insectos, en particular de la especie menos susceptible *P. guildinii*. Para lograrlo, en este trabajo de tesis se investigó la composición de CHC de individuos de *P. guildinii* colectados en cultivos de soja con manejo tradicional, es decir, tratados con insecticidas, como en cultivos sin tratamiento de insecticidas, se identificaron los CHC presentes en *P. guildinii* y ausentes en *T. peregrinus*, se evaluó su inclusión en estrategias que permitan aumentar la virulencia y se caracterizó la respuesta de algunas cepas a nivel de expresión de genes vinculados a la degradación de CHC y a la virulencia.

Hipótesis

La composición de hidrocarburos epicuticulares (CHC) de *P. guildinii* explicarían su baja susceptibilidad a los hongos entomopatógenos, existiendo diferencias en la virulencia entre cepas de *B. bassiana* debido a su capacidad de degradar estos compuestos. En este sentido, sería posible aumentar la virulencia mediante la activación de los mecanismos enzimáticos relacionados a la degradación de hidrocarburos.

Objetivo General

El objetivo de este trabajo es dilucidar la composición de hidrocarburos de la epicutícula de *Piezodorus guildinii*, evaluar cómo estos son afectados por las condiciones de cultivo de soja y su posible interacción con la virulencia de cepas del hongo entomopatógeno *Beauveria bassiana*.

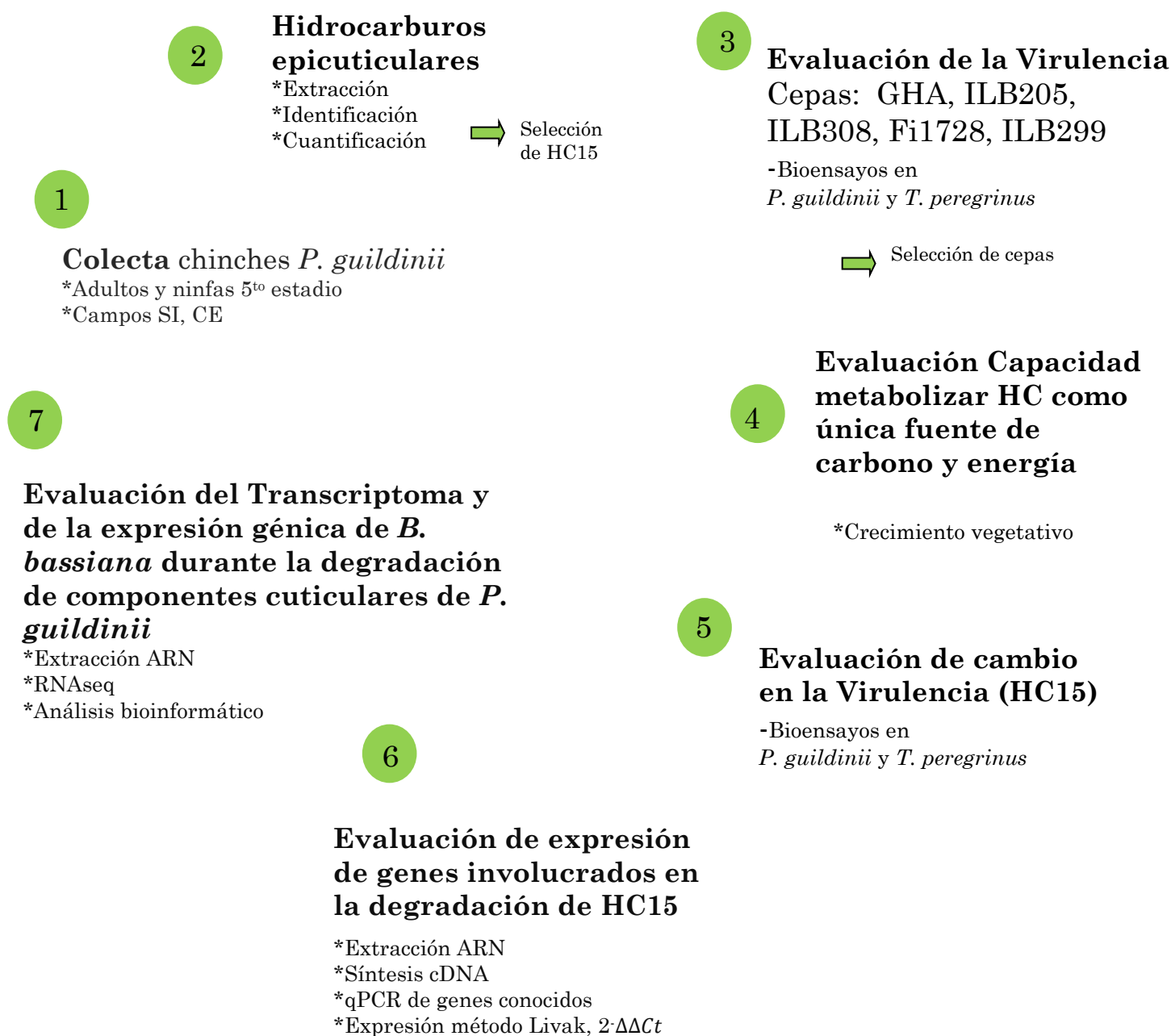
Objetivos Específicos

- 1) Caracterizar la composición de CHCs en *P. guildinii* e identificar diferencias entre los distintos estadios de los insectos y entre insectos colectados en campos de soja tratados con insecticida y en campos sin este tratamiento.
- 2) Evaluar el efecto de la nutrición fúngica con CHC en el crecimiento vegetativo y el efecto de la nutrición con *n*-pentadecano en la virulencia de cinco cepas de *B. bassiana* frente a *P. guildinii* y *T. peregrinus*, así como en la expresión de 10 genes relevantes para la infección de dos cepas de *B. bassiana* con diferente grado de virulencia.
- 3) Detectar la expresión diferencial de genes asociados a la degradación de los componentes de la cutícula de *P. guildinii* en cepas de *B. bassiana* de virulencia contrastante mediante el análisis de sus transcriptomas.

Estrategia de Trabajo

Con el objetivo propuesto se colectaron individuos de *P. guildinii* en plantaciones de soja con y sin previa aplicación de insecticida, se extrajeron los componentes lipídicos cuticulares de estos individuos y se obtuvo la identidad y composición relativa de los hidrocarburos que conforman su epicutícula mediante CGC-MS. Utilizando diversos HC sintéticos suplementados en el medio de cultivo como única fuente de carbono, análogos a los previamente identificados, se evaluó el crecimiento vegetativo de diferentes cepas del hongo entomopatógeno *B. bassiana*. Además, se evaluó el efecto sobre su virulencia, mediante bioensayos en los que se comparó la virulencia de los conidios producidos en medio suplementado con los HC de cada insecto, con los conidios obtenidos en medio no suplementado con HC. Aquella/s cepa/s cuya virulencia se vio aumentada fue seleccionada para analizar su perfil de expresión de genes durante la interacción con los HC. Se seleccionaron dos cepas de *B. bassiana* con virulencia contrastante sobre *P. guildinii* (hipervirulenta e hipovirulenta), que vieron aumentada su virulencia post exposición a HC sintético. Los genes analizados fueron aquellos previamente identificados en la literatura como involucrados en la patogénesis; incluyendo genes relacionados a la adhesión de esporas, degradación de hidrocarburos, degradación de otros componentes cuticulares y componentes de resistencia al estrés oxidativo, entre otros. Por otra parte, estas dos cepas fueron evaluadas a nivel de expresión sus transcriptomas completos mediante RNAseq, a los 4 días post inoculación en medios de cultivo con extractos cuticulares de *P. guildinii* y HC sintético (*n*-pentadecano), para evaluar nuevos genes relacionados con la virulencia que no fueron estudiados por RT-qPCR.

Esquema de Trabajo



Capítulo 1 _

Composición de
hidrocarburos
epicuticulares en *P.*
guildinii

Introducción

1.1 Cultivo de soja y principales plagas

El cultivo de soja [*Glycine max* (L.) Merrill], ha experimentado un drástico crecimiento en los últimos 20 años, consolidándose como el cultivo principal de la agricultura de secano en nuestro país tanto por su producción como por la superficie sembrada. Su superficie de siembra ha pasado de algo menos de 30.000 hectáreas (ha) en la zafra 2001/2002 a superar las 900.000 ha en la zafra 2020/2021, con una producción de 1.708 mil toneladas y un rendimiento 1.881 kg/ha (DIEA, 2021). Además, se ha ido incrementando el número de productores que ha incorporado la soja a su sistema productivo, siendo menor de 950 el número de productores en la zafra 2006, hasta superar los 2450 productores en la pasada zafra de 2020 (DIEA, 2021).

Este cultivo se ha extendido por toda la zona núcleo e incluso se ha desplazado hacia departamentos que no pertenecen a la zona tradicionalmente agrícola, incluyendo trece de los 19 departamentos del país, alcanzando la máxima superficie sembrada registrada por las encuestas de DIEA en la zafra 2014/15 con 1.330.000 hectáreas (DIEA, 2015). La soja se domesticó en el este de Asia y se convirtió en un cultivo importante durante la dinastía Zhou (ca. 2500 B.CP) en China (Lee et al., 2011). En regiones templadas a frías los problemas de insectos son casi inexistentes (Backman & Jacobsen, 1992), sin embargo, mientras su cultivo se va desarrollando hacia latitudes menores los problemas ocasionados por insectos plaga adquieren más importancia (Saulich & Musolin, 2018; Zerbino et al., 2020).

A nivel regional el cultivo de soja es susceptible al ataque de diversos insectos, siendo cuatro grupos los más importantes. El barrenador del brote *Crociosema (Epinotia) aporema* (Walsingham) (Lepidoptera: Tortricidae), las lagartas defoliadoras *Anticarsia gemmatalis* (Hübner) y *Rachiplusia nu* (Guenée) (Lepidoptera: Noctuidae) que dañan al cultivo en el período vegetativo, y el complejo de chinches fitófagas formado por *Piezodorus guildinii*

(Westwood), *Nezara viridula* (Linnaeus) y *Dichelops furcatus* (Fabricius) que son problema en el período reproductivo desde que se comienzan a formar las vainas hasta el fin del ciclo (Depieri & Panizzi, 2011).

1.2 Control químico de las principales plagas

El manejo integrado de plagas (MIP) incluye la identificación de los insectos, el monitoreo, la determinación de los niveles de daño que orientan a la toma de decisiones para mantener la población por debajo de un umbral determinado y la elección de diferentes tácticas de control que integradas de forma armónica permiten optimizar el control de manera sostenible. Dentro de las estrategias del MIP también se incluye el control químico, junto con el control biológico y las prácticas agrícolas adecuadas.

Dentro del manejo integrado de plagas en cultivos de soja, se debe inspeccionar regularmente cada chacra evaluando el nivel de ataque, con relación a la defoliación y al número y tamaño de los insectos. En los casos específicos de lagartas y chinches, los muestreos se deben realizar con un paño blanco de un metro de largo y un ancho adaptable a la distancia entre hileras de cultivo. El paño es colocado entre las dos hileras con cuidado de no perturbar los insectos, las plantas se inclinan sobre el paño y deben ser sacudidas vigorosamente sobre el paño de manera que los insectos se desprendan de las plantas y caigan en él. Luego se cuentan las lagartas y chinches, las que se discriminan en el caso de las lagartas por especie y por tamaño y en el de las chinches por estado ninfa y adulto. Posteriormente, en cada hilera en 25 plantas próximas a donde se colocó el paño se cuentan el número de plantas que tengan brotes frescos atacados. Este procedimiento debe ser repetido en varios lugares de la chacra, considerando como resultado la media de todos los puntos muestreados. En el caso de un espaciamiento reducido entre las hileras, se debe usar un paño golpeando las plantas de una hilera (Zerbino, 2002).

Para el caso particular de chinches los muestreos se deben iniciar desde la formación de las vainas (estadio fenológico R3) hasta madurez fisiológica (estadio fenológico R7) con una frecuencia semanal. Es conveniente que sean

realizados en las primeras horas de la mañana (antes de las 10:00 am), cuando los insectos se encuentran en las partes superiores de las plantas. Se recomienda, también realizar un mayor esfuerzo de muestreo en los bordes de las chacras porque allí es donde inician su ataque. En ciertas situaciones el control puede ser efectuado sólo en los bordes sin necesidad de aplicar el insecticida en toda la superficie de la chacra (Zerbino, 2002).

Los sitios de muestreo deben estar distribuidos en el cultivo de manera de ser representativos, el número mínimo de sitios de muestreo para chacras de 10 ha son seis, para las de 30 ha son ocho y para las más grandes de hasta 100 ha son 10 sitios. La información recabada en cada estación de muestreo se registra, se realiza el promedio y cuando se alcanzan los niveles de daño umbral se debe aplicar un insecticida, preferentemente debe ser selectivo de modo que la fauna benéfica (parasitoides y predadores) no sea afectada y de esta manera evitar los problemas de resurgencia de insectos-plaga (Zerbino, 2002).

En nuestro país se ha determinado que la decisión de aplicación de insecticida se debe realizar cuando la densidad poblacional alcanza valores entre 1 y 1,5 individuos mayores a 5mm (ninfas y adultos) por metro de surco desde el estado de fenológico R5 de la soja (Zerbino, 2010). Un estudio reciente sugiere que la especie vegetal sobre la cual deberían de concentrarse los esfuerzos de muestreo y control durante la primavera es la alfalfa, ya que éste es el cultivo alternativo de preferencia pre y pos-soja colonizado por *P. guildinii* (Zerbino et al., 2015).

En nuestro país normalmente es necesario realizar entre 3 y 5 aplicaciones de insecticidas para controlar estos insectos en el cultivo de soja. Casi la totalidad de la soja producida en nuestro país es transgénica, y el principal herbicida utilizado es el Glifosato, aunque también se utiliza la Atrazina para combatir las malezas menos susceptibles al Glifosato. Los insecticidas más utilizados han sido el Endosulfan para el control de chinches, que por lo general se utiliza en mezcla con Cipermetrina (Tabla 1). En el año 2007 el MGAP restringió el uso de Endosulfán, por lo que se comenzaron a utilizar los neonicotinoides y

piretroides como el Thiamethoxam y la Lambdacialotrina (Tabla 1). Para controlar las lagartas se utilizan insecticidas reguladores del crecimiento como Triflumron, Metoxifenocide y Diflubenzuron y Clorpirifos.

Tabla 1 Insecticidas recomendados para el control de chinches en cultivo de soja en Uruguay

Nombre técnico	Grupo Químico	Modo de acción	Clase Toxicológica (OMS/IPCS)
Endosulfan	Organoclorado	No sistémico, de contacto e ingestión y en su fase gaseosa a temperaturas mayores a 20 °C. Afecta el sistema nervioso de los insectos.	II
Thiamethoxam	Neonicotinoide	Sistémico, de contacto rápido y estomacal. Rápidamente absorbido por la planta y transportado acrópetamente vía xilema. Afecta los receptores de acetilcolina nicotínica en el sistema nervioso.	III
Lamdacialotrina	Piretroide, clorado, fluorado	No sistémico, de contacto, estomacal y residual. Con propiedades repelentes.	II
Cipermetrina	Piretroide, clorado	No sistémico, de contacto, estomacal y residual. Actúa sobre el sistema nervioso y disuade la alimentación.	II

I = extremadamente tóxico (DL50 oral = hasta 50); II = altamente tóxico (DL50 Oral = 50-500); III = medianamente tóxico (DL50 Oral = 500-5000); IV = poco tóxico (DL50 Oral = > 5000 mg/kg).

El control químico mediante el uso de insecticidas impone grandes dificultades debido a la tolerancia intrínseca a los principios activos que son utilizados comúnmente (Corso & Gazzoni, 1998), requiriéndose el uso de dosis cada vez más altas de insecticidas y observándose un incremento en la ineffectividad del control químico, además de las consecuencias que esto trae en la seguridad y salud del operador y la salud medioambiental de todo el ecosistema en su conjunto.

Debido a esto, dentro del MIP, complementario al control químico, se procura implementar el uso de hongos entomopatógenos para el control de insectos

plaga, ya que el uso de bioinsectidas puede resultar menos perjudicial que el uso de los insecticidas químicos tradicionales, con menor impacto sobre especies no blanco y el medio ambiente en general (Essiedu et al., 2020).

1.3 Características biológicas de *P. guildinii*

Piezodorus guildinii (Westwood) es el pentatómido fitófago más dañino y abundante en el cultivo de soja en nuestro país (Zerbino et al. 2016; Ribeiro & Castiglioni 2009; Zerbino & Alzugaray, 2003). Fue identificado por primera vez en 1969, no obstante, fue considerado plaga por Bourokhovitch y Morey en 1981. Esta chinche es pico-suctora y se alimenta de vainas y granos, generando como consecuencia el debilitamiento y la pérdida en la calidad y potencial germinativo del grano, con la consecuente pérdida de rendimiento del cultivo y el aumento en la susceptibilidad hacia la infección por microorganismos patógenos (Belorte et al. 2003; Panizzi 2000 y 2007; Panizzi & Silva, 2009; Medrano et al., 2007). La naturaleza de los daños en los tejidos vegetales y su severidad está asociada a las secreciones salivales, que pueden ser tóxicas y causar lesiones necróticas, y a la frecuencia y duración del período de alimentación (Panizzi, 2000).

Los adultos son de color verde y alcanzan a medir 10 mm, presentan una banda transversal en el pronoto, generalmente de color rojizo que en los machos es más clara (Figura 1). Los huevos son de color negro, tienen forma de barril y son depositados en hileras dobles, generalmente en las vainas de soja. Presenta cinco estadios ninfales y el tiempo en completar cada ciclo es muy dependiente de factores abióticos como el fotoperíodo y la temperatura, así como de factores bióticos como la calidad de su alimentación (Zerbino et al. 2013, 2014, 2015, 2016; Zerbino 2014; Panizzi & Silva 2009; Panizzi 1987, 1992, 1997, 2007; Panizzi et al. 2000a, 2002; Serra & La Porta 2001, Panizzi 1985, Panizzi & Slansky 1985a). Las ninfas recién eclosionadas (ninfa I) son muy pequeñas (1 mm), no se alimentan y tienen comportamiento gregario, permaneciendo próximas a la postura, probablemente como defensa física y química (Villas Bôas et al., 1985). Las ninfas entre el segundo y quinto estadio

tienen un largo de cuerpo entre 2,3 y 7,8 mm (Grazia et al., 1980). Inicialmente, son de color rojo y negro, y conforme maduran desarrollan una coloración verdosa con manchas rojas y negras en el abdomen (Fraga & Ochoa 1972; Panizzi 2007). A partir del segundo estadio se dispersan y comienzan a ingerir alimento (Villas Bôas et al., 1985). La duración media del ciclo de *P. guildinii* desde huevo a adulto es de 32 días en condiciones de laboratorio (24,9°C y 16 horas de luz) (Serra & La Porta, 2001) y de 30-40 días en condiciones de campo, en Londrina, Brasil (Villas Bôas et al., 1985). La proporción de sexos es de 1.3 hembras por macho (Serra & La Porta, 2001). La duración del ciclo fue de 25-33 días en condiciones de campo en Santa Fé, Argentina (Massoni & Frana, 2007).

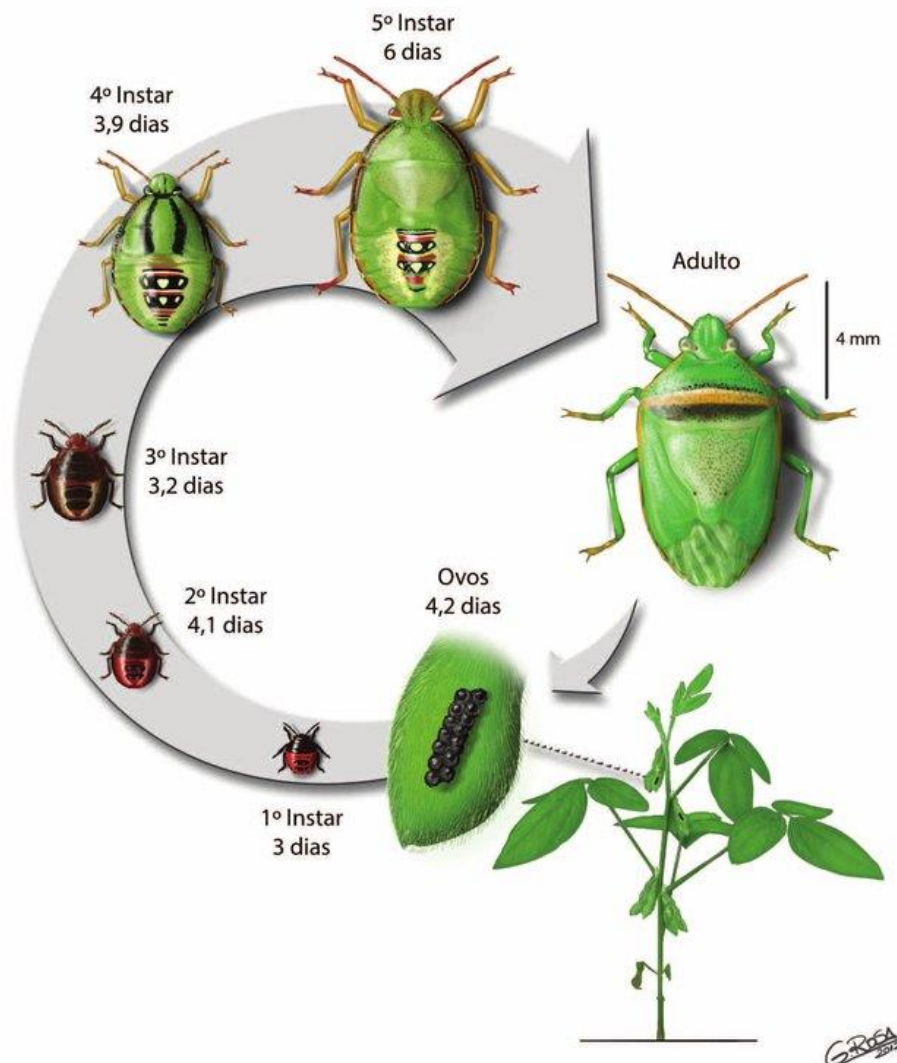


Figura 1 Ciclo de vida de la chinche de la soja *Piezodorus guildinii*. Adaptado de Panizzi, et al., 2012.

Entre cada estadio ninfal hasta llegar a la adultez los individuos mudan, desprendiendo en cada etapa la capa de exoesqueleto, denominada exuvia. Los adultos sobreviven en diapausa durante el invierno principalmente en alfalfa (Zerbino et al., 2020), y en primavera y verano completan el ciclo sobre este cultivo antes de colonizar los cultivos de soja en verano y otoño. *P. guildinii* desarrolla dos generaciones en el cultivo de soja en Colonia en Uruguay (Zerbino et al., 2014, 2015, 2016) y en Santa Fé Argentina (Massoni & Frana, 2007). En un estudio realizado en el noroeste de nuestro país, en Paysandú, en donde se estudiaron las fluctuaciones de poblaciones de *P. guildinii* en soja y alfalfa (Ribeiro & Castiglioni, 2009) se observó que los primeros adultos de *P. guildinii* fueron registrados en cultivos de soja estadio R4 en verano (febrero), observándose el máximo poblacional de adultos en otoño (finales de marzo), cuando la soja se encuentra en R5-R6 (Figura 2). El máximo poblacional de ninfas (tanto pequeñas como grandes) y de huevos también se registró en estadios R5 y R6, lo cual señalaría que es el estadio más adecuado para el desarrollo de esta especie. Esta especie permaneció en el cultivo de alfalfa durante todo el período de evaluación, disminuyendo su población mientras que se incrementaba en soja, a partir del estadio R4.

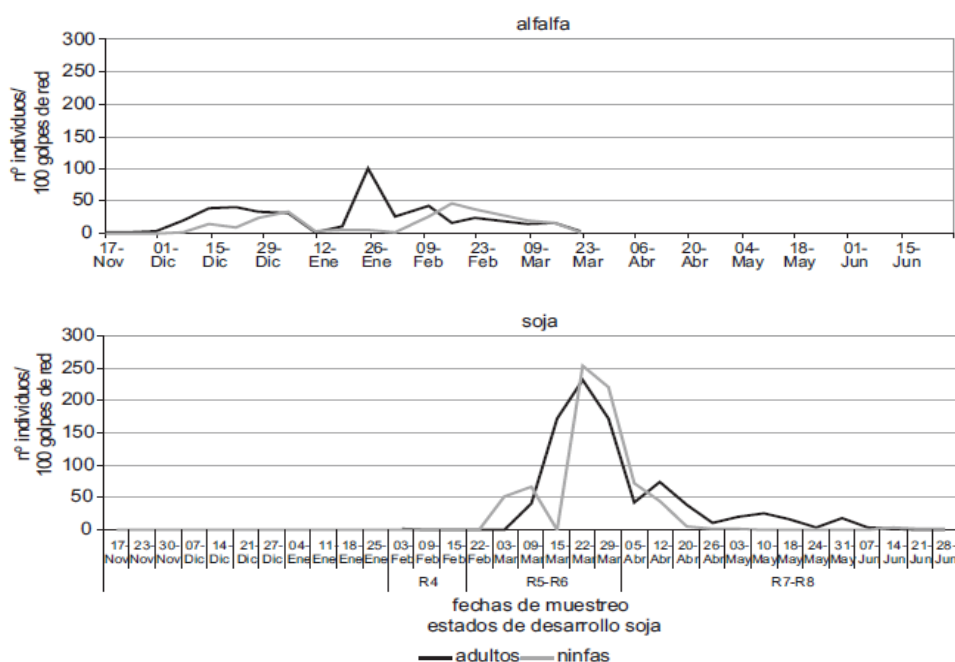


Figura 2 Fluctuación de poblaciones de ninfas y adultos de *P. guildinii* en alfalfa y soja. Adaptado de Ribeiro & Castiglioni, 2009.

1.4 Hidrocarburos cuticulares en insectos

El sistema tegumentario de los insectos está compuesto por la cutícula, la epidermis y la membrana basal (Figura 3). La epicutícula, capa más externa de la cutícula, se compone de múltiples capas, cada una de las cuales presenta una estructura química y propiedades diferentes. La capa más externa de la epicutícula es la capa lipídica, la cual es por sobre todo resistente a la degradación enzimática, restringe la pérdida de agua, controla la absorción de sustancias químicas y actúa en procesos de comunicación química (Juárez et al. 1994). Está compuesta por una mezcla compleja de alcoholes, ésteres, aldehídos, cetonas, ácidos grasos de cadena larga e hidrocarburos (CHC) (Wigglesworth, 1933; Blomquist et al. 1987). Estos últimos son uno de sus componentes principales y representan más del 90% de los componentes en la mayoría de la especies (Blomquist et al. 1972, Blomquist et al. 1987, Lockey 1988).

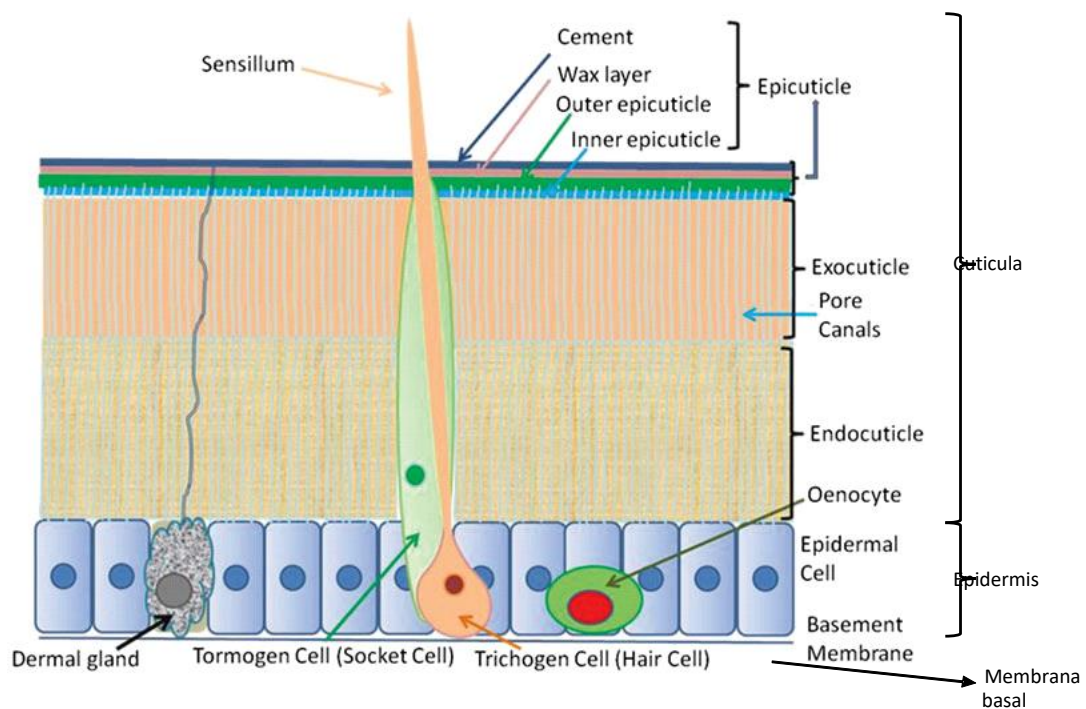


Figura 3 Esquema general del tegumento de insectos. Adaptado de Doucet & Retnakaran, 2016.

Su biosíntesis tiene como eje la síntesis de ácidos grasos y su posterior elongación mediante la acción de una ácido graso sintetasa acoplada a acil-CoA elongasas microsomales (Juárez & Brenner 1989, Juárez 1994, Juárez et al. 1996). El mecanismo fue descrito por primera vez en *Triatoma infestans*, y se demostró que las tres especies lipídicas principales de la cutícula de este insecto (hidrocarburos, alcoholes y ácidos grasos libres o esterificados) están relacionados biosintéticamente y que los ácidos grasos de 26, 28, 30, 32 y 34 carbonos en la cadena lineal son los sustratos para la síntesis de los principales hidrocarburos (*n*C25, *n*C27, *n*C29, *n*C31 y *n*C33, respectivamente). Este último proceso ocurriría mediante una vía de descarboxilación/descarbonilación reductiva (Reed et al., 1994, Schneider-Beahddad & Kolattukudy 2000, Blomquist, 2003).

Los hidrocarburos epicuticulares (CHC) varían cualitativa y cuantitativamente entre las especies, así como entre sexos y distintos estadios de desarrollo dentro de la misma especie e individuo. La proporción de hidrocarburos en los lípidos cuticulares varía considerablemente entre diferentes especies (Lockey, 1988), variando desde sólo el 3% en la ninfa acuática de la mosca de la piedra, *Pteronarcys californica* (Arnold et al., 1969) hasta el 95% en las especies de cucaracha, *Periplaneta japonica* y *P. americana* (Jackson, 1972).

El punto de fusión (temperatura a la que un compuesto cambia de estado sólido a líquido) varía dentro de cada clase de hidrocarburos según la cantidad de carbonos y según la cantidad y disposición de dobles enlaces o ramificaciones que contenga en su estructura. Por ejemplo, el punto de fusión aumenta conforme aumenta el número de carbonos en la cadena lineal saturada (*n*-alcanos), y disminuye si se agrega un doble enlace o un grupo metilo. El cambio será más o menos drástico según la posición y cantidad de dobles enlaces y según la posición y cantidad de los grupos metilo (Gibbs & Pomonis, 1995). Es por esto, que para mantener una cutícula flexible que permita cumplir sus funciones de permeabilidad y por lo tanto mantener la viabilidad del insecto en distintas temperaturas (Morgan, 2004), los hidrocarburos cuticulares usualmente se encuentran como mezclas complejas

de hidrocarburos saturados de cadena lineal (*n*-alcanos), hidrocarburos insaturados (*n*-alquenos) e hidrocarburos metil ramificados (*i*-alcanos).

Los CHCs ya sea específicamente o como subgrupo, tienen variadas funciones fisiológicas en el comportamiento de los insectos, por ejemplo, como agentes impermeabilizantes y como señales químicas de comunicación. Las funciones de permeabilidad de la membrana incluyen no sólo protección frente a la desecación mediante el flujo de agua trans-cuticular, sino también la actividad como barrera de defensa frente a agentes infecciosos, ya sea químicos, como los insecticidas, o biológicos, tales como los hongos entomopatógenos, bacterias y parásitos. Además, los CHC juegan un rol muy importante como señales de comunicación como el reconocimiento inter e intra-especie y género, señales específicas de la tarea, señales de dominancia y fertilidad, mimetismo químico, y también como feromonas cebadoras (Hölldobler & Wilson, 2009). En especies de insectos sociales, como las hormigas, los CHCs desempeñan papeles cruciales en reconocimiento, organización de las colonias y coordinación de actividades de búsqueda de alimento (Howard & Blomquist, 2005). Un ejemplo del rol que juega el perfil de hidrocarburos cuticulares en especies sociales como hormigas es el mecanismo de reconocimiento de áfidos compatibles, según su perfil de CHC. Se ha evidenciado que los endosimbiontes de áfidos pueden alterar la composición de CHC de su hospedero, a la vez que las hormigas pueden percibir las diferencias entre los perfiles de CHC de áfidos infectados o no infectados con endosimbionte (Hertaeg et al., 2021). Este es un ejemplo del mecanismo de reconocimiento inter-especie.

1.5 Identificación y cuantificación de hidrocarburos epicuticulares

Dada la complejidad y variedad de compuestos que hacen parte de la cutícula, específicamente de los CHCs, el análisis de su composición química es importante no solo evolutiva y taxonómicamente para el reconocimiento de una o más especies de difícil diferenciación, sino también en la ecología del comportamiento de insectos y en el estudio de la selectividad de la cutícula a compuestos naturales y artificiales, como ser los controladores biológicos y los insecticidas, respectivamente, y, por consiguiente, importante en el manejo y control de plagas (Liepert & Dettner, 1996; Doi et al., 1997; Anyanwu et al., 2000; Horne & Priestman, 2002; Pedrini et al. 2007).

La extracción de los compuestos epicuticulares (compuestos apolares) se realiza con solventes orgánicos como hexano y cloroformo. El solvente conteniendo el extracto se concentra en evaporador rotativo a baja presión y temperatura reducida, y se almacena a temperatura inferior a 0° C hasta su procesamiento. El análisis de los extractos se realiza mediante Cromatografía Gaseosa Capilar, acoplada a un detector de llama (FID) o de espectrometría de Masas (MS), obteniéndose como resultado diferentes cromatogramas. La identificación inicial de los compuestos cuticulares puede realizarse mediante la comparación de los tiempos de retención con patrones específicos, utilizando el índice de Kovats, y más exhaustivamente mediante los análisis de los espectros de masa obtenidos para cada pico y registrados en el cromatograma comparados con los espectros de masa de los compuestos presentes en la biblioteca del CGC-MS. El índice de Kovats es un índice de retención que describe el comportamiento de retención de un compuesto de interés comparativamente al de una mezcla de hidrocarburos saturados, alcanos, de diferentes números de carbono. El índice de Kovats expresa el número de átomos de carbono multiplicado por 100, de un alcano hipotético de cadena normal que tendría un tiempo de retención ajustado idéntico al del pico de interés cuando es analizado sobre condiciones idénticas.

Índice de Kovats (1965): $KI = 100 * (Ca + (Cb - Ca) * ((trX - tra) / (trb - tra)))$

donde: Ca: número de carbonos del estándar que eluye anteriormente, Cb: número de carbonos del estándar que eluye posteriormente, trX: tiempo de retención del hidrocarburo incógnita, tra: tiempo de retención del estándar que eluye anteriormente, trb: tiempo de retención del estándar que eluye posteriormente. La abundancia relativa de cada hidrocarburo en cada espécimen se obtiene dividiendo el área bajo cada pico del cromatograma sobre el área total. Estos datos proveen información sobre las variaciones cuantitativas entre los grupos analizados y constituyen los datos de entrada para los análisis posteriores.

Justificación

La identificación de la composición de hidrocarburos epicuticulares del insecto plaga *P. guildinii* supone un punto de partida hacia el conocimiento de aspectos de su ecología no explorados hasta el momento, concretamente en lo que respecta a su baja susceptibilidad frente al control químico con insecticidas, así como a la infección por hongos entomopatógenos.

Objetivo

Caracterizar la composición de CHCs en *P. guildinii* e identificar diferencias entre los distintos estadios de los insectos (ninfas y adultos), sexo de los adultos y entre insectos colectados de campos de soja tratados con insecticida y campos sin tratamiento.

Resultados

A continuación, se expone el artículo “**Epicuticular hydrocarbons of the redbanded stink bug *Piezodorus guildinii* (Heteroptera: Pentatomidae): sexual dimorphism and alterations in insects collected in insecticide-treated soybean crops**” el cual incluye la totalidad de los resultados del Capítulo 1 de este manuscrito, publicado en la revista Pest Management Science Q1, Impact factor 2022/2023 4.845.

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Artículo científico – Resumen en español

ANTECEDENTES: La chinche de la soja *Piezodorus guildinii* (Heteroptera: Pentatomidae) es una de las especies más importantes que afectan los cultivos de soja en el sur de América del Sur. La cromatografía de gases capilar acoplada a la espectrometría de masas se utilizó para caracterizar los perfiles de hidrocarburos epicuticulares de insectos recolectados en el campo y para identificar diferencias en la composición entre ninfas de quinto estadio y adultos, machos y hembras, y entre insectos recolectados en campos tratados con insecticida y campos sin tratamiento.

RESULTADOS: Las cadenas lineales saturadas de *n*-C27 y *n*-C29, y las cadenas de monometilo y dimetilo de C31 y C33 fueron los compuestos más abundantes. También se detectó un grupo de hidrocarburos volátiles con *n*-C13 y *n*-C15 como compuestos predominantes. El patrón de hidrocarburos fue diferente entre ninfas y adultos, machos o hembras. El heneicoseno fue casi exclusivamente detectado en machos adultos y fue el componente más importante para diferenciar entre ambos sexos, seguido del tricosadieno. La cantidad total de hidrocarburos fue significativamente mayor en ninfas, machos y hembras recolectadas en campos tratados con insecticida en comparación con los insectos obtenidos de campos no tratados.

CONCLUSIÓN: Se encontraron diferencias en el patrón de hidrocarburos epicuticulares entre ninfas y adultos, así como dimorfismo sexual en chinches adultas. Curiosamente, también se encontró una alteración en el perfil de hidrocarburos de los insectos recolectados en cultivos de soja tratados con insecticida y su relevancia se discute dentro de un contexto de manejo de plagas.

Epicuticular hydrocarbons of the redbanded stink bug *Piezodorus guildinii* (Heteroptera: Pentatomidae): sexual dimorphism and alterations in insects collected in insecticide-treated soybean crops

Lucía Sessa,^a  Gustavo M. Calderón-Fernández,^b  Eduardo Abreo,^a 
Nora Altier,^a Sergio J. Mijailovsky,^b Juan R. Girotti^{b*}  and Nicolás Pedrini^{b*} 

Abstract

BACKGROUND: The redbanded stink bug *Piezodorus guildinii* (Heteroptera: Pentatomidae) is one of the most important species affecting soybean crops in southern South America. Capillary gas chromatography coupled to mass spectrometry was used to characterize the epicuticular hydrocarbon profiles of field-collected insects, and to identify differences in their composition between fifth-instar nymphs and adults, males and females, and between bugs collected in insecticide-treated and insecticide-free soybean crops.

RESULTS: Straight chain saturated *n*-C27 and *n*-C29, and monomethyl and dimethyl chains of C31 and C33 were the most abundant compounds. A group of volatile hydrocarbons with *n*-C13 and *n*-C15 as the predominant compounds were also detected. The hydrocarbon pattern was different between nymphs and adults, either males or females. Heneicosene was almost exclusively detected in adult males and was the most important component to differentiate between both sexes, followed by tricosadiene. The total hydrocarbon amount was significantly higher in nymphs, males and females collected in insecticide-treated fields compared with insects obtained from untreated fields.

CONCLUSION: Differences were found in the epicuticular hydrocarbon pattern among nymphs and adults, as well as sexual dimorphism in adult stink bugs. Interestingly, an alteration was also found in the hydrocarbon profile of insects collected in insecticide-treated soybean crops and its relevance is discussed within a pest management context.

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Keywords: hydrocarbon pattern; insect cuticle; lipids; pesticide; resistance

1 INTRODUCTION

The outermost insect cuticle layer, the epicuticle, is covered by a complex mixture of lipids, mainly long-chain and very long-chain hydrocarbons and other apolar components.^{1,2} These are responsible for avoiding lethal desiccation,² but also are involved in chemical communication, usually as contact pheromones,³ and are known to regulate insecticide penetration.^{4,5} Epicuticle hydrocarbons (CHC) vary qualitatively and quantitatively between species as well as between sexes and different stages of development within the same species.^{6,7} CHC chain lengths vary from about 20 to more than 50 carbons and are usually found as complex mixtures of straight chain saturated (*n*-alkanes), unsaturated (*n*-alkenes) and methyl-branched components.⁶

Regarding communication between sexes, CHC are known to function with short-range activity, facilitating recognition of sexual partners, and in some cases act as a courtship inhibitor to reduce the attractiveness of mated females.^{8–11} Increased CHC amounts and changes in CHC patterns have also been reported

to lead to contact insecticide resistance by reducing insecticide penetration.^{12–15} Nonetheless, there is scarce information about CHC profile alterations after direct insecticide exposure.¹⁶ Why insects can alter their hydrocarbon profile by contact insecticide pressure and how these alterations may impact on behavior and insecticidal resistance are relevant topics worth investigating.

* Correspondence to: J. R. Girotti and Nicolás Pedrini, Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), CCT La Plata Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) - Universidad Nacional de La Plata (UNLP), calles 60 y 120, 1900 La Plata, Argentina. E-mail: jgirotti@med.unlp.edu.ar and npedrini@med.unlp.edu.ar

a Laboratorio de Bioproducción, Plataforma de Bioinsumos, Instituto Nacional de Investigación Agropecuaria (INIA), Las Brujas, Uruguay

b Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), CCT La Plata Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) - Universidad Nacional de La Plata (UNLP), La Plata, Argentina

The redbanded stink bug *Piezodorus guildinii* (Westwood) and the southern green stink bug *Nezara viridula* (Linnaeus) are key hemipteran pests of soybean crops (*Glycine max* (L.) Merrill). They can be found from southern North America to southern South America.^{17,18} The redbanded stink bug is the most important species affecting soybean crops, and is the most damaging in Argentina, Brazil and Uruguay.^{19,20} It feeds on soy seeds and also colonizes other legume hosts according to its phenological stage.²¹ Both nymphs and adults of *P. guildinii* generally utilize these other legumes as host plants where populations can build up before migrating as adults to soybean fields in the summer. The redbanded stink bug prefers crimson clover (*Trifolium incarnatum* L.) and white clover (*Trifolium repens* L.) as early season reproductive hosts.^{22,23} In Uruguay, it was recently determined that *P. guildinii* prefers alfalfa (*Medicago sativa* L.) as the main host,^{24,25} prior to and after soybean infestation.^{26–28} A variety of chemical control options are available for soybean growers in Argentina and Uruguay, although the redbanded stink bug is less susceptible to insecticides than the southern green stink bug, *N. viridula* L.²⁹ Knowledge of the CHC pattern of *P. guildinii*, and its potential modification in insects collected from insecticide-treated soybean crops will help to provide clues on the tolerance to insecticides reported in this species and its implication in insect control strategies.

The aims of this study were to characterize the CHC pattern in *P. guildinii*, and to identify differences in CHC between: (i) nymphs and adults; (ii) males and females, to detect sexual dimorphism; and (iii) bugs collected in insecticide-treated and insecticide-free soybean crops, to detect changes in the CHC pattern related to insecticide exposure. In this regard, the hypothesis is that contact insecticides can induce a phenotype with larger amounts of CHC, and thus alter insecticide penetration and contribute to insecticide tolerance or resistance in this species.

2 MATERIALS AND METHODS

2.1 Sampling of insects

Prospecting for *P. guildinii* insects was performed on 24 March 2017 and 5 April 2018 from late-planted soybean fields located at INIA La Estanzuela, Colonia, Uruguay (34°19'42"S, 57°43'30"W), during the grain-filling period corresponding to phenological stages R5–R6.³⁰ Fifth-instar nymphs and adults were from a field previously treated with insecticides (treated field) and from a field with no insecticide application (untreated field), both 1850 m (2017) and 250 m (2018) distant from each other. Engeo® (Syngenta Agro) containing thiamethoxam (plant systemic neonicotinoid precursor of clothianidin, which is active on insect nicotinic acetylcholine receptors) and lambda-cyhalothrin (contact synthetic pyrethroid that penetrates the insect cuticle, disrupting nerve conduction) was applied in both years on the treated fields to control stink bugs. Quintal® Xtra, (Corteva Agriscience) containing methoxyphenozide and spinetoram, and Alsystin® (Bayer) containing triflumuron were also applied in combination with Engeo® in 2017 and 2018, respectively, for Lepidoptera control.

Insects were collected using a sweep net, put in a tinfoil-covered recipient and kept for 30 min at 4°C until sex sorting. Fifth-instar nymphs, and adult males and females were weighed individually and kept separate in the laboratory at $-20 \pm 2^\circ\text{C}$ until analysis. In the first year, 36 redbanded stink bugs were collected from the insecticide-treated field (15 fifth-instar nymphs, 6 males and 15 females) and 45 specimens were collected from the untreated field (18 fifth-instar nymphs, 15 males and 12 females). In the

second year, 36 bugs were collected from the treated field (21 males and 15 females), and 30 insects were collected from the untreated field (15 males and 15 females). No nymphs were found in the second-year field collection. From each year's collection and insect class, at least five subsamples of three individuals each were taken for hydrocarbon extraction, as detailed below.

2.2 Sample extraction and hydrocarbon purification

Epicuticular lipids were extracted from subsamples of each insect stage (fifth-instar nymphs, males and females) and each treatment (insects collected in soybean crops either with or without insecticides application) by immersing them in three successive portions (1 ml each) of redistilled *n*-hexane (6 ml g^{-1}) (Carlo Erba) for 5 min each. *n*-Docosane (300 ng) was added to subsamples as the internal standard to allow future hydrocarbon quantification. The total number of subsamples (each containing three insects) extracted was five treated and six untreated nymphs, nine treated and ten untreated males, and ten treated and nine untreated females. The hydrocarbon fraction was purified by adsorption chromatography on a mini-column ($2.5 \times 0.5\text{ cm}$) of activated Supelcosil A (Supelco), eluted with redistilled *n*-hexane (6 ml mg^{-1} lipid), then concentrated to an appropriate volume under a nitrogen stream.³¹

2.3 Epicuticular hydrocarbon analysis

CHC analysis by capillary gas chromatography–mass spectrometry (CGC–MS) was performed using a Hewlett Packard 6890 gas chromatograph equipped with a Zebtron ZB-5HT Inferno capillary column (30 m long $\times$ 0.25 mm internal diameter $\times$ 0.25 μm stationary phase; Phenomenex), and coupled with a mass selective detector (5975C VL; Agilent). The injection port (splitless mode) was set at 360°C, and the temperature program for CGC was set-up as follows: 50°C for 1 min (initial), from 50 to 200°C at a rate of $50^\circ\text{C min}^{-1}$ (ramp 1), from 200 to 360°C at a rate of 7°C min^{-1} (ramp 2), and then held at 360°C for 20 min. The ion source, transfer line and quadrupole were maintained at 280, 360 and 150°C, respectively. Interpretation of mass spectra was performed.^{32,33} Shorthand nomenclature was used in text and tables to identify the hydrocarbons as follows: CXX, the total number of carbons in the straight chain; *n*-CXX, linear alkanes; CXX:x, the number of unsaturations in unsaturated alkanes. The location of methyl branches was described as: x-Me, monomethyl alkanes; x,x-DiMe, dimethyl alkanes; and x,x,x-TriMe, trimethyl alkanes. Methyl branching assignments were based on their chromatographic retention index (KI) values and mass fragmentation patterns.^{32,34,35}

Hydrocarbons were also analyzed by CGC coupled to a flame ionization detector, using helium as a mobile phase (2 ml min^{-1}), and setting the flame ionization detector at 360°C. Extracts obtained from each subsample were dissolved in *n*-hexane (10 μl) and 1 μl was later injected into CGC. The KI value was calculated for each hydrocarbon peak,³⁶ after measuring the elution times of commercially available *n*-alkane standards of 22–44 carbons (Sigma Aldrich) run under similar conditions. Quantitative amounts were estimated by co-injection of *n*-C22 as an internal standard (30 ng per sample).

2.4 Statistical analysis

Significant differences between samples were assessed by a *t*-test or, alternatively, by a one-way analysis of variance (ANOVA) in SPSS v.15.0.1 (SPSS Inc) after log-e transformation of CHC amount data. The hypotheses of normality and homoscedasticity were

Table 1. Epicuticular hydrocarbons of the redbanded stink bug *Piezodorus guildinii*

Peak ^a	KI	Full name ^b	Shorthand nomenclature ^b	Diagnostic ions, <i>m/z</i> ^c
1	1200	<i>n</i> -Dodecane	<i>n</i> -C12	170
2	1292	<i>x</i> -Tridecene	<i>x</i> -C13:1	182
3	1300	<i>n</i> -Tridecane	<i>n</i> -C13	184
4	1392	<i>x</i> -Tetradecene	<i>x</i> -C14:1	196
5	1400	<i>n</i> -Tetradecane	<i>n</i> -C14	198
6	1492	<i>x</i> -Pentadecene	<i>x</i> -C15:1	210
7	1500	<i>n</i> -Pentadecane	<i>n</i> -C15	212
8	1592	<i>x</i> -Hexadecene	<i>x</i> -C16:1	224
9	1600	<i>n</i> -Hexadecane	<i>n</i> -C16	226
10	1700	<i>n</i> -Heptadecane	<i>n</i> -C17	240
11	1800	<i>n</i> -Octadecane	<i>n</i> -C18	254
12	2082	<i>x</i> -Heneicosene	<i>x</i> -C21:1	294
13	2100	<i>n</i> -Heneicosane	<i>n</i> -C21	296
14	2250	<i>x,x</i> -Tricosadiene	<i>x,x</i> -C23:2	320
15	2254	<i>x</i> -Tricosene	<i>x</i> -C23:1	322
16	2300	<i>n</i> -Tricosane	<i>n</i> -C23	324
17	2316	9-; 11-Methyl-tricosane	9-; 11-MeC23	140/141, 224/225; 168/169, 196/197; 323
18	2323	7-Methyl-tricosane	7-MeC23	112/113, 252/253; 323
19	2396	Unknown	Unknown	
20	2404	3,13-Dimethyl-tricosane	3,13-DiMeC23	56/57; 323; 168/169; 211; 337
21	2429	Unknown	Unknown	
22	2467	<i>x,x</i> -Pentacosadiene	<i>x,x</i> -C25:2	348
23	2471	<i>x</i> -Pentacosene	<i>x</i> -C25:1	350
24	2479	<i>x</i> -Pentacosene	<i>x</i> -C25:1	350
25	2500	<i>n</i> -Pentacosane	<i>n</i> -C25	352
26	2533	9-; 11-; 13-Methyl-pentacosane	9-; 11-; 13-MeC25	140/141; 252/253; 196/197; 168/169, 224/225; 351
27	2540	7-Methyl-pentacosane	7-MeC25	112/113, 280/281; 351
28	2600	<i>n</i> -Hexacosane	<i>n</i> -C26	366
29	2607	3,13-; 3,15-Dimethyl-pentacosane	3,13-; 3,15-DiMeC25	56/57, 351; 168/169, 239; 196/197, 211; 365
30	2666	<i>x,x</i> -Heptacosadiene	<i>x,x</i> -C27:2	376
31	2673	<i>x</i> -Heptacosene	<i>x</i> -C27:1	378
32	2700	<i>n</i> -Heptacosane	<i>n</i> -C27	380
33	2727	9-; 11-; 13-Methyl-heptacosane	9-; 11-; 13-MeC27	140/141, 280/281; 168/169, 252/253; 196/197, 224/225; 379
34	2736	7-Methyl-heptacosane	7-MeC27	112/113, 308/309; 379
35	2772	3-Methyl-heptacosane	3-MeC27	56/57, 364/365; 379
36	2800	<i>n</i> -Octacosane	<i>n</i> -C28	394
37	2807	Unknown	Unknown	
38	2823	Unknown	Unknown	
39	2829	Unknown	Unknown	
40	2837	7-Methyl-octacosane	7-MeC28	112/113, 322/323, 393
41	2853	4-Methyl-octacosane	4-MeC28	70/71, 364/365, 393
42	2866	<i>x,x</i> -Nonacosadiene	<i>x,x</i> -C29:2	404
43	2900	<i>n</i> -Nonacosane	<i>n</i> -C29	408
44	2932	9-; 11-; 13-; 15-Methyl-nonacosane	9-; 11-; 13-; 15-MeC29	140/141, 308/309; 168/169, 280/281; 196/197, 252/253; 224/225; 407
45	2940	7-Methyl-nonacosane	7-MeC29	112/113, 336/337; 407
46	2963	Unknown	Unknown	
47	2974	3-Methyl-nonacosane/7,11-; 7,17-; 7,19-Dimethyl-nonacosane	3-MeC29/7,11-; 7,17-; 7,19-DiMeC29	56/57, 392/393; 407/112/113, 351, 280/281, 183; 196/197, 267, 295; 421
48	3000	<i>n</i> -Triacontane	<i>n</i> -C30	422
49	3007	3,23-Dimethyl-nonacosane	3,23-DiMeC29	56/57, 407; 126/127, 337; 421
50	3032	9-, 10-, 11-, 12-, 13-, 14-Methyl-triacontane	9-, 10-, 11-, 12-, 13-, 14-MeC30	140/141, 322/323; 154/155, 308/309; 168/169, 294/295; 196/197, 266/267; 182/183, 280/281; 210/211; 252/253; 421
51	3040	7-Methyl-triacontane	7-MeC30	112/113, 350/351; 421

Table 1. Continued

Peak ^a	KI	Full name ^b	Shorthand nomenclature ^b	Diagnostic ions, <i>m/z</i> ^c
52	3065	10,20; 7,19-Dimethyl-triacontane	10,20; 7,19-DiMeC30	154/155, 323; 168/169; 309; 435/112/113, 365; 182/183, 295; 435
53	3100	<i>n</i> -Hentriacontane	<i>n</i> -C31	436
54	3139	9-; 11-; 13-; 15-Methyl-hentriacontane	9-; 11-; 13-; 15-MeC31	140/141, 336/337; 168/169, 308/309; 196/197, 280/281; 224/225, 252/253; 435
55	3143	7-Methyl-hentriacontane	7-MeC31	112/113, 364/365; 435
56	3165	<i>x,y</i> -Dimethyl-hentriacontane (<i>x</i> = 7, 9, 11, 13; <i>y</i> = 19, 21, 23, 25)	<i>x,y</i> -DiMeC31 (<i>x</i> = 7, 9, 11, 13; <i>y</i> = 19, 21, 23, 25)	112/113, 379; 140/141, 351; 168/169, 323; 196/197, 295; 449
57	3175	3-Methyl-hentriacontane	3-MeC31	56/57, 420/421, 435
58	3200	7,11,21-; 7,13,21-Trimethyl-hentriacontane	7,11,21-; 7,13,21-TriMeC31	112/113, 393; 183, 323, 211, 295; 168/169, 337; 463
59	3230	9-, 10-, 11-, 12-, 13-, 14-, 15-, 16-Methyl-dotriacontane	9-, 10-, 11-, 12-, 13-, 14-, 15-, 16-MeC32	140/141, 350/351; 154/155, 336/337; 168/169, 322/323; 182/183, 308/309; 196/197, 294/295; 210/211, 280/281; 224/225, 266/267; 238/239, 252/253; 449
60	3262	11,21-Dimethyl-dotriacontane	11,21-DiMeC32	168/169, 337; 182/183, 323; 463
		9,21-; 11,19-; 10,22-; 12,20-Dimethyl-dotriacontane	9,21-; 11,19-; 10,22-; 12,20-DiMeC32	140/141, 365; 182/183, 323; 168/169, 337; 210/211, 295; 154/155, 351; 196/197, 309; 463
		9,19-; 9,23-; 10,20-; 13,19-Dimethyl-dotriacontane	9,19-; 9,23-; 10,20-; 13,19-DiMeC32	140/141, 365; 210/211, 295; 154/155, 351; 196/197, 309; 463
61	3287	<i>x</i> -Tritriacontane	<i>x</i> -C33:1	462
62	3300	<i>n</i> -Tritriacontane	<i>n</i> -C33	464
63	3344	9-, 11-, 13-, 15-, 17-Methyl-tritriacontane	9-, 11-, 13-, 15-, 17-MeC33	140/141, 364/365; 168/169, 336/337; 196/197, 308/309; 224/225, 280/281; 252/253; 463
64	3377	11,21-; 11,23-Dimethyl-tritriacontane	11,21-; 11,23-DiMeC33	168/169, 351; 196/197, 323; 477
		9,21-; 9,23-; 13,21-Dimethyl-tritriacontane	9,21-; 9,23-; 13,21-DiMeC33	140/141, 379; 196/197, 323; 168/169, 351; 477
		9,19-; 9,25-; 11,19-; 13,19-; 15,19-Dimethyl-tritriacontane	9,19-; 9,25-; 11,19-; 13,19-; 15,19-DiMeC33	140/141, 379; 224/225, 295; 168/169, 351; 196/197, 323; 477
65	3394	9,13,23-Trimethyl-tritriacontane	9,13,23-TriMeC33	140/141, 393, 211, 323, 168/169, 295; 491
66	3426	11-; 12-; 13-; 14-; 15-; 16-; 17-Methyl-tetratriacontane	11-; 12-; 13-; 14-; 15-; 16-; 17-MeC34	168/169, 350/351; 182/183, 336/337; 196/197, 322/323; 210/211, 308/309; 224/225, 294/295; 238/239, 280/281; 252/253, 266/267; 477
67	3455	10,22-; 10,24-; 12,22-Dimethyl-tetratriacontane	10,22-; 10,24-; 12,22-DiMeC34	154/155, 379; 196/197, 337; 168/169, 365; 182/183, 351; 491
68	3481	Unknown	Unknown	
69	3492	Unknown	Unknown	
70	3520	11-; 13-; 15-; 17-Methyl-pentatriacontane	11-; 13-; 15-; 17-MeC35	168/169, 364/365; 196/197, 336/337; 224/225, 308/309; 252/253, 280/281; 491
71	3549	11,21-; 11,23-; 11,25-; 13-21-; 13,23-Dimethyl-pentatriacontane	11,21-; 11,23-; 11,25-; 13-21-; 13,23-DiMeC35	168/169, 379; 196/197; 351; 224/225, 323; 505
72	3570	<i>x,x,x</i> -Trimethyl-pentatriacontane	<i>x,x,x</i> -TriMeC35	519

x, unidentified position.

^aNumbers correspond to peaks from Figure 1. The capillary gas chromatography peaks are listed in order of elution. Some minor components may not be evident in Figure 1.

^bStraight chain hydrocarbons were identified by comparison of their Kovats index (KI) with that of the corresponding standards. Methyl branching was estimated based on their KI values and their mass fragmentation pattern.

^cDiagnostic ions from chemical ionization mass spectral analysis.

tested, and the samples followed the criteria to carry out parametric tests. Principal component analysis (PCA) was carried out to select the CHC components that best contributed to differentiate between sexes and developmental stages in MVSP v.3.13b (Kovach Computing Services), hydrocarbons with variable loading values equal to or higher than 0.20 were selected as the best contributing components.

3 RESULTS

3.1 Volatile and epicuticular hydrocarbon patterns in juvenile and adult stages

Odd-numbered saturated straight chains, and isomeric mixtures of odd-chains with the methyl groups inserted at odd carbon numbers were mostly detected (Table 1). Chromatograms exhibited two different groups of peaks (Figure 1).

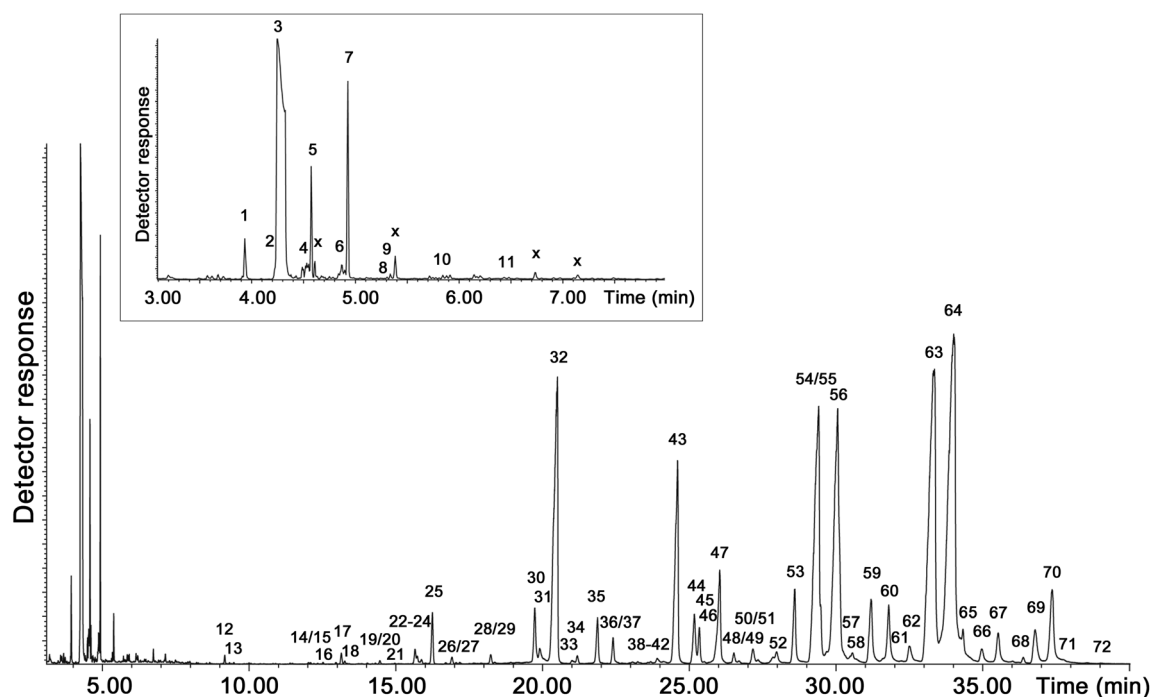


Figure 1. Chromatographic hydrocarbon profile of adult female of the redbanded stink bug *Piezodorus guildinii*. Numbers indicating hydrocarbon peaks correspond to numbers in Table 1. Inset: Volatile hydrocarbons, x, unidentified compounds.

The first group is represented by volatile hydrocarbons with a short to medium chain length (from C11 to C16), with *n*-C13 and *n*-C15 the predominant compounds detected after CGC-MS analysis (Table 1). Traces of the corresponding unsaturated hydrocarbons were also detected (Figure 1, inset). Compounds of this first group are released from glandular reservoirs and are often not regarded as CHCs⁹; thus, they were not considered for further quantitative analysis. Hydrocarbons that are true components of the epicuticle were the majority of the very long-chain compounds detected (from C21 to C35), and the corresponding peaks are shown in the second group of the chromatogram. Among them, the *n*-alkanes *n*-C27 and *n*-C29, and the monomethyl and dimethyl chains of C31 and C33 were the most abundant compounds (Table 1 and Figure 1).

Because of the different whole-body weights found for the different development stages (mean $\pm$ SD) (fifth-instar nymphs: 48.7 ± 3.6 mg, adult males: 54.7 ± 3.7 mg, adult females: 71.4 ± 6.5 mg), the total hydrocarbon quantities were referred by insect fresh body weight. After one-way ANOVA followed by Tukey's post-hoc test, the total hydrocarbon amount was significantly higher in adult males (86.9 ± 12.1 ng CHC per mg insect) than in adult females (54.7 ± 10.2 ng CHC per mg insect) ($P < 0.0001$) and fifth-instar nymphs (39.5 ± 4.4 ng CHC per mg insect) ($P < 0.0001$) collected from insecticide-free crops.

Differences in the hydrocarbon pattern between developmental stages of insects collected from insecticide-free crops, that is nymphs versus adults, and males versus females (Figure 2), were also evident. After PCA (Figure 3A), 21 of 49 CHC were selected as the key components to differentiate between nymphs and adults (Table 2). Most of these CHC were present in higher amounts in adults than nymphs, except for KI 2666, which was more abundant in nymphs than in adult males.

3.2 Sexual dimorphism

As detailed before, significant differences ($P < 0.0001$) were observed in the total amount of CHC between males and females collected in insecticide-free soybean fields, with males having higher amounts than females. Regarding the CHC pattern, 42 components were quantitatively different between both sexes; thus, PCA was used to select those which contributed most to sexual dimorphism (Figure 3B and Table 3). Heneicosene (KI 2098) was almost exclusively detected in

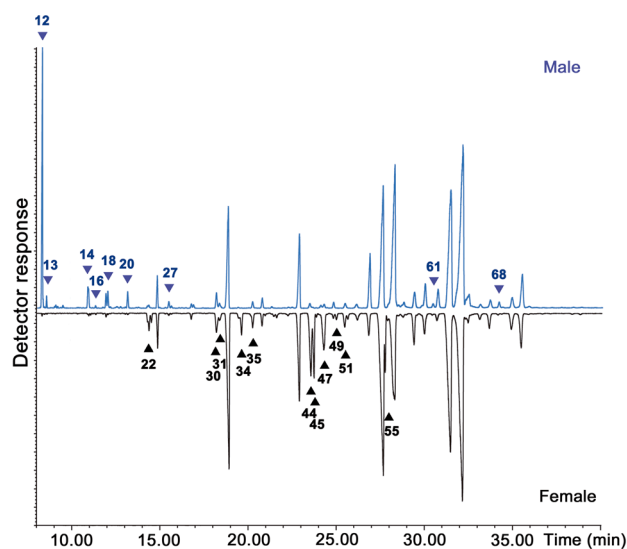
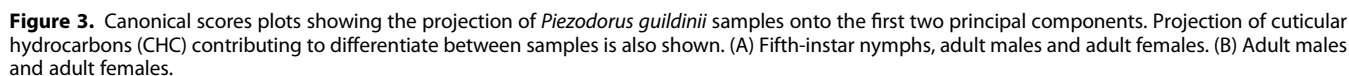


Figure 2. Chromatograms of male (upper) and female (lower) *Piezodorus guildinii*. The peaks with significant differences ($P < 0.05$) in the total amount of cuticular hydrocarbons (CHC) that mostly contribute to differences between males and females are marked with an arrow. Peak numbers correspond to Table 1.



Different whole-body weights between different development stages were also found in insects collected from insecticide-treated crops (mean $\pm$ SD) (fifth-instar nymphs: 37.6 ± 0.8 mg, adult males: 52.2 ± 2.2 mg, adult females: 79.5 ± 3.9 mg). Then,

Table 2. Selected epicuticle hydrocarbons of the redbanded stink bug *Piezodorus guildinii* with statistically significant differences between nymphs and adults

KI	Nymphs	Males	Females
2082	0.32 ± 0.03	548.40 ± 62.29	13.16 ± 2.58
2100	0.70 ± 0.02	59.70 ± 10.90	2.43 ± 0.43
2250	0.34 ± 0.03	129.62 ± 9.77	11.33 ± 3.36
2254	0.25 ± 0.01	1.09 ± 0.13	1.43 ± 0.61
2316	0.54 ± 0.11	34.56 ± 5.24	16.02 ± 5.23
2323	0.70 ± 0.19	43.15 ± 5.49	4.22 ± 0.41
2404	1.13 ± 0.19	43.66 ± 6.22	4.93 ± 0.82
2467	4.63 ± 1.66	9.05 ± 1.35	42.11 ± 8.37
2479	0.62 ± 0.22	1.83 ± 0.44	9.07 ± 2.36
2500	3.25 ± 0.35	28.77 ± 2.56	59.51 ± 21.12
2533	1.10 ± 0.17	23.57 ± 2.44	8.10 ± 1.62
2666	37.25 ± 10.39	18.63 ± 4.94	125.03 ± 20.43
2673	13.51 ± 3.35	23.04 ± 5.94	66.00 ± 8.35
2772	20.49 ± 2.87	24.18 ± 4.85	51.30 ± 4.84
2932	3.32 ± 0.30	55.79 ± 6.37	145.28 ± 33.97
2940	0.46 ± 0.05	11.39 ± 2.58	91.85 ± 22.75
2973	17.45 ± 0.98	35.42 ± 6.96	159.40 ± 33.32
3032	2.91 ± 0.22	37.99 ± 2.88	27.80 ± 5.51
3065	3.61 ± 0.16	41.90 ± 4.23	30.51 ± 3.71
3394	21.40 ± 1.66	88.74 ± 5.25	33.72 ± 4.78
3481	1.08 ± 0.08	39.85 ± 7.40	4.48 ± 1.41

Values are mean ng hydrocarbon per insect ± standard error of mean; five replicates (three insects each).

Insects were collected in insecticide-free soybean crops.

Significant differences in hydrocarbon amounts between samples were first determined by a one-way ANOVA ($\alpha \leq 0.05$), followed by a PCA analysis to select those which contributed the best to differentiation.

KI, Kovats index.

after one-way ANOVA followed by Tukey's post-hoc test, significant differences in their total hydrocarbon amounts were also detected between adult males (115.0 ± 38.0 ng CHC per mg insect) and adult females (78.9 ± 18.0 ng CHC per mg insect) ($P < 0.0001$), as well as between adult males and fifth-instar nymphs (75.4 ± 11.5 ng CHC per mg insect) ($P < 0.0001$).

Comparison of CHC relative total amounts between bugs collected from insecticide-treated soybean fields and those obtained from untreated fields showed significantly higher values in the former situation for nymphs, males and females ($P < 0.0001$, $P = 0.0036$ and $P = 0.0008$, respectively) (Figure 4). Analysis of the CHC pattern showed that 13, 7 and 4 hydrocarbons significantly varied in their amount among nymphs, males and females, respectively (Table 4 and Figure 5). Monomethyl and dimethyl CHC chains from C27 to C33, together with some *n*-alkanes predominated in this list (Table 4).

4 DISCUSSION

The hydrocarbon profile extracted from the epicuticle of one of the most important soybean pests in South America, the redbanded stink bug *P. guildinii*, was characterized. The main conclusions of the study were: (i) there are significant differences in hydrocarbon composition between insect stages and sexes; and (ii) there are significant alterations in the amounts of CHCs in fifth-instar nymphs and adults, both male and female, collected

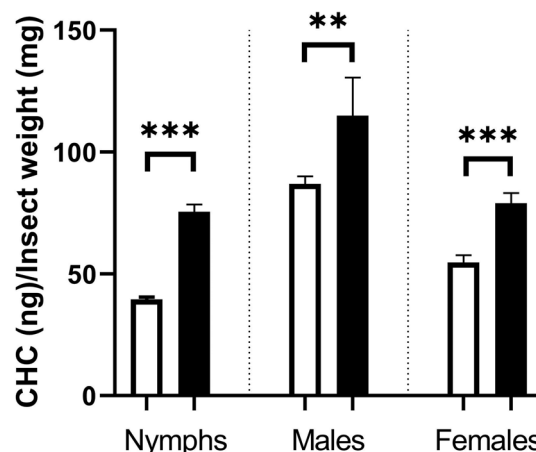


Figure 4. Total hydrocarbon amounts per insect fresh body in different stages of *Piezodorus guildinii* collected in soybean crops either treated (black bars) or not treated (white bars) with insecticides. ** $P < 0.005$, *** $P < 0.0005$.

in insecticide-treated soybean fields compared with those collected from insecticide-free crops.

Regarding the hydrocarbon profile, two different groups of hydrocarbons were found: one containing volatile compounds of short/medium chain length with *n*-tridecane and *n*-pentadecane as the predominant compounds, and the other containing the major constitutive components of the epicuticle comprising very long-chain hydrocarbons. A similar profile was described in the southern green stink bug *N. viridula*.³⁷ However, in *N. viridula* *n*-pentadecane is absent in the first group of volatile hydrocarbons, as well as monomethyl and dimethyl chains of C33 in the second group. Also, peaks corresponding to branched chains of C31 are not well resolved in that study, probably due to the sensitivity of the equipment used more than 20 years ago. A new re-analysis with current methodology would help to solve accurately the very long-chain compounds of the southern green stink bug, and thus establish whether or not there are real differences between these two related stink bugs. In summary, we found mostly *n*-alkanes and branched chain alkanes, except for an unsaturated CHC found mostly in males (see below). These results are in concordance with reports from all insect orders, because unsaturated hydrocarbons are much less common than saturated hydrocarbons in insect cuticles.⁹

It is well known that some stink bugs such as *N. viridula*, *P. guildinii*, *Cosmopepla bimaculata* and *Tibraca limbativentris* secrete a blend containing *n*-tridecane and a mix of aldehydes, such as (*E*)-2-hexenal, (*E*)-2-octenal and (*E*)-2-decenal from their paired metathoracic glands.^{38–40} Differences in the composition of these secretions were reported between nymphs, males and females of five species of neotropical stink bugs, including *P. guildinii*.⁴¹ In *N. viridula*, these aldehydes function as adult alarm pheromones,³⁸ to attract female egg parasitoids (*Trissolcus basalis*),⁴² and protect *N. viridula* and *T. limbativentris* against infection by entomopathogenic fungi.^{37,40} Similar glandular secretion containing unsaturated medium-chain hydrocarbons (mainly 1-pentadecene) and alkyl-branched ketones was found in the coleopterans *Tribolium castaneum* and *Ulomoides dermestoides*.^{43–45} Although methyl- and ethyl-benzoquinones secreted by both beetles have also been reported as alarm pheromones and have fungistatic effects,^{46,47} similar to the aldehydes from

Table 3. Selected epicuticle hydrocarbons of adults of the red-banded stink bug *Piezodorus guildinii* with statistically significant differences contributing to sexual dimorphism

KI	Males	Females
2082	548.40 ± 62.29	13.16 ± 2.58
2100	59.70 ± 10.90	2.43 ± 0.43
2250	129.62 ± 9.77	11.33 ± 3.36
2300	7.48 ± 1.12	2.33 ± 0.54
2323	43.15 ± 5.49	4.22 ± 0.41
2404	43.66 ± 6.22	4.93 ± 0.82
2467	9.05 ± 1.35	42.11 ± 8.37
2540	10.37 ± 1.45	3.87 ± 0.37
2666	18.63 ± 4.94	125.03 ± 20.43
2673	23.04 ± 5.94	66.00 ± 8.35
2736	4.09 ± 0.99	52.31 ± 12.89
2772	24.18 ± 4.85	51.30 ± 4.84
2932	55.79 ± 6.37	145.28 ± 33.97
2940	11.39 ± 2.58	91.85 ± 22.75
2973	35.42 ± 6.96	159.40 ± 33.32
3007	1.16 ± 0.25	6.71 ± 2.39
3040	1.31 ± 0.19	7.86 ± 2.29
3143	2.89 ± 1.31	40.13 ± 13.00
3287	46.55 ± 10.75	3.99 ± 1.17
3481	39.85 ± 7.40	4.48 ± 1.41

Values are mean ng hydrocarbon per insect ± standard error of mean; five replicates (three insects each).

Insects were collected in insecticide-free soybean crops.

Significant differences in hydrocarbons amounts between samples were first determined by a t-test ($\alpha \leq 0.05$), followed by a PCA analysis to select those which contributed the best to differentiation.

KI, Kovats index.

T. limbativentris,⁴⁰ it seems that 1-pentadecene has only a carrier role for these biological active carbonyls.⁴⁵ However, *n*-tridecane induces nymphal aggregation in *N. viridula* and also insect dispersal at higher concentrations³⁸; and it plays a defensive role against predators in *N. viridula*,⁴⁸ and *C. bimaculata*.⁴⁹ The biological role of this compound in *P. guildinii* needs to be further studied. The pheromone-producing gland in *P. guildinii* is similar to that reported for *N. viridula*, that is, it is not discrete but made up of numerous minute glands in the ventral subcuticular tissues.^{50,51} This gland structure, together with the chain length of the components belonging to the first group, suggest that they were collected from the insect glands during extraction procedures rather than being constitutive components of the epicuticle.

We found significant differences in constitutive hydrocarbons between fifth-instar nymphs and adults, with 21 CHC proposed as the most important to differentiate between both developmental stages. Although it is well-known that the qualitative hydrocarbon pattern is species specific and does not significantly change as a consequence of aging, significant quantitative changes related to insect development are widely reported for many insect orders.⁹ These changes provide insects with mechanisms to adapt to different environmental conditions; for example, a higher ratio of short hydrocarbon chains facilitates higher water loss and, conversely, a greater abundance of long carbon chains correlates well with lower cuticle permeability.⁵² In the hemipteran *Triatoma infestans*, nymph-adult transition brings

together a major switch in the relative amount of the major straight chain epicuticular components, with *n*-C31 and *n*-C33 predominating in nymphs and *n*-C29 predominating in adults.¹⁰

Regarding sex recognition, there is extensive literature on the pheromonal role of cuticular hydrocarbons; among them, chain lengths like those here reported, and much longer chains, have been described as contact sex pheromones in several insects.⁹ In this study, we also found sexual dimorphism in the CHC components of adults of *P. guildinii*. Of the 42 CHC with significant differences between sexes, the unsaturated hydrocarbon heneicosene (KI 2098) was almost exclusively detected in males and was the most important component that contributed to sexual dimorphism. The occurrence of unsaturated hydrocarbons with pheromonal role have been described in *Musca domestica* and *Bactrocera oleae* [(*Z*)-9-tricosene]^{53,54} and *Drosophila virilis* [(*Z*)-10-heneicosene].⁵⁵ Interestingly, in the stink bug *C. bimaculata* (*E*)-8-heneicosene was found only in undisturbed males.⁴⁹ Although the function of this compound remains uncertain, it has been suggested to have a possible role as a male sex pheromone because it was isolated from field-collected males during the breeding season, but it was not detected in males from newly emerged adults collected when copulation was not observed.⁴⁹ Thus, the potential pheromonal role of heneicosene in *P. guildinii* needs to be further investigated.

We also found significant differences in the CHC pattern of bugs collected in soybean fields previously treated with insecticides compared with bugs collected from untreated fields. As far as we know, the only study available on CHC profile alterations after direct insecticide exposure was done in the leaf beetle *Phaedon cochleariae*, in which one hydrocarbon was only detected in insects exposed to lambda-cyhalothrin, and another hydrocarbon was detected in insecticide-treated males but not in treated females or untreated beetles¹⁶; however, their identities were not provided (only their retention index) and thus comparison with our results cannot be made. Surface hydrocarbons are discarded after each molt; thus, they are not reusable from one developmental stage to another. The biosynthesis of hydrocarbons, their transport to and their deposition on the new cuticle start under the old cuticle in the previous stage.¹⁰ However, the process can continue during the current stage because it has been shown that CHC are newly deposited on the surface after being mechanically removed.⁶ It is well-known that redbanded stink bug adults move unidirectionally and bidirectionally between soybean and alfalfa crops.²⁸ Adults move into soybean fields as the plants mature during the R2 and R3 stages, with peak populations observed at maturity stage R7.²³ Nymph dispersal is restricted because they lack wings, and egg clusters laid in the top 35 cm of the plant are also stationary. Considering both the incapacity of nymphs and eggs to fully escape insecticide action and the history of field applications in this case, the changes observed in the epicuticular composition could be the result of the insecticide exposure at sublethal doses of eggs and nymphs that later turn into adults. This might be occurring since chemical treatment often results inefficient due to issues with canopy penetration.⁵⁶

One of the mechanisms proposed to contribute to insecticide resistance is known as 'cuticular factor', and involves a thickening of insect surface that leads to reduced insecticide penetration across the cuticular barrier.^{13,57} In concordance with our results, an increase in the amount of CHC has also been reported in pyrethroid-resistant kissing bugs^{12,13} and mosquitoes.^{15,58} Knockdown of the genes involved in CHC

Table 4. Epicuticle hydrocarbons (ng per insect) with significant differences between nymphs, males, and females of the redbanded stink bug *Piezodorus guildinii* collected from insecticide-free (–) or insecticide-treated (+) soybean crops

KI	Nymphs		Males		Females	
	–	+	–	+	–	+
2300	0.90 ± 0.10	0.52 ± 0.10				
2396	1.51 ± 0.15	0.68 ± 0.22				
2700	147.37 ± 10.71	251.65 ± 20.54				
2736			4.09 ± 0.99	0.97 ± 0.19		
2800	8.41 ± 0.56	10.26 ± 0.48				
2823					0.70 ± 0.09	1.21 ± 0.08
2853			5.23 ± 0.35	2.41 ± 0.24		
2932	3.32 ± 0.30	4.77 ± 0.33	55.79 ± 6.37	37.31 ± 4.59		
2940	0.46 ± 0.05	1.00 ± 0.17	11.39 ± 2.58	2.81 ± 0.72		
3007			1.16 ± 0.25	0.55 ± 0.12		
3032	2.91 ± 0.22	4.71 ± 0.42				
3040			1.31 ± 0.20	0.55 ± 0.06		
3065	3.61 ± 0.16	4.47 ± 0.35	41.90 ± 4.23	24.76 ± 2.53	30.51 ± 3.71	18.76 ± 0.56
3100	102.75 ± 3.51	158.21 ± 12.24				
3139	138.53 ± 6.08	233.29 ± 20.60				
3165	228.47 ± 10.19	305.05 ± 14.83				
3300	40.64 ± 1.78	83.20 ± 9.26				
3344					618.00 ± 88.65	943.71 ± 69.93
3492	0.00 ± 0.00	2.06 ± 0.24			2.51 ± 0.26	1.64 ± 0.1

Values are mean ng hydrocarbon per insect ± standard error of mean; five replicates (three insects each).

Significant differences between samples were determined by a *t*-test ($\alpha \leq 0.05$).

KI, Kovats index.

biosynthesis caused a reduction in CHC, which led to enhanced cuticular penetration of insecticides in *Blattella germanica* and *Locusta migratoria*.^{59,60}

Müller *et al.* hypothesized that insecticide effects on CHC profiles might be caused by changes in CHC precursors but also in enzymes involved in their biosynthesis, mostly CYP genes

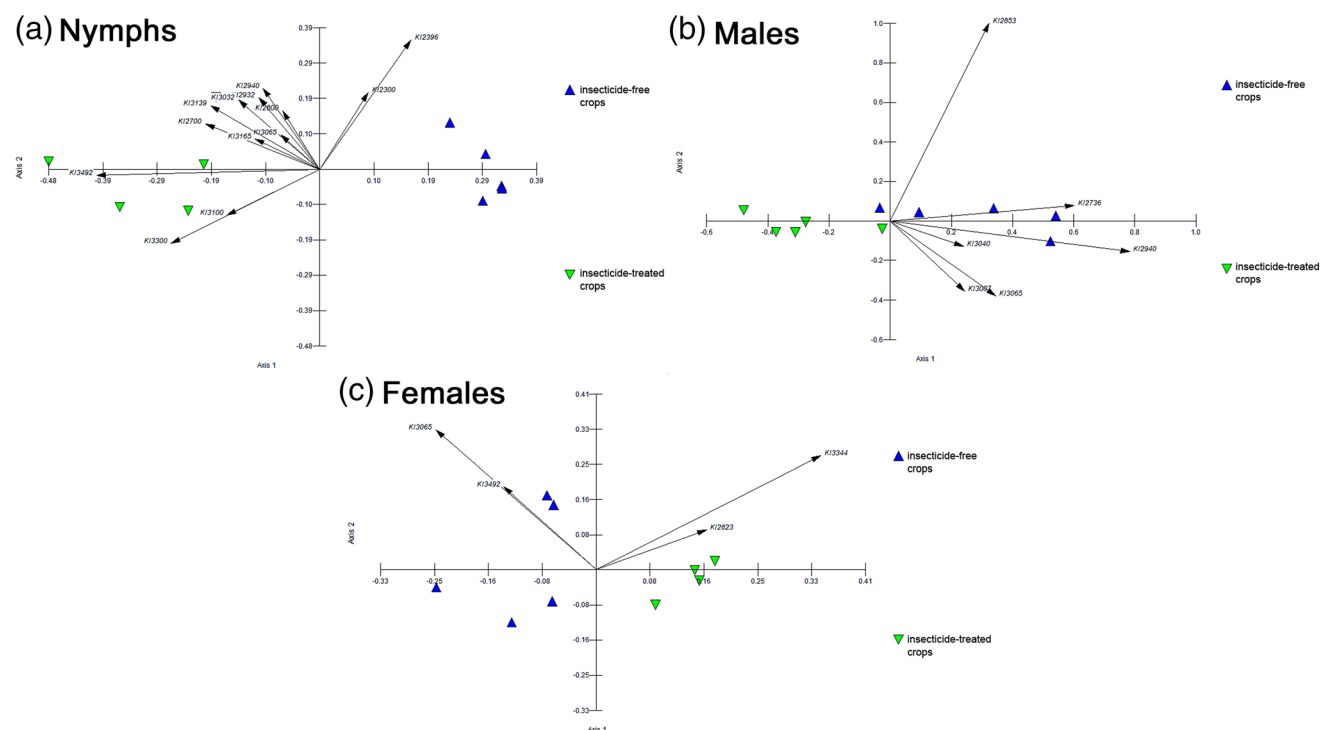


Figure 5. Canonical scores plots showing the projection of *Piezodorus guildinii* samples onto the first two principal components. Projection of cuticular hydrocarbons (CHC) contributing to differentiate between each set of samples is also shown. (A) Fifth-instar nymphs. (B) Adult males. (C) Adult females. Samples were collected either from insecticide-free or insecticide-treated soybean crops.

encoding cytochrome P450s, which are often induced by insecticide treatment. We have previously reported a role for CYP genes from *T. infestans* in the detoxification of deltamethrin in pyrethroid-resistant insects.^{61,62} We could speculate that insecticide applications for controlling stink bugs (particularly the pyrethroid component of Engeo whose mode of action is by contact penetrating the insect cuticle) might be forcing the insect plasticity to a phenotype with larger amounts of CHC, and this could contribute, ultimately, to insecticide tolerance or resistance.⁶³ Additional research, especially functional characterization of *P. guildinii* genes involved in CHC biosynthesis, is needed to confirm or refute this hypothesis. In practical terms, this could lead to the deployment of strategies like pyrethroid avoidance to reduce CHC build-up, or the use of compounds targeting the epicuticular CHC, like the inclusion of petroleum spray oils, which contain similar CHC to those present in the epicuticle and thus readily interact and modify its structure, leading to the death of insects.⁶⁴ Other questions have arisen with this research that should be further investigated: what is the function of heneicosene only detected in males? Why do insects in close contact with pesticides increase the amount of cuticular hydrocarbons? And whether this phenomenon is related to failures in the control of these insects frequently detected in soybean crops.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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Capítulo 2_

Control Biológico de *P.*
guildinii y *T. peregrinus* e
interacción de *B. bassiana* con
HC sintéticos

Introducción

2.1 Control Biológico de insectos: hongos entomopatógenos

El control de insectos plaga con pesticidas químicos ha sido la estrategia más ampliamente utilizada a lo largo de los años como estrategia de control, sin embargo, su uso ha traído como consecuencias la prevalencia de poblaciones resistentes a insecticidas, brotes de plagas secundarias controladas por enemigos naturales, riesgos de seguridad para humanos y animales domésticos, contaminación de aguas subterráneas, disminución de la biodiversidad y otras preocupaciones ambientales. Todo esto ha traído un mayor interés en el manejo integrado de plagas (MIP), que propone la utilización de productos químicos más selectivos, menos tóxicos, aplicaciones en dosis mínimas y complementación con otras estrategias de control ecológicamente amigables. Entre estas estrategias se encuentra el control biológico, basado en el empleo de bioplaguicidas: productos en base a animales y microorganismos para prevenir, repeler, eliminar o reducir los daños causados por las plagas (Lecuona, 1996; Lacey et al., 2001; Kumar & Kalita, 2017). En este sentido, distintos microorganismos son empleados como agentes de control biológico para controlar insectos, entre los que se encuentran los hongos, los virus, los protozoarios, nematodos y las bacterias. A diferencia de virus y bacterias, los hongos presentan una ruta de infección directa a través de la cutícula del insecto, actuando como un insecticida de contacto, pudiendo además potencialmente causar epizootias de una manera dependiente de la densidad (Hajek & St. Leger, 1994).

Los microorganismos más utilizados para el control microbiano de insectos plaga son los hongos entomopatógenos (HEP) de los géneros *Beauveria* y *Metarhizium* (Ascomycota: Hypocreales) (Faria & Wraight, 2007; Lacey et al., 2015; Lovett & St. Leger, 2017).

Ejemplos de bioinsecticidas a base de hongos entomopatógenos registrados para el control de insectos plaga son BioPower (*B. bassiana*), Osho Chemical Industries Ltd (Kenia), para el control de gusanos barrenadores y cortadores, BotaniGard®22WP y Mycotrol®ESO, (*B. bassiana*), Bioworks Inc, (Estados Unidos), para el control de Ortópteros, mosca blanca, áfidos, trips, psílidos, pseudocóccidos, Lepidópteros, Heterópteros, etc., ALMID (*M. anisopliae*) y ALMITE (*P. fumosoroseus*), AMRUTH ORGANIC FERTILIZERS (India) para el control de gusanos barrenadores y cortadores y termitas y en nuestro país CREBIO 1, (*B. bassiana*) (Ñangapiré, Uruguay), para el control de hormigas cortadoras.

Si bien se reconoce que la chinche plaga de la soja *P. guildinii* naturalmente es poco susceptible a la infección por hongos entomopatógenos, posiblemente debido al efecto de los compuestos volátiles que segregan sus glándulas metatorácicas (Lopes et al., 2015) se ha reportado su infección por hongos entomopatógenos como *Beauveria bassiana* y *Metarhizium anisopliae* (Sosa-Gomez et al., 1997). En nuestro país no se han registrado epizootias atribuibles a HEP y el número de individuos con micosis es bajo (Castiglioni com. pers.). Sin embargo, estudios recientes indican que el control biológico de *P. guildinii* utilizando cepas de *B. bassiana* parece una estrategia prometedora (Parys & Portilla, 2020).

2.2 Hongo entomopatógeno: *Beauveria bassiana*

Beauveria bassiana (Bals.) Vuilles (Ascomycota: Hypocreales) es un hongo cosmopolita perteneciente a los Ascomycetes filamentosos. El estado sexual de *Beauveria* reconocido (*Cordyceps*) solo se encuentra ocasionalmente en la naturaleza y es muy difícil su formación en el laboratorio (Lee et al., 2010). Presenta un amplio rango de hospedadores incluyendo más de 200 especies de insectos, dentro de 9 órdenes entre los cuales se encuentran Hemíptera, Lepidóptera y Coleóptera (Feng et al., 1994; Steinkraus & Tugwell, 1997; Mascarín & Jaronski, 2016). El género *Beauveria* se caracteriza

morfológicamente por células conidiógenas (fiálides) simpodiales, cortos y globosos en forma de matraz, que terminan en un raquis en zig-zag, a partir de los cuales se producen conidios holoblásticos unicelulares (Kendrik & de Hoog, 1972; Rehner & Buckley, 2005; Rehner et al., 2011; Chen et al., 2013). Las características de los conidios son las principales características morfológicas utilizadas para distinguir entre especies de *Beauveria* y su forma puede ser globosa, elipsoidal, cilíndrica o en forma de coma. (Petch, 1926; Rehner et al., 2011; Chen et al., 2013). Molecularmente se utilizan las filogenias basadas en la región de ADNr de ITS, factor de elongación de traducción 1 α (TEF), subunidad más grande de ARN polimerasa II (RPB1), segunda subunidad más grande de ARN polimerasa II (RPB2) y la región intergénica nuclear Bloc (Khonsanit et al., 2020) para la asignación de especie. Se encuentran descritas 23 especies para los anamorfos y algunas de estas especies se han conectado a teleomorfos de *Cordyceps*. En cultivo en placa con medio papa dextrosa agar (PDA) *B. bassiana* típicamente se presenta como una colonia de color blanco con escaso micelio aéreo, siendo mayoritariamente un hongo pulverulento, sus conidios son esféricos, globosos, hialinos, típicamente entre 1,7 y 5,5 μ m de diámetro, y paredes lisas (Rehner & Buckley, 2005).

Según Faria y Wraight (2007) el 40% del total de los mico-insecticidas se encuentra formulado a base de *B. bassiana*, de los cuales aproximadamente el 75% se encuentran registrados, en proceso de registro o disponibles comercialmente (en algunos casos sin registro), mientras que el 15% ya no están disponibles. En nuestro país se encuentra registrado un único producto fitosanitario a base de *B. bassiana* (CREBIO 1) en la DGSA, del MGAP, cuyo registro se encuentra en renovación, sin embargo, el uso de productos no registrados a base de *B. bassiana* en diferentes cultivos en nuestro país es de uso común a nivel doméstico.

En este trabajo se evaluaron 3 cepas pertenecientes a la colección del Laboratorio de Bioproducción de INIA Las Brujas (ILB205, ILB308 e ILB299), una cepa perteneciente a la colección del Laboratorio de Micología de Facultad de Ciencias -Ingeniería (FI1728) y la cepa comercial GHA. Las cepas provenían

de diversos orígenes y algunas de habían sido utilizadas en proyectos de investigación con énfasis en el control biológico.

La cepa ILB205 fue seleccionada por su alta virulencia frente al hemíptero plaga del eucaliptus *Thaumastocoris peregrinus* (proyecto FO_18 INIA-LB) y su buena capacidad de ser multiplicada en forma masiva en medio líquido y sólido (Abreo et al., 2019). La cepa FI1728 fue seleccionada por su baja virulencia sobre *T. peregrinus* y la cepa ILB299 fue seleccionada para obtener información respecto a su virulencia, dado que no había sido evaluada previamente en el proyecto FO_18. La cepa GHA es una cepa comercial de referencia utilizada ampliamente en estudios de *Beauveria bassiana*. La cepa ILB308 fue seleccionada debido a que fue aislada a partir de un individuo de *P. guidinii* que exhibía micosis en condiciones de cautiverio.

2.3 Mecanismo de acción de *B. bassiana*

Los hongos entomopatógenos, a diferencia de otros organismos entomopatógenos como bacterias o virus, no necesitan ser ingeridos por el hospedador para comenzar el ciclo infectivo, ya que generalmente invaden al hospedador mediante la penetración de su cutícula (Pedrini et al., 2007). A continuación, se describe en el ciclo infectivo de *B. bassiana*, el cual involucra una batería de enzimas las cuales actúan a lo largo de todo el ciclo, desde su comienzo, vinculando genes y rutas metabólicas específicas.

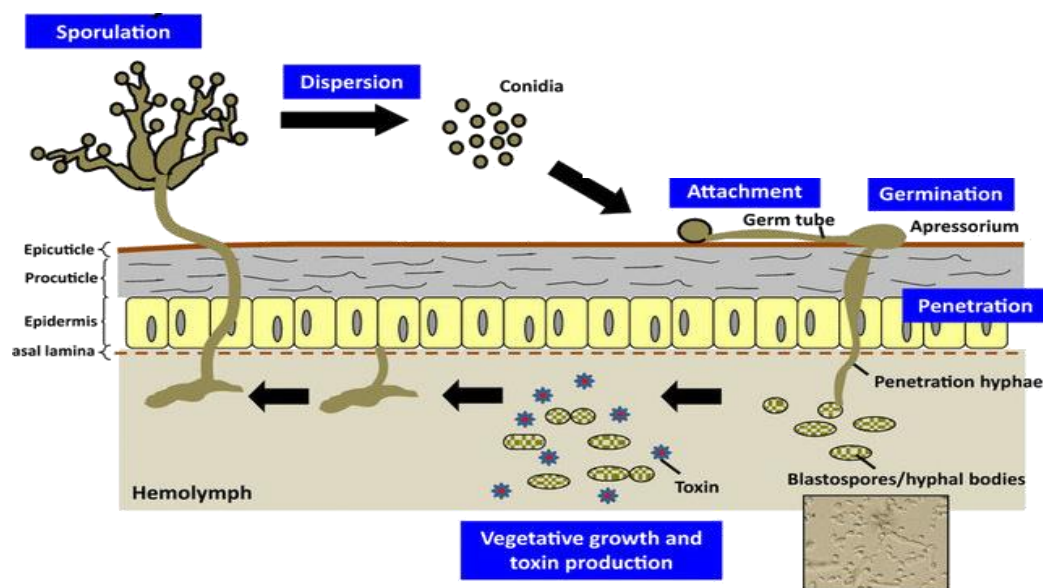


Figura 4 Descripción general del ciclo de infección básico representado por *Beauveria bassiana* en invertebrados. Adaptada de Mascarin & Jaronski, 2016.

Los conidios son dispersados por el viento, las salpicaduras de lluvia o por vectores artrópodos que ayudan al hongo a establecer infecciones en huéspedes susceptibles. En primer lugar, los conidios se adhieren a la cutícula del insecto mediante fuerzas químicas y electrostáticas y, tras la rehidratación y los estímulos químicos, germinan y forman el tubo germinativo. Así el hongo se abre paso penetrando todas las capas de la cutícula utilizando una batería de enzimas hidrolíticas, presión mecánica y otros factores hasta alcanzar la hemolinfa. En la hemolinfa, el hongo se multiplica como blastosporas. Durante esta etapa de la infección, el hongo también puede secretar metabolitos tóxicos que ayudan en la inmunosupresión del huésped, lo cual culmina en su muerte. Tras la muerte del insecto hospedador, los conidióforos emergen del cadáver (cadáver momificado) después de unos días y producen nuevos conidios infecciosos (esporulación) para su diseminación y para continuar el ciclo de vida del entomopatógeno. Las flechas indican la dirección del crecimiento del HEP (adaptado de Humber, 2008; Ortiz-Urquiza & Keyhani, 2013; Valero-Jiménez et al., 2014).

En primera instancia, los conidios aéreos toman contacto y se adhieren a la superficie de la cutícula del huésped mediante interacciones hidrófobas y electrostáticas inespecíficas (Boucias et al., 1988) junto con la expresión de los genes *Bbhyd1* y *Bbhyd2*, que codifican para las proteínas hidrófobas llamadas hidrofobinas, que forman la estructura protectora de la capa de las esporas, y facilitan la fijación de las mismas a la superficie de la cutícula (Figura 4 y 5) (Zhang et al., 2011). Las hidrofobinas son proteínas fúngicas anfipáticas que

facilitan la interacción entre interfases hidrofílicas-hidrofóbicas (Holder & Keyhani, 2005; Vigueras et al., 2014). Además, existen otras proteínas de la pared celular importantes en el proceso de adhesión (Wang & Wang, 2017) como las adhesinas codificadas por los genes *Mad-1* y *Mad-2*, característicos del género *Metarhizium* (Barelli et al., 2011) y sus análogos *Adh1*, *Adh2* y *Adh3* de *Beauveria bassiana* (Zhou et al., 2021).

Luego, cuando las condiciones de temperatura, humedad y fuentes de carbono externas son favorables, los conidios germinan formando el apresorio que ayuda a penetrar la cutícula mediante una combinación de presión mecánica y degradación enzimática de los componentes cuticulares (St. Leger et al., 1991; Valero-Jiménez et al., 2016). En algunos casos, el proceso de penetración puede desencadenarse sin la formación de apresorios (Butt et al., 2016). En *B. bassiana* los principales genes estudiados son los que codifican para citocromos P450 (varias familias de BbCYP), proteasas (*Bbcdep1*, *Bbpr1* y *Bbpr2*) y quitinasas (*Bbchit1*), enzimas que contribuyen al proceso de invasión de las hifas al hemocele (Fang et al., 2005 y 2008; Zhang et al., 2009, 2010, 2011, 2012; Pedrini et al., 2010).

Finalmente, una vez en el hemocele, los conidios sufren una diferenciación morfogénica cambiando de un crecimiento filamentoso a cuerpos hifales unicelulares similares a levaduras o blastosporas que se multiplican por gemación. Las blastosporas tienen una alta proporción superficie-volumen que mejora la absorción de nutrientes, evaden el sistema inmunológico del hospedador y colonizan los tejidos internos. Para tolerar la alta presión osmótica dentro del hemocele estas células expresan algunos genes que actúan como osmosensores (*Mos1*) o que codifican una proteína de recubrimiento defensiva (*Mcl1*), que ayudan a los cuerpos hifales a la adaptación al entorno hipertónico de la hemolinfa (Butt et al., 2016). Además, los sistemas antioxidantes se activan para mitigar las especies reactivas de oxígeno y nitrógeno (ROS y RNS) liberadas por el hospedador (Pedrini, 2018; Butt et al., 2016). Así, evaden el sistema inmunológico, se nutren de los componentes de la hemolinfa, proliferan, diseminan y secretan toxinas dentro del hemocele y

varios tejidos, lo que lleva a la muerte del insecto huésped (Pedrini, 2018). Estas toxinas son metabolitos secundarios de bajo peso molecular tóxicos para el hospedador (Valero-Jiménez et al., 2016; Pedrini, 2018) y tienen un papel principal en este escenario, ya que son cruciales para la supervivencia de los hongos entomopatógenos y su interacción con otros organismos (Pichersky & Gang, 2000). *Beauveria bassiana*, por ejemplo, produce metabolitos secundarios que actúan como inmunosupresores, facilitando la infección, como beauvericina (Bea), bassianolida (Bsl), oosporeína (Op), tenellina (Ten), bassiantina y beauverolides (Pichersky & Gang, 2000; Pedrini 2022). Los metabolitos secundarios poseen propiedades antibacterianas y antifúngicas, evitando el crecimiento de hongos y bacterias saprofitas oportunistas (Singh & Kaur, 2014). Tras la muerte del insecto, cuando se han agotado las fuentes de nutrientes en el hemocele, las células alcanzan la cutícula desde adentro, escapan y esporulan sobre la superficie exterior del cadáver, lo que permite que los conidios se dispersen y comiencen el proceso de infección en un nuevo individuo, lo que podría potencialmente causar una epizootia (Valero-Jiménez et al., 2016).

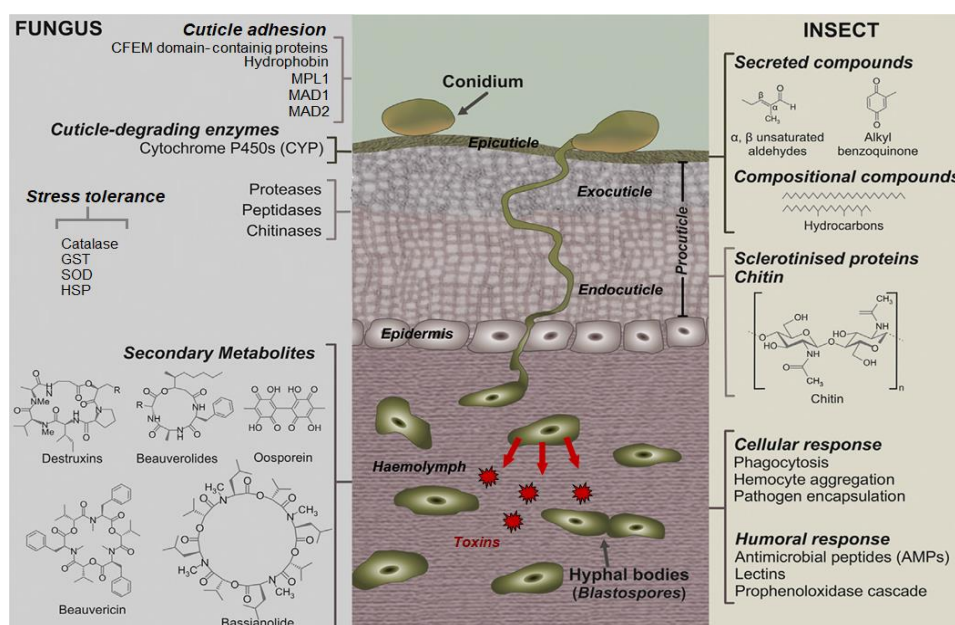


Figura 5 Esquema de infección. Ejemplos de enzimas degradadoras de la cutícula y metabolitos secundarios tóxicos, así como la composición de la superficie de barrera del insecto y su respuesta inmune ante la invasión fúngica. Adaptada de Pedrini, 2018.

En la tabla 2 se resumen los genes involucrados en la virulencia de *B. bassiana* mencionados anteriormente y su posible función

Tabla 2 Genes involucrados en la virulencia de *B. bassiana* y su Posible función

Nombre	Tipo	Posible función	Referencia
<i>Hyd1</i>	Hidrofobina	Adhesión a la cutícula	Zhang et al., 2011
<i>Hyd2</i>	Hidrofobina	Adhesión a la cutícula	Zhang et al., 2011
<i>Mad1</i>	Adhesina	Adhesión, germinación formación de blastosporas	Wang & St. Leger 2007
<i>Mad2</i>	Adhesina	Adhesión a la cutícula	Wang & St Leger, 2007
<i>Cdep1</i>	Subtisilin-endoproteasa	Degradación cutícula	Fang et al., 2008
<i>Pr1</i>	Subtisilin-Proteasa	Degradación cutícula	St Leger et al., 1994
<i>Pr2</i>	Tripsin-Proteasa	Degradación cutícula	Joshi et al., 1995
<i>Chit1</i>	quitinasa	Degradación quitina	Fang et al., 2005
<i>Hog1</i>	Protein quinasa	Viabilidad de espora, adhesión, apresorio y resistencia osmolaridad	Zhang et al., 2009
<i>MPK1</i>	MAP kinasa	Formación apresorio, adhesión, conidiación, salida de la cutícula	Zhang et al., 2010
<i>Cyp52x1</i>	Citocromo P450 monooxigenasa	Oxidación de lípidos. Germinación y salida de la cutícula	Zhang et al., 2012
<i>pacC</i>	Factor de transcripcion	Adaptación al pH	Peñalva et al., 2008
<i>Beas</i>	Beauvericina (toxina)	Insecticida, antifúngico y citotóxico	Xu et al., 2008
<i>catA</i>	Catalasa especifica de esporas	Rermotolerancia, resistencia UV	Wang et al., 2012
<i>catB</i>	Catalasa secretada	Eliminación de H ₂ O ₂	Wang et al., 2012
<i>catC</i>	Catalasa citoplasmática	Eliminación de H ₂ O ₂	Wang et al., 2012
<i>catD</i>	Catalasa/Peroxidasa (secretada)	Eliminación de H ₂ O ₂	Wang et al., 2012
<i>catP</i>	peroxisomica	Beta-oxidación	Wang et al., 2012
<i>GPx</i>	Glutación peroxidasa		
<i>Sod1</i>	Superoxido Dismutasa Metaloproteína	Tolerancia al stress oxidativo	Li et al., 2015
<i>Sod2</i>	Superoxido Dismutasa	Tolerancia al stress oxidativo	Li et al., 2015
<i>Sod3</i>	Superoxido Dismutasa	Tolerancia al stress oxidativo	Xie et al; 2012
<i>GsT</i>	Glutación S- transferasa	Tolerancia al stress oxidativo	Li et al., 2015

2.4 Interacción entre *B. bassiana* e hidrocarburos cuticulares CHC

Un resultado exitoso del proceso de infección descrito anteriormente implica la muerte del insecto, y depende en primera instancia de la penetración de la cutícula. La composición lipídica epicuticular de los insectos difiere entre especies y entre estadios de la misma especie pudiendo influenciar el proceso de infección ya que distintos hidrocarburos pueden inhibir o estimular la adhesión de conidios, germinación y crecimiento (Valero-Jiménez et al., 2016; Pedrini, 2018; Lopes et al., 2015).

Beauveria bassiana puede degradar una variedad de estructuras de hidrocarburos cuticulares o sus análogos sintéticos, utilizándolos para la producción de energía, pero también incorporando sus productos de degradación en diferentes lípidos fúngicos (Napolitano & Juárez, 1997; Pedrini et al., 2007).

Mediante el uso de hidrocarburos radiomarcados se pudo investigar y dilucidar la ruta catabólica de degradación de los hidrocarburos en la interfase entre la célula infectiva (espora, conidio, blastospora) y la epicutícula (Pedrini et al., 2006, 2007, 2013) y la incorporación de subproductos en componentes celulares fúngicos.

Los peroxisomas son los orgánulos donde sucede el último paso de las reacciones de degradación de hidrocarburos en *B. bassiana* (Pedrini et al., 2007). La ruta catabólica de CHC (Figura 6) involucra la oxidación mediante el sistema enzimático del citocromo P450, seguida por sucesivas transformaciones para producir el acilCoA graso apropiado, y la posterior beta oxidación de este en el peroxisoma (Pedrini et al., 2006, 2007). La degradación comienza con la hidroxilación del grupo metilo terminal de *n*-alcanos en el RE por citocromos P450 (específicos) que se acoplan a reductasas de NADPH-citocromo P450. El alcohol resultante también se puede catabolizar para activar el ácido graso en la mitocondria además del

RE y el peroxisoma. Los ácidos grasos activados se catabolizan a acetil-CoA por β -oxidación en el peroxisoma y/o la mitocondria.

Además, se ha evidenciado que la nutrición en hidrocarburos sintéticos como única fuente de carbono aumenta la virulencia en algunas cepas de *B. bassiana*, probablemente favorecida por la afinidad aumentada hacia los componentes de la cutícula de los insectos (Crespo et al., 2002; Pedrini et al., 2009; Pedrini et al., 2010), como resultado de la inducción de genes implicados en la asimilación cuticular de hidrocarburos (Pedrini et al., 2010, 2013; Zhang et al., 2012).

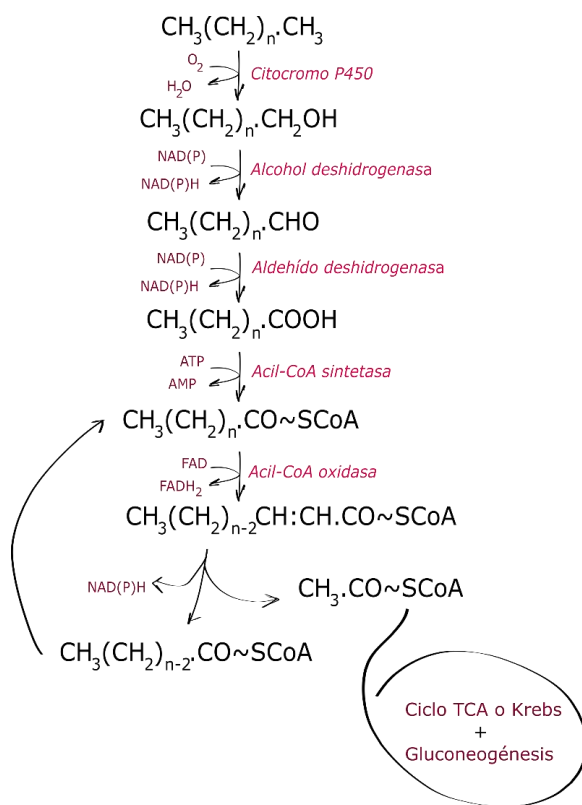


Figura 6 Vía catabólica principal propuesta para la degradación de alcanos. Adaptado de Pedrini et al., 2013.

Justificación

La evaluación del efecto de la nutrición en HC sintéticos análogos a los identificados en *P. guildinii*, en el crecimiento vegetativo y la virulencia de las diferentes cepas de *B. bassiana*, de manera *in vivo* mediante bioensayos como *in vitro* mediante la evaluación de expresión génica en placa, permitirá evaluar, al menos en condiciones de laboratorio, el potencial biocontrolador de estas cepas, concretamente en lo que respecta a un posible aumento en la virulencia hacia esta chinche debido a la nutrición en uno de sus hidrocarburos característico: *n*-pentadecano.

Objetivo

Evaluar el efecto de la nutrición de CHC sobre el crecimiento vegetativo y el efecto de la nutrición en *n*-pentadecano sobre la virulencia de cinco cepas de *B. bassiana* hacia *P. guildinii* y *T. peregrinus*, y sobre la expresión de 10 genes relevantes para la infección de dos cepas de *B. bassiana* con virulencia contrastante mediante RT-qPCR.

Resultados

A continuación, se expone el artículo “**Alkane-priming of *Beauveria bassiana* strains to improve biocontrol of the redbanded stink bug *Piezodorus guildinii* and the bronze bug *Thaumastocoris peregrinus***” el cual incluye la totalidad de los resultados del Capítulo 2 de este manuscrito, publicado en la revista Journal of Invertebrate Pathology. Q1, Impact factor 2022/2023 2.795.

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Artículo científico – Resumen en español

Los hidrocarburos de la epicutícula de insectos (CHC) son determinantes importantes en el grado de susceptibilidad de los insectos hacia los entomopatógenos fúngicos. Se analizaron fenotípicamente cinco cepas de *Beauveria bassiana* con respecto a su respuesta a la nutrición de CHC y su patogenicidad y virulencia hacia *Thaumastocoris peregrinus* altamente susceptible a hongos y *Piezodorus guildinii* poco susceptible a hongos, ambos hemípteros plagas importantes en las plantaciones de eucalipto y soja, respectivamente. Dos de estas cepas, que resultaron más (ILB308) y menos (ILB299) virulentas para *P. guildinii*, también se evaluaron a nivel de expresión génica después del crecimiento en *n*-pentadecano, un hidrocarburo epicuticular de *P. guildinii*. La cepa hipervirulenta ILB308 de *Beauveria bassiana* mostró el crecimiento más bajo en la mayoría de los medios CHC evaluados. Sin embargo, esta cepa indujo de forma distintiva la mayoría de los genes analizados implicados en la asimilación de CHC, la degradación de la cutícula y la tolerancia al estrés. La virulencia hacia *P. guildinii* de baja susceptibilidad se incrementó en cepas de ILB308 hipervirulentas e ILB299 hipovirulentas después del crecimiento en *n*-pentadecano como única fuente de carbono, mientras que el aumento de virulencia hacia *T. peregrinus* de alta susceptibilidad solo se observó en la cepa hipervirulenta. El aumento de la virulencia hacia *P. guildinii* podría explicarse principalmente por un efecto de cebado producido por CHC sobre la inducción de algunos genes relacionados con la asimilación de hidrocarburos en ILB299 e ILB308, como los genes del citocromo P450 (*BbCyp52g11* y *BbCyp52x1*), junto con los genes de adhesión y tolerancia al estrés, como hidrofobina (*Bbhyd2*) y catalasa (*Bbcatc*) y glutatión peroxidasa (*Bbgpx*), respectivamente.



Alkane-priming of *Beauveria bassiana* strains to improve biocontrol of the redbanded stink bug *Piezodorus guildinii* and the bronze bug *Thaumastocoris peregrinus*

Lucía Sessa^{a,*}, Nicolás Pedrini^b, Nora Altier^a, Eduardo Abreo^{a,*}

^a Laboratorio de Bioproducción, Plataforma de Bioinsumos, Instituto Nacional de Investigación Agropecuaria, estación experimental Wilson Ferreira Aldunate, Ruta 48, km 10, Canelones, Uruguay

^b Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), CCT La Plata Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) - Universidad Nacional de La Plata (UNLP), calles 60 y 120, 1900 La Plata, Argentina

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ABSTRACT

Insect epicuticle hydrocarbons (CHC) are known to be important determinants in the susceptibility degree of insects to fungal entomopathogens. Five *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales; Clavicipitaceae) strains were phenotypically analyzed regarding their response to CHC nutrition and their pathogenicity and virulence towards high fungal-susceptible *Thaumastocoris peregrinus* (Carpintero and Dellapé) (Heteroptera: Thaumastocoridae) and low fungal-susceptible *Piezodorus guildinii* (Westwood) (Hemiptera: Pentatomidae), which are important hemipteran pests in eucalyptus and soybean plantations, respectively. Two of these strains, which were the most (ILB308) and the least (ILB299) virulent to *P. guildinii*, were also evaluated at gene expression level after growth on *n*-pentadecane, a *P. guildinii* epicuticular hydrocarbon. *Beauveria bassiana* hypervirulent strain ILB308 showed the lowest growth on most evaluated CHC media. However, this strain distinctively induced most of the analyzed genes involved in CHC assimilation, cuticle degradation and stress tolerance. Virulence towards low susceptibility *P. guildinii* was enhanced in both hypervirulent ILB308 and hypovirulent ILB299 strains after growth on *n*-pentadecane as the sole carbon source, whereas virulence enhancement towards high susceptibility *T. peregrinus* was only observed in the hypervirulent strain. Virulence enhancement towards *P. guildinii* could be mostly explained by a priming effect produced by CHC on the induction of some genes related to hydrocarbon assimilation in ILB299 and ILB308, such as cytochrome P450 genes (*BbCyp52g11* and *BbCyp52x1*), together with adhesion and stress tolerance genes, such as hydrophobin (*Bbhyd2*) and catalase (*Bbcac*) and glutathione peroxidase (*Bbgpx*), respectively.

1. Introduction

Entomopathogenic fungi, such as *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales; Clavicipitaceae), are used worldwide as bio-pesticides for pest control due to their capacity to infect arthropods by direct penetration through the cuticle. The overall infection process can be described in three phases. First, aerial conidia take contact with and adhere to the surface of the host cuticle by non-specific hydrophobic and electrostatic interactions (Boucias et al., 1988) supported by the expression of a set of hydrophobin genes (*Bbhyd1* and *Bbhyd2*), which encode for the hydrophobic proteins that contribute to the protective spore coat structure known as rodlet layer (Zhang et al., 2011). Secondly, conidia germinate forming the appressorium that helps to

penetrate the cuticle by a combination of mechanical pressure and enzymatic degradation (St. Leger et al., 1991). The main genes expressed are those encoding for cytochrome P450s (several BbCYP families), proteases (*Bbcdep1*, *Bbpr1* and *Bbpr2*), and chitinases (*Bbchit1*), enzymes that contribute to the process of invasion of the hyphae into the hemocoel (Fang et al., 2005, 2008; Zhang et al., 2009, 2010, 2011, 2012; Pedrini et al., 2010). Finally, once in the hemocoel, conidia switch to develop as hyphal bodies evading the immune system, nurturing of hemolymph components, proliferating, disseminating and secreting toxins inside the haemocoel and various tissues, leading to the death of the insect host (Pedrini, 2018). After the insect's death, fungal cells in the hemocoel reach the cuticle from within, escape and sporulate on the cadaver, allowing conidia to disperse and start the infection

* Corresponding authors.

E-mail addresses: lsessa@inia.org.uy (L. Sessa), eabreo@inia.org.uy (E. Abreo).

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process in a new individual, potentially causing an epizootic (Zhang et al., 2010; Valero-Jiménez et al., 2016).

A successful infection outcome, which implies the death of the insect, depends in first instance on the penetration of the cuticle, which is composed of a thin outer layer (epicuticle) containing mainly lipids, and a thick layer (procuticle) containing chitin and proteins. The epicuticular lipids are mostly hydrocarbons, i.e., *n*-alkanes, *n*-alkenes and methyl branched chains (Juárez et al., 1985; Blomquist et al., 1987; Lockey, 1988).

Beauveria bassiana can degrade a variety of hydrocarbon structures similar or identical to those of their insect host, utilizing them for energy production but also incorporating their degradation products into different fungal lipids (Napolitano and Juárez, 1997; Pedrini et al., 2007). Moreover, it is reported that nutrition on hydrocarbons enhances virulence in some *B. bassiana* strains probably favored by the augmented affinity to insect cuticle components (Crespo et al., 2002; Pedrini et al., 2009), as result of the induction of genes involved in cuticular hydrocarbon assimilation (Pedrini et al., 2010, 2013; Zhang et al., 2012). This approach seems to be getting attention in pest control management strategies with microbial control agents, and virulence gene profiling together with virulence enhancement through adaptation to growth on insect-like culture media appears to be the next step.

In Uruguay two of the most economically important insect pests are the redbanded stink bug *Piezodorus guildinii* (Westwood) (Heteroptera: Pentatomidae) and the bronze bug *Thaumastocoris peregrinus* (Carpintero and Dellapé) (Heteroptera: Thaumastocoridae). These pests cause significant yield losses in plantations of soybean *Glycine max* (L.) Merrill (Fabales: Fabaceae) and eucalyptus, *Eucalyptus obliqua* (L'Her) (Myrtales: Myrtaceae), respectively (Zerbino 2010; Martínez and Bianchi, 2010). Whereas chemical pesticides applied to reduce *P. guildinii* insect populations are not very successful (Catchot, 2019), chemical control of *T. peregrinus* is not suitable due to the wide distribution of the insect within the canopy of the tree (Martínez and Bianchi, 2010), and because eco-certification programs for international trade of wood products impose restrictions to the use of chemicals (<https://us.fsc.org/en-us/certification>).

Biological control by *B. bassiana* seems to be an appropriate alternative for the control of both pests, and its effectiveness has been previously reported (Corallo et al., 2019; Abreo et al., 2019; Parys and Portilla, 2020). However, unlike for the bronze bug, no epizootics have been reported for *P. guildinii*. It is known that other members of the hemipteran family Pentatomidae produce volatile metathoracic secretions with fungistatic activity that could explain the low susceptibility of pentatomids to fungal pathogens (Lopes et al., 2015). Recent research has indicated that *n*-pentadecane is one the most abundant semivolatile CHC in *P. guildinii* (Sessa et al., 2021).

In this study, we hypothesize that growth on hydrocarbon-containing media may serve as a virulence enhancement method for obtaining an effective *B. bassiana*-based biopesticide against *P. guildinii*. The aims of the present work were to evaluate i) the effect of hydrocarbon nutrition on vegetative growth and virulence of five *B. bassiana* strains towards *P. guildinii* and *T. peregrinus*, and ii) the effect of *n*-pentadecane -used for virulence enhancement- on gene expression regarding genes relevant to infection of two *B. bassiana* strains with contrasting virulence.

2. Materials and methods

2.1. Entomopathogen fungal strains

Five *B. bassiana* strains were evaluated in this study. Strains ILB308, ILB299 and ILB205 were retrieved from INIA-Las Brujas Fungal Collection (ILB), Fi1728 was provided by Laboratorio de Micología-Universidad de la República (Montevideo, Uruguay), and strain GHA is a commercial strain (Laverlam International, Butte, MT). Strains were revitalized on mealworms (*Tenebrio molitor*) and maintained on PDA

plates at 4 °C. The process of revitalization consisted on the inoculation of a spore suspension prepared from a colony of each fungal strain into five *Tenebrio molitor* mealworms, followed by incubation and the re-isolation of the fungal entomopathogen from the mummified mealworms onto fresh PDA plates.

2.2. Fungal cultures and spore suspension preparation

Every spore suspension used in this study was obtained from aerial spores harvested and resuspended with Tween-saline solution (0.1% Tween 80 and 0.85% NaCl) and filtered using autoclaved gauze. Conidia were counted in a Neubauer chamber (Heinz Herenz, Hamburg, Germany) and suspension concentration was adjusted to 2×10^7 spores/ml for virulence and gene expression assays and to 6×10^7 spores/ml for growth on *n*-alkanes assays. Spore viability (%) of each strain suspension was assessed by plating an aliquot of 150 µl of a 1/10 diluted suspension (2×10^6 spores/ml) on a water agar plate (WA) and incubated for 24 h at 26 °C. After the incubation period, viability was evaluated as the number of viable spores out of the total number of counted spores, multiplied by 100. Viability was measured in three areas per plate, with one hundred total spores counted each time. Spores were produced either on potato dextrose agar (PDA), complete media (CM) or on minimal media (MM) supplemented with *n*-alkanes. CM plates contained 0.4 g KH₂PO₄, 1.4 g Na₂HPO₄, 0.6 g MgSO₄·7H₂O, 1.0 g KCl, 0.7 g NH₄NO₃·7H₂O, 10 g glucose, 5 g yeast extract and 15 g agarose in 1000 ml of distilled water and MM contained the same as CM without the glucose and yeast extract and supplemented with *n*-alkanes (Thermo Fisher Scientific, USA) as carbon source (Pedrini et al., 2010).

2.3. Rearing of insect colonies

Individuals of *P. guildinii* were obtained from an indoor mass rearing maintained at 24 ± 1 °C, 80 ± 10% RH, 14 h of photophase on fresh soybeans, green beans, and water. Nymphs were placed on transparent plastic boxes and checked daily until adult emergence. Adults were sexed and placed on plastic cages prior to each bioassay.

Individuals of *T. peregrinus* were obtained from an indoor mass rearing maintained on fresh shoots of *E. tereticornis* in Erlenmeyer flasks (Martínez et al., 2014). Nymphs were placed on aluminum framework cages covered with a mesh screen and were checked weekly for adult emergence. Adult individuals were sexed and relocated into similar cages prior to the inoculation.

2.4. Pathogenesis and virulence assay towards *P. guildinii* and *T. peregrinus*

A preliminary pathogenicity assay was conducted using PDA-grown fungal conidia. Strains ILB308, ILB299, ILB205, Fi1728 and GHA were grown on PDA plates and conidia were harvested after 14 days of incubation at 26 °C. Suspension concentration was adjusted to 2×10^7 viable spores/ml.

Twenty laboratory-reared adults of *P. guildinii* insects (10 males and 10 females) were immersed in each *B. bassiana* spore suspension for 5 s, and immediately placed individually on a 55 mm Petri dish containing sterile paper, water, and surface-disinfected soybean grains for insect nutrition during incubation. Grains were immersed in 4 % NaOCl for 90 s, later washed with autoclaved tap water and air dried. Controls were immersed in water/Tween-saline solution. In total 120 insects were used. All treated insects were incubated at 24 ± 1 °C for 12 days and insect mortality was assessed and recorded daily. Each insect cadaver was placed on a humid chamber at 25 °C for fungal development. The entire trial was repeated three times, evaluating a total of 360 *Piezodorus* insects.

Fifty laboratory-reared adults of *T. peregrinus* (25 males and 25 females) were placed on bouquets of fresh leaves of *Eucalyptus tereticornis* Sm. and kept in voile mesh cages, according to Martínez et al. (2014).

Each conidial suspension was applied by spraying onto the bouquets to the point of run-off. Three bouquets were sprayed per fungal strain. Control bouquets were sprayed with water/tween-saline solution. A total of 18 cages/bouquets, containing 900 insects were evaluated. Cages were kept for 10 days under controlled conditions of temperature and moisture ($23 \pm 2^\circ\text{C}$ and 80% relative humidity) under 12-h light/dark period. Mortality of insects was assessed and recorded daily. Each insect cadaver was superficially disinfected in alcohol 70% for 5 s, washed twice with sterile distilled water, dried on sterile filter paper, placed in individual humid chambers, and incubated at 26°C for fungal development.

Cumulative survival curves were constructed for both bioassays. Survival curves were plotted using Kaplan-Meier survival analysis and compared with the Log-Rank test at $\alpha = 0.05$ using MedCalc statistical Software trial version 20.009 (MedCalc Software bv, Ostend, Belgium; www.medcalc.org; 2021). The median lethal time (MLT) was the parameter used for assessing virulence when all treated insects reached 100% mortality. MLT was estimated as $\Sigma (\text{days}_n \times \text{dead insects}_n) / \text{total dead insects}$ (Moore et al., 1995).

2.5. *Beauveria bassiana* growth on *n*-alkanes

The preference of the *B. bassiana* strains for *n*-alkanes was evaluated by assessing vegetative growth on MM culture media supplemented with different *n*-alkanes and on CM. Four *n*-alkane substrates were evaluated, which were selected according to their relative abundance on the epicuticular layer of each of the two insects. These were *n*-tridecane, *n*-pentadecane and two blends. The long chain hydrocarbon blend (LC-HC) resembled the relative abundance of epicuticular hydrocarbons found in the soybean stink bug and consisted of a mixture of *n*-tridecane, *n*-pentadecane and *n*-octacosane in proportion 1:0.9:0.1. The very long chain hydrocarbon blend (VLC-HC) resembled the relative abundance of epicuticular hydrocarbons found in the bronze bug and consisted of a mixture of *n*-tetracosane and *n*-octacosane in equal proportions (1:1). To assess vegetative growth, a 20 μl droplet containing 6×10^7 viable spores/ml of each suspension was inoculated in the center of a petri dish with either CM or alkane-added MM solid media. Each strain and condition were repeated three times. Plates were incubated at 26°C and diameter growth was recorded on the sixth day post inoculation.

2.6. Expression pattern of genes involved in conidia adhesion, hydrocarbon assimilation, pathogenesis and oxidative stress tolerance using qRT-PCR

Differential expression of 10 genes relevant to infection (Table 1) in fungi grown on CM and on MM supplemented with *n*-pentadecane were investigated. Strains were grown on solid MM supplemented with *n*-pentadecane (HC-15, 10 % final concentration) and CM plates, covered with a cellophane membrane to avoid interference with the qRT-PCR protocol (Pedrini et al., 2010). Plates were incubated at 26°C and total RNA was extracted on day 3, 6 and 9 employing the RNeasy plant

mini kit (Qiagen). RNA was quantified using Nanodrop (Thermo Fisher) and integrity and purity were evaluated on an agarose gel 1% electrophoresis. Samples were treated with Turbo DNase (Invitrogen, Thermo Fisher Scientific) and EDTA. Purified RNA was transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad) and stored at -20°C until qRT-PCR analysis. The assay was performed three times (three biological replicates).

qRT-PCR analyses were performed using iQ SYBR Green Supermix (Bio-Rad). PCRs were performed using Quant Studio 3 (Applied Biosystems, Thermo Fisher), using the following conditions: denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Data was analyzed using the Quant Studio design and analysis software version 1.4.3. Transcripts were amplified using 10 gene-specific primers detailed in Table 1. For normalization the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene from *B. bassiana* was used (Table 1). The qRT-PCR analyses were performed in triplicate for each of the three independent biological replicates.

To assess the primer's amplification efficiency, standard curves were constructed by plotting the log of the cDNA dilution values versus CT values. Serial 5-fold dilutions of cDNA samples were used as templates for each qRT-PCR reaction. Each reaction efficiency was calculated as $E = 10^{(-1/\text{slope})}$, using the slope of each curve (Table 1). The relative expression levels of each gene were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

2.7. Virulence enhancement of *B. bassiana* strains grown on *n*-pentadecane towards *P. guildinii* and *T. peregrinus*

The virulence enhancement bioassays were conducted using 2×10^7 viable spores/ml solutions obtained from aerial conidia of ILB308, ILB299, ILB205, Fi1728 and GHA strains grown on CM and MM amended with *n*-pentadecane plates. Because ILB299 showed reduced sporulation, it was evaluated at a lower concentration (2×10^6 viable spores/ml) on *T. peregrinus*. Inoculation and evaluation procedures, including humid chambers, were as described on the previous bioassays, and each assay was repeated twice. Cumulative survival curves were constructed for both bioassays, using the same parameters as described in Section 2.4.

3. Results

3.1. *Beauveria bassiana* virulence towards *P. guildinii* and *T. peregrinus*

Pathogenicity baseline of the five fungal strains was determined to select a high and a low virulence strain (hyper and hypo virulent) for future evaluations and to define the time frame for the gene expression experiments.

Piezodorus guildinii insects showed low susceptibility towards *B. bassiana* infection, although all strains proved to be pathogenic as insect survival curves differed from the curve observed in the control treatment (Fig. 1a). From the comparison of survival curves (Log-rank

Table 1
Primer sequences and PCR amplification efficiency of genes related with infection.

Gene name	Forward 5'–3'	Reverse 5' – 3'	Source/reference	Amplification efficiency (%)	R ²
<i>Bbhog1</i>	ATACTACCGCGCTCCTGAGA	CTGGCAATGGTGTGATGAC	Romón et al., 2017	114	0.99
<i>Bbhyd2</i>	TGTCAGAGACTGGCGACATTGCG	TCGATGGGGACAAGCTGGTTGA	Huarte-Bonnet et al., 2018a	101	0.99
<i>Bbchit1</i>	TTGTTGGCTTCCACCATGGTCA	GATGACGTTGTAGCGGTGCT	Romón et al., 2017	106	0.99
<i>Bbcdp1</i>	ACAAGGTGGGTTTTTGATGC	TGCATGATCCTCTCAACGAC	Romón et al., 2017	112	0.97
<i>Bbcyp52x1</i>	TCGGTTACGTTATGCAACACC	TTCTCCAGGGCAGTTGGTCAGG	Huarte-Bonnet et al., 2018b	114	0.99
<i>Bbcyp52g11</i>	CGCTCACTGCTATCCTCATCGG	TTCTTAGGCGCATGGTGGTGAC	Huarte-Bonnet et al., 2018b	122	0.98
<i>Bbcyp52t1a</i>	TTATTGGTGCATTCACCAACG	TCCATGAGCATGCCCTTGAAAC	Huarte-Bonnet et al., 2018b	114	0.97
<i>Bbsod1</i>	ACAACACCAACGGCTGCACC	ACGGCCAAACACGCTGTGAG	Xie et al., 2012	107	0.99
<i>Bbgpx</i>	CAAGGTGCTCCTCGTCGTCAC	CTTGTCGCCATTGACCTCCACC	Forlani et al. 2014	100	0.99
<i>Bbcatc</i>	TGCTGGACGATGTGTCTGAC	CACGACCGTATCGCTAGAG	Forlani et al. 2014	120	0.98
<i>GAPDH</i>	ATAAGGTCAGGACACGGCG	TGAGAAGGGTGCCAGCTACG	Mantilla et al 2012	102	0.99

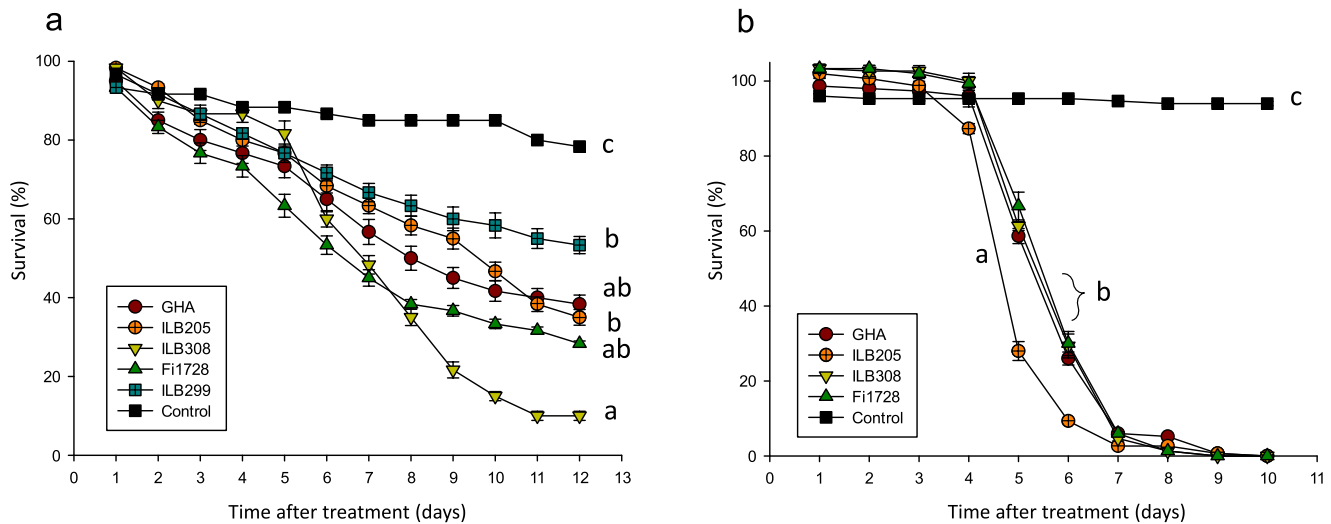


Fig. 1. Cumulative daily survival of (a) *Piezodorus guildinii* and (b) *Thaumastocoris peregrinus* exposed to different PDA grown *Beauveria bassiana* strains. Strain ILB299 was inoculated at a lower concentration in *T. peregrinus* so it is not shown in the comparative survival curves in figure b. Data show means $\pm$ SD from corresponding replicates. Different letters designate significant differences between survival curves (Log-rank test, $p < 0.05$).

test) strain ILB308 had the highest virulence and ILB299 had the lowest.

Thaumastocoris peregrinus proved to be very susceptible to all *B. bassiana* strains, as survival in all fungus-treated insects was null by the end of the evaluated period (Fig. 1b). From the comparison of survival curves (Log-rank test), strain ILB205 had the highest virulence of all evaluated strains. Median lethal time was estimated and strain ILB205, GHA and ILB308 were the most virulent with MLT values of 5.21 ± 0.05 , 5.84 ± 0.06 and 5.89 ± 0.12 days, respectively.

3.2. *Beauveria bassiana* growth on *n*-alkanes

All strains evaluated were able to utilize synthetic *n*-alkanes as the sole carbon source, as evidenced on the vegetative growth evaluation. As expected, the preferred culture media was CM as all strains showed higher total growth (Table 2), as well as more abundant aerial mycelia (Fig. 2). Overall, the strains with higher vegetative growth on any hydrocarbon or their combinations were Fi1728, ILB205 and ILB299, and strain GHA together with ILB308 had the lowest (Fig. 2, Table 2). Morphologically strain ILB308 differed from the rest of the *B. bassiana* strains, showing scarce aerial mycelial development (Fig. 2).

Table 2

Colony diameter (cm) of *Beauveria bassiana* strains measured after 6 days of growth on complete media (CM) and minimal media (MM) supplemented with different *n*-alkanes and blends.

Strain	HC-supplemented MM				CM
	<i>n</i> -tridecane	<i>n</i> -pentadecane	LC-HC	VLC-HC	
Fi1728	2.33 $\pm$ 0.23 c AB	2.68 $\pm$ 0.08 bc A	2.76 $\pm$ 0.12 b A	2.56 $\pm$ 0.12 bc A	3.36 $\pm$ 0.03 a BC
ILB299	2.53 $\pm$ 0.09 b A	2.45 $\pm$ 0.27 b AB	2.56 $\pm$ 0.03 b A	2.53 $\pm$ 0.03 b A	2.98 $\pm$ 0.03 a D
ILB308	2.13 $\pm$ 0.11 b BC	1.91 $\pm$ 0.24 b B	1.90 $\pm$ 0.43 b A	1.98 $\pm$ 0.03 b B	3.53 $\pm$ 0.03 a B
ILB205	2.53 $\pm$ 0.04 b A	2.40 $\pm$ 0.35 b AB	2.44 $\pm$ 0.36 b A	2.42 $\pm$ 0.15 b A	3.16 $\pm$ 0.06 a CD
GHA	1.98 $\pm$ 0.06 b C	2.00 $\pm$ 0.21 b B	2.00 $\pm$ 0.51 b A	1.99 $\pm$ 0.17 b B	3.97 $\pm$ 0.17 a A

LC-HC: long chain hydrocarbon blend, VLC-HC: very long chain hydrocarbon blend.

Means ($\pm$ SD) followed by different letters indicate statistical differences (Tukey's HSD test, $p < 0.05$). Capital letters indicate differences within columns, lower case letters indicate differences within rows.

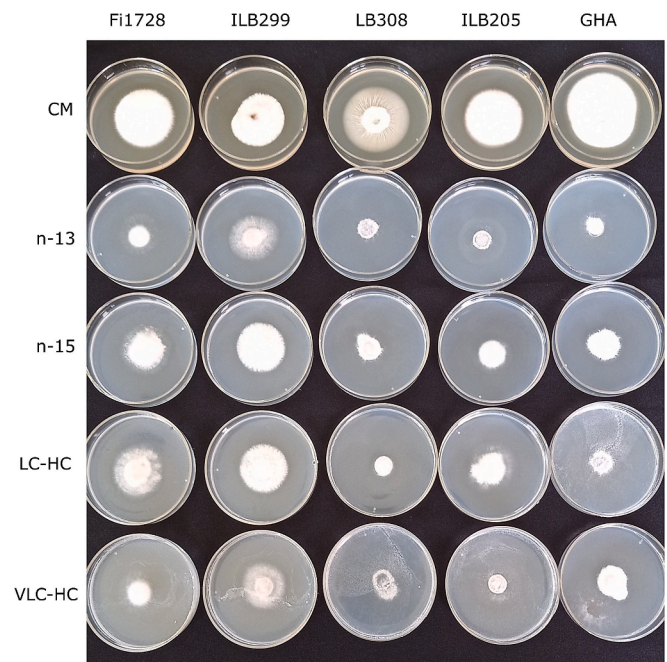


Fig. 2. Vegetative growth evaluation of *Beauveria bassiana* strains after 6 d of incubation at 26 ± 2 °C. A 20 μ l droplet of *B. bassiana* conidial suspensions were inoculated on complete medium and minimal medium supplemented with different *n*-alkanes and two blends. n-13: *n*-tridecane, n15: *n*-pentadecane, LC-HC: long chain hydrocarbon blend, VLC-HC: very long chain hydrocarbon blend.

3.3. Expression of *B. bassiana* genes involved in hydrocarbon assimilation (*Cyp52g11*, *Cyp52t1a* and *Cyp52x1*)

Regarding genes involved in hydrocarbon assimilation, a delay in the induction was observed in the low virulence strain ILB299. This strain showed under expression of the three genes on the first day of the evaluation (Fig. 3a). Later, on the 6th day *Bbcyp52g11* and *Bbcyp52x1* genes exhibited very low overexpression (1.61 ± 0.28 and 1.96 ± 0.66 foldchange, respectively), while *Bbcyp52t1a* remained under-expressed (Fig. 3a). On the 9th day *Bbcyp52g11* and *Bbcyp52t1a* reached maximum overexpression of 2.21 ± 0.73 and 5.78 ± 4.92 foldchange,

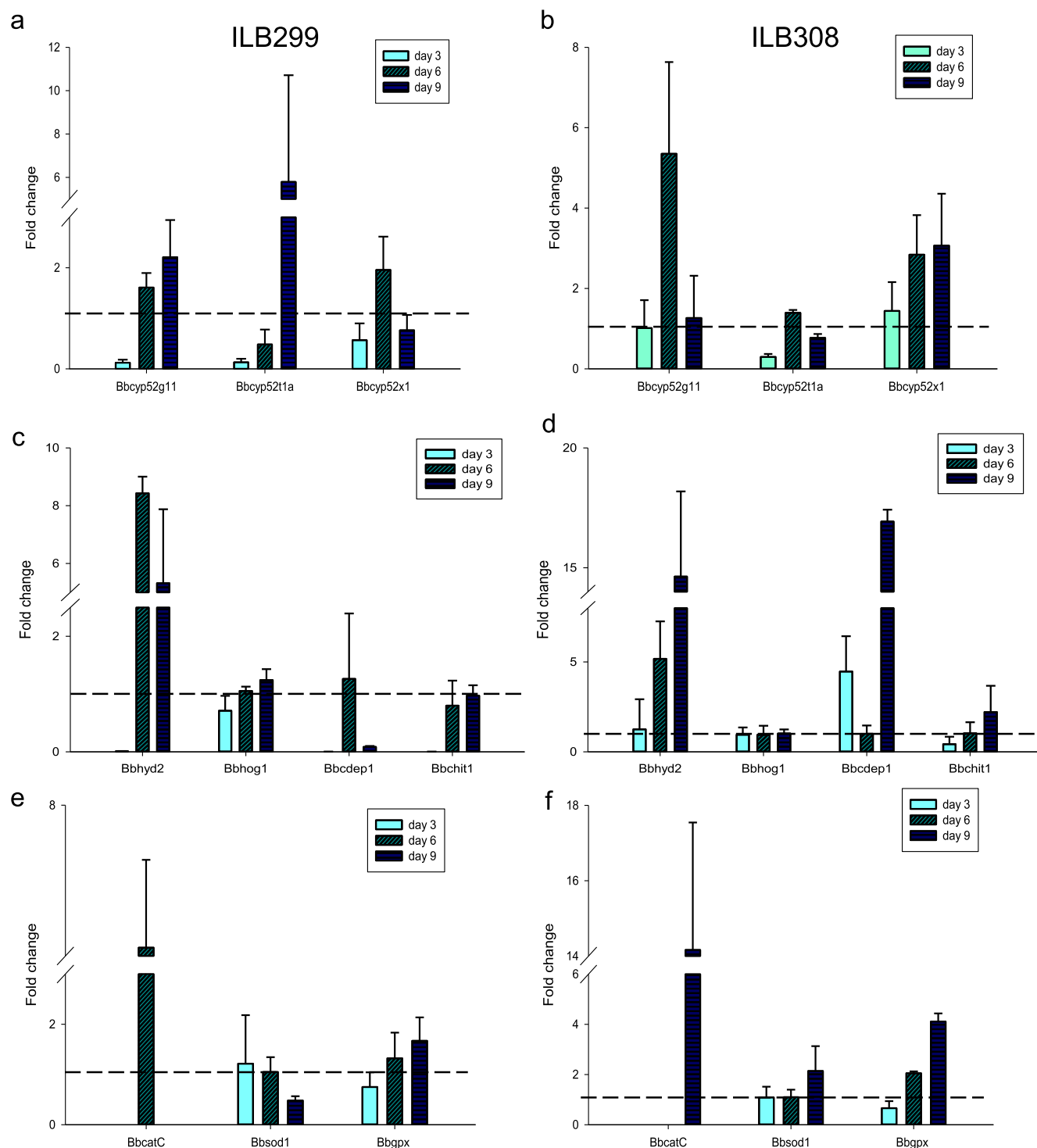


Fig. 3. Expression pattern of (a-b) hydrocarbon degradation and assimilation genes, (c-d) host adhesion, appressorium formation and cuticle degradation genes and (e-f) antioxidant defense system genes in *Beauveria bassiana* strains ILB299 and ILB308 grown on *n*-pentadecane. The dashed line shows an expression ratio = 1, indicating no expression change due to *n*-pentadecane nutrition compared with fungi grown in complete medium. Values are means $\pm$ SE of three biological replicates.

respectively, while *Bbcyp52x1* decreased to 0.76 ± 0.30 (Fig. 3a). At the beginning of the evaluation hypervirulent strain ILB308 exhibited overexpression of *Bbcyp52x1* gene involved in hydrocarbon assimilation in 1.43 ± 0.71 foldchange (Fig. 3b). Later, expression of this gene together with *Bbcyp52g11* and *Bbcyp52t1a* reached a maximum, with foldchange values of 5.35 ± 2.28 , 1.39 ± 0.07 and 2.89 ± 0.98 , respectively (Fig. 3b). Gene *Bbcyp52x1* remained overly expressed in the

9th day of the evaluation (Fig. 3b).

3.4. Expression of *B. bassiana* genes involved in host adhesion and appressorium formation (*Bbhyd2* and *Bbhog1*) and cuticle degradation (*Bbchit1*, *Bbcdep1*)

The four evaluated genes (*Bbhyd2*, *Bbhog1*, *Bbchit1* and *Bbcdep1*)

were found under-expressed in the hypovirulent strain ILB299 on day 3 of evaluation (Fig. 3c). In fact, cuticle degradation genes remained under-expressed during the entire evaluation. The highest over-expression was observed in the host adhesion gene *Bbhyd2* on day 6 which remained so until day 9 (8.43 ± 0.57 and 5.32 ± 2.56 on day 6 and day 9, respectively). In contrast, the hypervirulent strain ILB308 had not only the hydrophobin gene (*Bbhyd2*), but also the cuticle degradation gene *Bbdcep1* overexpressed at the evaluation start point (Fig. 3d). On day 9, *Bbhyd2* reached a maximum expression (14.6 ± 3.55 fold-change) together with *Bbdcep1* (16.9 ± 0.50) and the chitinase *Bbchit1* gene (2.23 ± 1.46). The gene involved in appressorium formation (*Bbhog1*) had similar expression than control throughout the entire evaluation in both strains, meaning that *n*-pentadecane culture media had no effect on the expression of these gene in either hypo- or hypervirulent strains.

3.5. Expression of *B. bassiana* genes involved in the antioxidant defense system (*Bbcatc*, *Bbsod1* and *Bbgpx*)

Catalase gene (*Bbcatc*) expression showed a peak on day 6 and on day 9 of the evaluation with foldchange values of 6.58 ± 0.87 and 14.16 ± 3.38 in ILB299 and ILB308, respectively (Fig. 3e-f). The glutathione peroxidase gene (*Bbgpx*) was under-expressed on the first day of the evaluation, later showing an increased induction in both strains (Fig. 3e-f). Superoxide dismutase gene (*Bbsod1*) expression remained unaffected during the entire evaluation period in strain ILB299 (Fig. 3e) and slightly induced in strain ILB308 on day 9 (Fig. 3f).

3.6. Virulence enhancement evaluation of alkane-grown *B. bassiana* strains towards *T. peregrinus* and *P. guildinii*

In *T. peregrinus*, all *B. bassiana* strains proved to be highly virulent, as insects showed very low survival percentages by the end of the assay

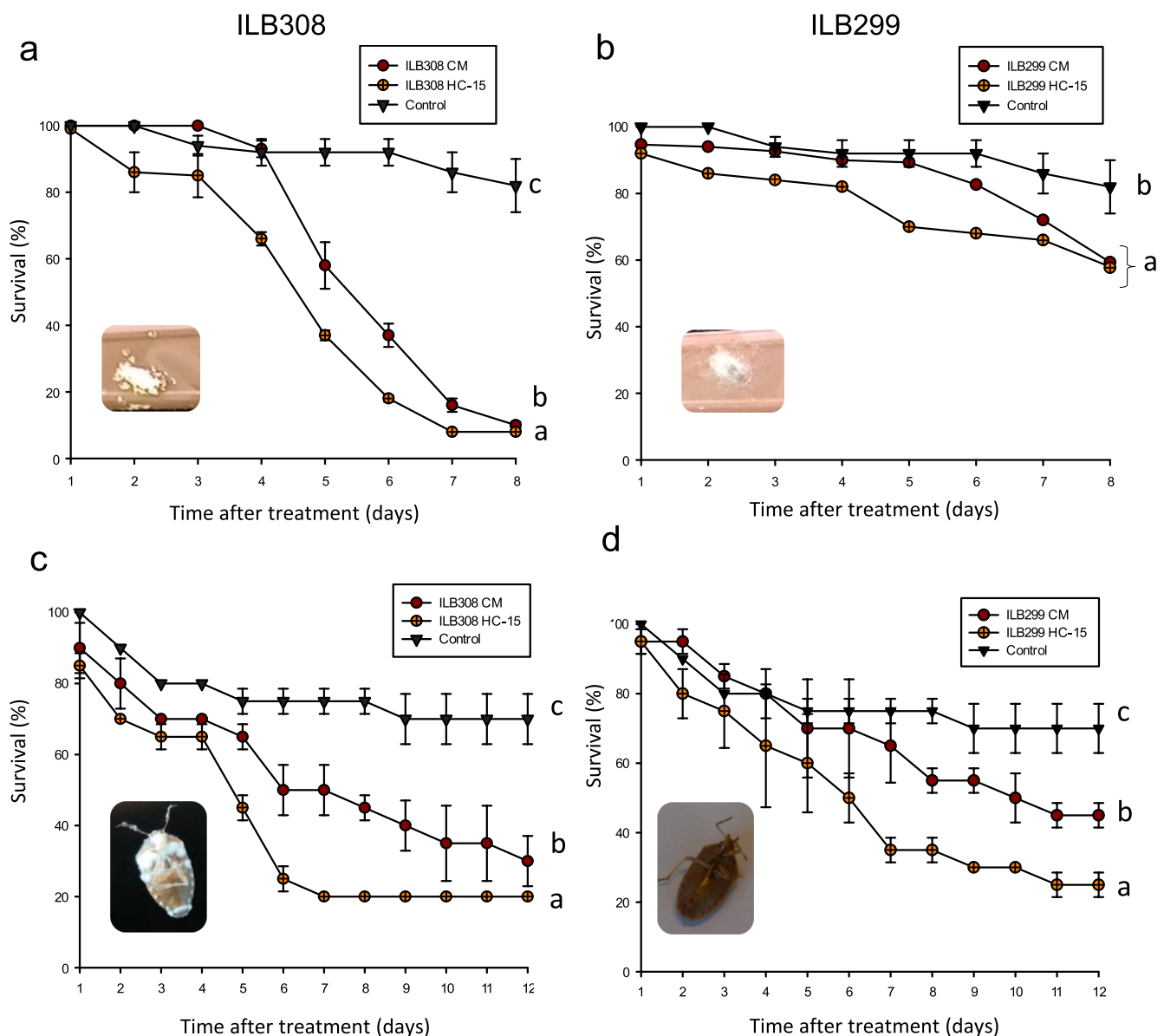


Fig. 4. Survival of insects inoculated with (a-c) ILB308 and (b-d) ILB299 strains of *B. bassiana* grown on *n*-pentadecane (HC-15) and CM. Insects of (a-b) *T. peregrinus* and (c-d) *P. guildinii* infected with *B. bassiana*. (a, c, d) conidial suspension of 2×10^7 conidia/ml. (b) conidial suspension of 2×10^6 conidia/ml. Insets: Result of humid chamber treatments showing cadavers of insects after 10 days post-inoculation with *B. bassiana* strains. Different letters designate significant differences between survival curves (Log-rank test, $p < 0.05$).

compared with the mock control (Fig. S1). Only for strain ILB308, a virulence enhancement caused by growth on *n*-alkanes was observed compared with the same strain grown on CM medium (Fig. 4a, Fig. S1 right). Fig. 4b shows the hypovirulent strain survival curve less pronounced due to a lower concentration of the conidial suspension inoculated. No virulence enhancement was observed in the hypovirulent strain grown on *n*-pentadecane.

In *P. guildinii*, an effect of *n*-alkane nutrition on virulence enhancement was evidenced. Strains ILB308 and ILB299 showed an augmentation on virulence, being significantly higher in strains grown on *n*-pentadecane than CM medium (Fig. 4c and 4d). Strains GHA, Fi1728 and ILB205 showed no significant differences in mortality between conidia grown on *n*-pentadecane and conidia grown on CM medium (Fig. S1 left).

Sporulation on dead cadavers of *T. peregrinus* after the humid chamber treatment was evident in almost all treated insects inoculated with ILB308, ILB205 and GHA grown on both culture media. Cadavers on humid chambers of ILB299 and Fi1728 inoculated insects showed aerial mycelial growth rather than conidia (Fig. 4a and 4b, insets). On *P. guildinii* dead insects, sporulation in cadavers was rather scarce. Strain ILB308 showed the highest levels of mummified cadavers covered in *B. bassiana* conidia while ILB299 mycelium was barely observed on dead insects (Fig. 4c and 4d, insets).

4. Discussion

The interaction of the entomopathogen *B. bassiana* with insect pests leads to insect death when the fungal spore is firstly able to adhere and penetrate the insect cuticle and then progress to the haemocoel, where it must avoid host response, multiply and invade the insect cavity, ultimately killing the host (Pedrini et al., 2007; Ortiz-Urquiza & Keyhani, 2013; Valero-Jiménez et al., 2016). The redbanded stink bug *P. guildinii* and the bronze bug *T. peregrinus*, two hemipteran insect pests with different epicuticle composition (Sessa et al., 2021; Abreo et al., 2015), were studied in their interaction with five *B. bassiana* strains showing a range of virulence. We focused on the response of a hyper- and a hypovirulent *B. bassiana* strain to nutrition with hydrocarbons that are an integral part of the insect's epicuticle and a first defense barrier against fungal infection. The ultimate objective was to explore the possibility of virulence enhancement through manipulation of the interaction between the fungus and the insect epicuticle components, to obtain a biocontrol agent capable of infecting both insect pests. This is important for the soybean bug *P. guildinii*, which is known for its low susceptibility towards fungal infection under field conditions (Sosa-Gómez and Moscardi, 1998).

Both hemipterans showed different susceptibilities towards the *B. bassiana* strains used in this study. Entomopathogen-inoculated *T. peregrinus* insects reached 100 % total mortality with all evaluated *B. bassiana* strains. Similar results were previously noted by Corallo et al. (2019) when comparing with other fungal entomopathogen species, such as *Isaria farinosa*, *Lecanicillium lecanii* and *Paecilomyces variotii*, who found that *B. bassiana* showed the greatest virulence, killing more individuals in shorter time. On the contrary, the redbanded stink bug *P. guildinii* showed to be less susceptible to fungal infection, with different strains exerting different mortality percentages. The highest mortality percentage recorded was 90%, reached by the hypervirulent strain ILB308, and the lowest was 46.7% shown by the hypovirulent strain ILB299. Similar results were obtained by Parys and Portilla (2020), where *P. guildinii* insects inoculated with *B. bassiana* showed maximum mortality percentage of 88%.

The main hypothesis tested in this study was that the capacity to grow on CHC is positively co-related with virulence, since CHCs are the first barrier spores must overcome to invade the insect host. Because growth on hydrocarbons is known to impose cellular stress (Huarte-Bonnet et al., 2014; Cray et al., 2015), the lower vegetative growth as well as less abundant mycelia observed on all strains grown on MM

supplemented with *n*-alkanes compared with CM medium was expected (Crespo et al., 2000). However, when growth on different CHCs was evaluated, ILB308 -regarded as the most virulent strain against the most resistant insect *P. guildinii*- showed the lowest vegetative growth, together with the commercial strain GHA. Strains Fi1728 and ILB205, of intermediate virulence towards *P. guildinii*, showed higher radial growths, but in general lower than the least virulent strain ILB299. Morphologically, ILB299 and Fi1728 produced more abundant aerial mycelia on all evaluated culture media. In conclusion, no positive correlation between vegetative growth on CHCs and virulence was found. Other studies have also evidenced that no positive correlation existed between morphological characteristics such as germination speed or growth rates of virulent strains and increased virulence (Liu et al., 2003; Romón et al., 2017).

Interestingly, when analyzing the gene expression of the hypervirulent strain ILB308 and hypovirulent strain ILB299 grown in *n*-alkane, in relation with cuticle adhesion and degradation and antioxidant defense, it was clearly seen that in general, in the hypervirulent strain the genes involved in those functions were up-regulated earlier and in a more pronounced manner than the hypovirulent strain. These results are in concordance with previous reports where early infection reported genes are up regulated in most virulent strains (Zhang et al., 2011; Wang and Wang, 2017). The apparent contradiction between colony growth in CHC, which was lower in ILB308, and the expression of genes related with CHC assimilation, which was higher, could be explained by the effect of the expression of other genes that could be more directly related to growth or sporulation that were not evaluated in this experiment (Dias et al., 2008; Zhang et al., 2010; Khoury et al., 2019).

Regarding the effect of *n*-alkane nutrition on the expression of virulence related genes, the most virulent strain overexpressed genes related to virulence earlier and in a more sustained fashion than the hypovirulent strain when grown on *n*-pentadecane. Strain ILB308 overexpressed the *Bbhyd2* gene -encoding hydrophobin- at the beginning of the evaluation, with constant increase until the end of the assay, while overexpression in the hypovirulent strain ILB299 was evidenced on day 6, and it later decreased by day 9. Hydrophobins are necessary for the fungus to adapt to the hydrophobic wax-rich epicuticular surface and their early overexpression in strain ILB308 might imply better probabilities for the progression of the pathogenesis for this strain than the hypovirulent ILB299. This result is in concordance with Huarte-Bonnet et al. (2018a) where high induction of the genes coding for hydrophobins were found in hydrocarbon-grown fungi.

Enzymes involved in terminal hydroxylation of *n*-alkanes and fatty acids (P450alk) are expressed from genes belonging to the CYP52 family (Tanaka et al., 1982; Pedrini et al., 2013; Huarte-Bonnet et al., 2018b), which act on a wide variety of endogenous and xenobiotic molecules, including alkanes. Here we evaluated the expression of *Cyp52g11*, *Cyp52t1a* and *Cyp52x1* genes involved in hydrocarbon assimilation. Results show that *BbCyp52g11*, *Cyp52t1a* and *BbCyp52x1* genes were induced in both fungal strains, although induction levels were higher and earlier in strain ILB308.

Genes *Bbcdep1* (Fang et al., 2005; Romón et al., 2017), encoding a subtilisin-like protease, and *Bbchit1* encoding a chitinase (Bogo et al., 1998) were overexpressed in strain ILB308 at day 3 and 9 of the evaluation, respectively. These two genes were not induced in strain ILB299. As single copy genes, it seems reasonable that their relative expression levels were directly correlated with virulence. Therefore, when ILB308 grew on *n*-pentadecane, a priming of virulence genes related with degradation of proteins and chitin was observed, which could explain the baseline virulence of this strain towards *P. guildinii* and partially its increased virulence after growth on pentadecane. Interestingly, a fusion protein with protease and chitinase activity based on these two genes was designed to enhance the virulence of *B. bassiana* (Fang et al., 2009). With proteins and chitin being the most abundant components of the cuticle, the overexpression of these two genes in ILB308 grown on *n*-pentadecane could be reflecting the virulence enhancement effect

obtained by the transformed strain in that study.

Regarding *Bbcdp1* overexpressed in ILB308 and not in ILB299, a recent study by Gao et al. (2020) found CDEP1 to be correlated to higher fungal outgrowths on insect cadavers and their conidial yield, implying a link of this protease to fungal survival, dispersal, and infection cycle in host habitats. Thus, the differences observed in our study in the expression of this gene, particularly the under-expression observed in ILB299 may be correlated to the incapacity of this strain to sporulate on dead cadavers.

Because growth on alkanes causes major changes in fungal metabolism (Crespo et al., 2000) and a scenario of oxidative stress is caused by the accumulation of reactive oxygen species, the induction of anti-oxidant genes and enzymes to overcome this situation can be understood as another example of fungal higher fitness (Huarte-Bonnet et al. 2014). In this study both strains showed an induction of the stress tolerance gene *Bbcac* and the GSH system *Bbgpx* gene, although induction levels were higher in hypervirulent strain ILB308. The *Bbgpx* gene (selenium-glutathione peroxidase enzyme) expression is regulated to maintain catalase activity (Baud et al., 2004), so the higher induction of this gene together with *Bbcac*, may offer an advantage in the oxidative response of the hypervirulent strain ILB308.

Showing the previously described differences in original virulence, growth on CHC and gene expression, it is remarkable that the hyper- and hypo- virulent strains were similarly affected in their capacity to infect and kill *P. guildinii* insects after growth on *n*-pentadecane. The HC-15 priming effect on virulence in both strains seems linked to the over-expression of the genes belonging to the CYP52 family evaluated on this work *Bbcyp52g11* and *Bbcy52px1*, together with adhesion gene (*Bbhyd2*) and stress tolerance genes (*Bbcac* and *Bbgpx*), and relatively independent from the observed induction of *Bbchit* and *Bbcdp1* genes, which were clearly overexpressed in the hypervirulent ILB308 and not in the hypovirulent strain ILB299. This finding is in concordance with reports that suggest that the higher virulence observed in hydrocarbon grown strains is due to induction of alkane assimilation genes (Pedrini et al., 2010, 2013; Zhang et al., 2012). In other words, the genes whose overexpression could explain the difference in original virulence between ILB308 and ILB299 are not the ones that can be signaled as the responsible for the virulence enhancement observed in both strains after growth on *n*-pentadecane. Rather, the overexpression of genes related to CHC assimilation, observed in ILB308 and ILB299 when grown on *n*-pentadecane, could be responsible of the virulence enhancement observed in both strains.

In the case of *T. peregrinus*, a rather susceptible insect pest, conidia harvested from the hypervirulent strain ILB308 grown on *n*-pentadecane showed an increase in virulence, whereas differences in virulence in the hypovirulent strain were not statistically significant.

We have shown that hypervirulent strain ILB308 can increase its virulence towards both hemipteran pests *P. guildinii* and *T. peregrinus* by growth on HC-15, and that exposure to this short chain alkane induces not only genes related to the degradation of epicuticular hydrocarbons but also primes the activity of genes that are needed before or after epicuticle penetration. The virulence of ILB299 was also enhanced but to a lower extent only in the highly resistant insect *P. guildinii*. In concordance with previous studies (Crespo et al., 2002; Pedrini et al., 2009, 2010, 2013; Huarte-Bonnet et al., 2018a,b), these results suggest that growth on CHC, in a Petri plate, can trigger the expression of genes related to CHC metabolism, adhesion and virulence, and that this early priming can lead to virulence enhancement against insects that are highly resistant to entomopathogens. Because other genes are involved in the pathogenicity process of entomopathogenic fungi, including genes indirectly involved in virulence such as genes related to adaptation to the hemolymph and nutrient assimilation genes, the transcriptome profiling of strains ILB308 and ILB299 grown on cuticle extracts is being evaluated with a RNAseq approach, in order to identify all genes expressed under CHC nutrition and to obtain a better understanding of the process.

5. Conclusion

n-pentadecane-priming of *B. bassiana* strains can be used to enhance their virulence towards low fungal-susceptible insects, such as the soybean pest *P. guildinii*. In the present study, we studied the expression of relevant to infection genes, involved in virulence of *n*-pentadecane grown strains. We have shown that the induction of some genes related to hydrocarbon assimilation, adhesion, and stress tolerance, may be responsible for the virulence enhancement observed both in the hyper- and hypovirulent strains.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jip.2021.107700>.

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Capítulo 3_

Evaluación del transcriptoma
de *B. bassiana* en su interacción
con diferentes componentes
cuticulares de *P. guildinii*

Introducción

El término secuenciación de nueva generación, por sus siglas en inglés Next Generation Sequencing (NGS) hace referencia a las tecnologías diseñadas para analizar gran cantidad de ADN de forma masiva y paralela. Una de estas tecnologías es el RNAseq, mediante la cual se pueden conocer los perfiles transcriptómicos de muestras individuales, permitiendo conocer la totalidad de los genes que se están expresando a nivel celular bajo una condición dada en un momento determinado; más aún, los niveles de detección son tan bajos que hasta es posible detectar y cuantificar transcritos no codificantes, isoformas, nuevos transcritos, corte y empalme alternativos y sitios de interacciones proteína-ARN. Comparando perfiles transcriptómicos, es decir cambios en la expresión génica, se pueden conocer diferentes funciones y características de interés biológico a nivel de cepa, es decir cepa-específicos, ya sea comparando diferentes cepas, o la misma cepa en diferentes condiciones. Además, el potencial de estas técnicas radica en la posibilidad de dilucidar nuevos genes o rutas metabólicas relacionadas directa o indirectamente a la virulencia. El esquema general de trabajo para un experimento de expresión génica se puede dividir en dos etapas. La primera es la etapa del secuenciado, la cual incluye la extracción del ARN, la preparación de las librerías de ADN complementario (cADN) resultante de la transcripción reversa de la población de ARN y el secuenciado del cADN. La segunda etapa es la Bioinformática, la cual incluye el procesamiento de las lecturas o “reads” obtenidos, la estimación de expresión individual de genes, normalización y la identificación de genes diferencialmente expresados.

Finalmente, luego de los análisis bioinformáticos, se suelen validar los resultados obtenidos mediante RT-qPCR. Para realizar esta etapa, se suelen seleccionar diferentes genes de interés, por ejemplo, genes sobre-expresados, se obtienen primers específicos para éstos y se realiza una amplificación mediante PCR real time utilizando las mismas muestras de ARN, para validar los resultados de secuenciación obtenidos.

3.1 Obtención del ARN y Secuenciado

Para la obtención del ARN se pueden utilizar diferentes metodologías de extracción, las cuales resultarán en diferentes niveles de rendimiento y de calidad. Se pueden utilizar tanto kits comerciales como protocolos caseros, a los cuales a su vez se les puede ajustar determinadas etapas para obtener resultados variables, ya sea que resulten con mayores cantidades o calidades de ARN. Sea cual sea la metodología elegida, la estrategia radica en utilizar para todas las muestras siempre el mismo protocolo estandarizado. Una vez obtenido el ARN se debe evaluar su cantidad y calidad, para determinar qué tan íntegro y puro se ha obtenido. En primera instancia se suele medir la concentración en el espectrofotómetro Nanodrop en donde también se obtienen algunos parámetros que dan cuenta de la calidad y luego se realiza una electroforesis en gel de agarosa al 1%. Por otra parte, la empresa Agilent desarrolló un software (Agilent Bioanalyzer) que permite calcular el número que se conoce como RIN, de su sigla en inglés RNA Integrity Number, a partir de una representación digital de la distribución de tamaño de las moléculas de ARN (Figura 7 a). El número RIN se acota entre 1 y 10, siendo 1 el estado más degradado y 10 el estado más íntegro (Figura 7c). Además, los electroferogramas (Figura 7b) obtenidos permiten visualizar la presencia de contaminación por ADN en las muestras.

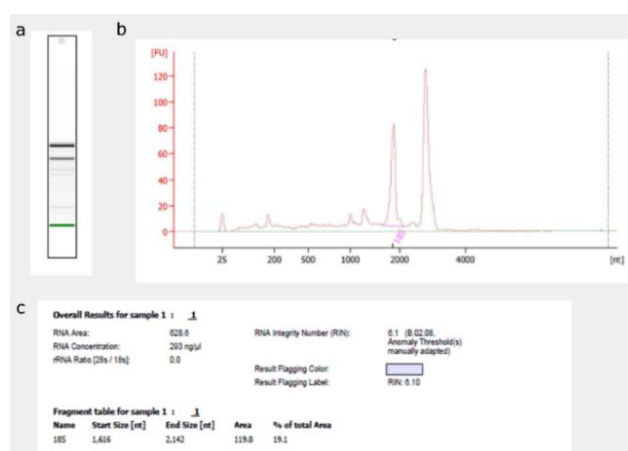


Figura 7 RIN obtenido mediante Agilent Bioanalyzer. a: electroforesis, b: electroferograma, c: resumen de la muestra y valor de RIN.

La ausencia de ADN genómico en la muestra de ARN es de suma importancia, dado que incluso cantidades ínfimas pueden afectar negativamente los resultados; para evitar la contaminación de ADN es aconsejable realizar el tratamiento con DNasas.

En términos de NGS “librería” hace referencia a una colección aleatoria de fragmentos de ADNc que están prontos para ser secuenciados mediante un protocolo específico. Uno de los métodos más utilizados, y el utilizado en este trabajo es la secuenciación por Illumina. El método de Illumina se basa en la propiedad de la ADN polimerasa de identificar el orden preciso en el que se necesitan los nucleótidos específicos para crear una copia complementaria perfecta de una sola hebra de ADN determinada y garantiza que haya un registro específico de la base cada vez que la ADN polimerasa agrega un nuevo nucleótido a una copia de ADN en evolución.

El registro en las plataformas Illumina es una señal fluorescente, es decir, cada nucleótido está marcado con un fluoróforo específico para que la incorporación de A, C, T, G produzca una emisión de luz distinta. Estas señales se registran a través de cámaras automatizadas en microscopios sofisticados. Esto significa que para que los fragmentos de ADNc se secuencien en un secuenciador de Illumina, estos tienen que ser monocatenarios, no pueden exceder un cierto tamaño, tienen que ser inmovilizados en un portaobjetos, y las propiedades de secuencia inherentes de un fragmento determinado (longitud, contenido de GC, homopolímeros) influirán en la fidelidad de la ADN polimerasa y, por lo tanto, en la señal final.

Por lo tanto, la preparación de la librería para los protocolos de Illumina tiene como objetivo generar fragmentos de ADNc entre 150 y 300 pb, que se hibridan a un portaobjetos de vidrio con cámaras microfluídicas (flowcell). De esos fragmentos, solo se secuenciarán los extremos (50 a 150 pb).

El protocolo de preparación de las librerías dependerá en el tipo de ARN con el que se esté trabajando, en el caso de este trabajo, y el más común en los

trabajos de expresión diferencial de genes mediante RNAseq, se trabajó con el ARN mensajero (m-ARN). El ARN total que se extrajo de las muestras contiene típicamente un 80-85% de r-ARN y un 10-15% de t-ARN (Farrell, 2010), lo cual es un problema cuando el ARN que nos interesa es el m-ARN. Por lo tanto, el primer paso es el enriquecimiento del m-ARN mediante la selección positiva de ARN mensajeros mediante la captura de ARN poliadenilado “polyA-capture” (Cui et al., 2010).

Luego se aísla el m-ARN enriquecido mediante perlas magnéticas oligo(dT), diseñadas para que se unan a las colas poli(A) complementarias del ARN mensajero. Entonces tras la purificación, el ARN se fragmenta en trozos pequeños por medio de cationes divalentes a temperatura elevada. Los fragmentos de ARN segmentados se copian en el ADNc de la primera cadena mediante cebadores aleatorios y transcriptasa inversa y, seguidamente, se produce la síntesis de ADNc de la segunda cadena mediante ADN polimerasa I y ARNasa H. Entonces, se añade una base única “A” en estos fragmentos de ADNc y se produce la posterior ligadura del adaptador. Los productos se purifican y se enriquecen mediante PCR. El proceso de ligación de los adaptadores utiliza una enzima para conectar los adaptadores a ambos extremos de los fragmentos de ADN. Se agrega una base 'A' a los extremos romos de cada hebra, preparándolos para la ligadura a los adaptadores de secuenciación. Cada adaptador contiene un saliente de base en 'T', que proporciona un saliente complementario para ligar el adaptador al ADN fragmentado con cola A. Los fragmentos de ADN se hibridan a la celda de flujo a través de los adaptadores y cada uno se amplifica masiva y clonalmente, formando grupos de ADN de doble cadena, llamados clusters. De esta manera se genera la biblioteca definitiva de ADNc (Dündar et al., 2019).

Este paso es necesario para garantizar que la señal de secuenciación sea lo suficientemente fuerte como para detectarse sin ambigüedades para cada base de cada fragmento. A las secuencias cortas que se obtienen a partir de estos fragmentos de ADNc se les conoce como lecturas (“reads”), las cuales

se pueden generar en los formatos de lecturas simples (“single-end”) cuando la secuenciación se realiza sólo en alguno de los extremos de cada fragmento, o bien como lecturas apareadas (“paired-end”) cuando a partir del mismo fragmento se secuencian los dos extremos. Los protocolos de secuenciación de Illumina más utilizados solo cubrirán de 50 a 100 pb de cada fragmento (dependiendo de la longitud de lectura que se haya elegido). La secuenciación de los extremos de los fragmentos se basa en dNTP marcados con fluoróforo con elementos terminales reversibles que serán incorporados y excitados por un láser uno a la vez y, por lo tanto, permitirán la identificación óptica de cada base individual (Figura 8)

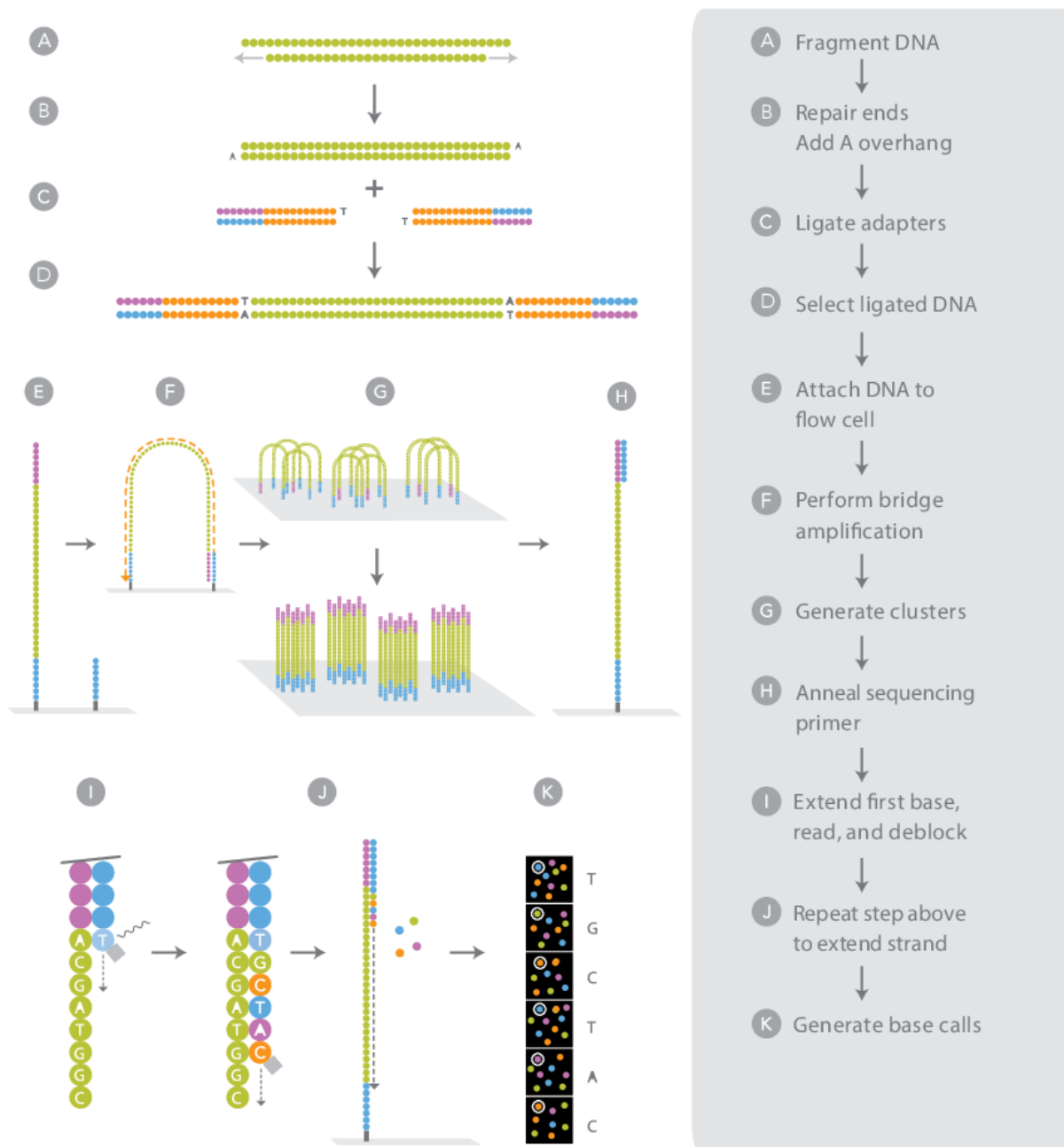


Figura 8 Diagrama del Flujo de Trabajo de NGS Illumina. Preparación de la librería (A-D), Generación de clusters (E-H), Secuenciado (I-K).

3.2 Análisis Bioinformático

Las lecturas se solapan contra un genoma de referencia generando un número de transcritos probables (“contigs”), Cuando existe un genoma de referencia, las lecturas apareadas permiten además detectar isoformas o eventos de empalme (“splicing”) alternativo de RNAs mensajeros. Una vez que se ensamblaron los contigs, las lecturas se mapean sobre ellos y se cuantifican; al normalizar el número de lecturas mapeadas sobre cada transcrito con base en la longitud de cada contig y el tamaño de la biblioteca de secuenciación, se puede calcular la abundancia relativa de cada transcrito en las muestras biológicas que se secuenciaron. Los análisis posteriores al ensamblaje del transcriptoma estarán determinados por los objetivos particulares de cada estudio, aunque el uso más común del RNAseq es la detección de expresión diferencial entre muestras biológicas.

Comúnmente los resultados de secuenciación, es decir el conjunto de lecturas o “reads” se reciben como archivos muy pesados y comprimidos llamados FASTQ. Aunque el formato específico de dichos archivos FASTQ puede variar según la plataforma elegida para realizar la secuenciación, por lo general todos son derivados de archivos FASTA (archivos de texto de secuencias nucleotídicas o aminoacídicas) es decir van a incluir la secuencia nucleotídica de cada lectura individual junto con los puntajes de calidad correspondientes. Para las secuenciaciones pair-end (PE), se obtendrá un archivo FASTQ para cada caso, por lo tanto, por cada muestra pair-end recibiremos 2 archivos.

En la figura 9 se muestra un esquema del flujo de trabajo en el análisis bioinformático de expresión diferencial de genes mediante RNAseq, el cual generalmente implica varios pasos: corte de adaptadores, alineamiento, conteo y normalización de las lecturas secuenciadas y análisis de expresión diferencial (DE).

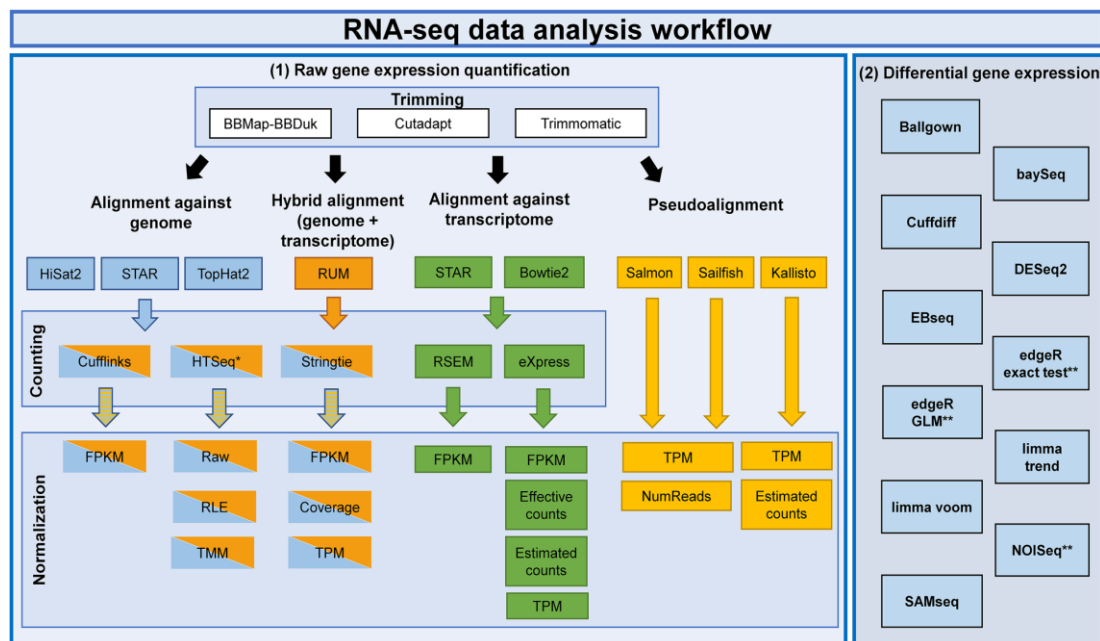


Figura 9 Diagrama del Flujo de Trabajo en el análisis bioinformático de RNAseq. Cada cuadro contiene los algoritmos y métodos usados para el corte de adaptadores, alineamiento, conteo, normalización y pseudoalineamiento. El panel de la derecha muestra los algoritmos usados para DGE. Adaptado de Corchete et al., 2020.

El primer paso a nivel bioinformático es el análisis de la calidad de las secuencias crudas, es decir los reads tal y como los hemos recibido. Para esto utilizamos la herramienta *FastQC* (Andrews, 2010). Luego se realiza el recorte de los adaptadores o “trimming” a cada archivo FASTQ para aumentar la tasa de mapeo de lecturas mediante la eliminación de las secuencias adaptadoras y la eliminación de nucleótidos de mala calidad (Figura 9). Existen diversos algoritmos para realizar el “trimming”, entre los cuales se encuentra *Trimmomatic* (Bolger et al., 2014), el cual fue utilizado en este trabajo. Luego se han de mapear o alinear las secuencias con un genoma de referencia del organismo en cuestión o en caso de no haberlo reportado con un transcriptoma de referencia. En este trabajo se realizó el alineamiento con el genoma de referencia de *Beauveria bassiana* ARSEF8028. Nuevamente existen diversos algoritmos para realizar el alineamiento (Figura 9), en este trabajo se utilizó *STAR* (Dobin, et al., 2013). Una vez que se han mapeado los reads deben de asignarse a un gen o transcripto, en un proceso llamado conteo y/o cuantificación. En este trabajo

se utilizó *RSEM* (Li & Dewey, 2011). Posteriormente se realiza la normalización para eliminar el posible sesgo de secuenciación. El paso final en la mayoría de los estudios de RNAseq es el análisis de genes diferencialmente expresado DEG. En este trabajo se realizó dicho análisis utilizando el algoritmo *EdgeR* (Robinson et al., 2010) (Figura 9, derecha).

Justificación

El análisis transcriptómico comparativo de cepas de *B. bassiana* de virulencia contrastante creciendo en medio de cultivo suplementado con extractos cuticulares de *P. guildinii*, permitirá conocer el perfil de genes expresados por una cepa de *B. bassiana* con potencial biocontrolador bajo condiciones de cultivo *in vitro*, que se asemejen lo más posible a las condiciones *in vivo*, lo cual es de particular interés para identificar genes o rutas metabólicas cepa-específicos.

Objetivos

Detectar la expresión de genes específicos asociados a la degradación de los componentes de la cutícula de *P. guildinii* en cepas de *B. bassiana* de virulencia contrastante mediante el análisis del transcriptoma por RNAseq.

Resultados

A continuación, se expone el manuscrito que será enviado a una revista internacional arbitrada, titulado “**Transcriptome analysis of *Beauveria bassiana* during assimilation of exuviae and cuticular lipids of the redbanded stink bug *Piezodorus guildinii* (Heteroptera: Pentatomidae)**” en el cual se incluye la totalidad de los resultados del Capítulo 3 de este manuscrito.

Artículo científico – Resumen en español

La chinche de la soja *Piezodorus guildinii* (Westwood) (Heteroptera: Pentatomidae) es el insecto plaga de la soja de mayor importancia económica en América del Sur y es tolerante tanto a los insecticidas químicos como a las infecciones naturales por hongos entomopatógenos. Recientemente, la virulencia hacia esta plaga de cepas hipo e hipervirulentas (ILB299 e ILB308, respectivamente) del hongo entomopatógeno *Beauveria bassiana*, así como el aumento de su virulencia por un efecto de cebado cuando se cultivan en el hidrocarburo epicuticular *n*-pentadecano como única fuente de carbono fueron observados. En este estudio, evaluamos y comparamos estas dos cepas de *B. bassiana* con respecto a los genes expresados diferencialmente (DEG), la ontología génica (GO) y las vías KEGG mediante tecnología RNAseq en respuesta a dos extractos de cutícula de *P. guildinii* y a *n*-pentadecano. Encontramos un perfil de expresión diferente de los genes relacionados con la virulencia, así como los parámetros GO y KEGG a los 4 días post inoculación, lo que podría ser responsable de su virulencia intrínseca pero también del efecto de cebado observado. La cepa hipovirulenta ILB299 mostró sobreexpresión predominante de genes de penetración de la cutícula, como quitinasas, proteasas y citocromo P450 (CYP), y predominantemente los términos GO 'unión de hemo' y 'actividad de monooxigenasa', así como la ruta metabólica del 'peroxisoma' enriquecida. La cepa hipervirulenta ILB308 mostró sobreexpresión de genes de remodelación de la pared celular y del ciclo celular, y también adhesión de la cutícula y CYP, junto con el término GO de 'actividad del factor de transcripción de unión al ADN' y las vías KEGG de 'Meiosis-levadura' y 'Ciclo celular' enriquecidas. Estos resultados revelaron un "efecto retardado" observado en la cepa hipovirulenta ILB299 en comparación con la hipervirulenta ILB308, que se correlaciona con la menor virulencia de la cepa anterior.

**Transcriptome analysis of *Beauveria bassiana* during assimilation of exuviae
and cuticular lipids of the redbanded stink bug *Piezodorus guildinii*
(Heteroptera: Pentatomidae)**

Lucia Sessa, Héctor Oberti, Eduardo Abreo, Nicolas Pedrini

a Laboratorio de Bioproducción, Plataforma de Bioinsumos, Instituto Nacional de Investigación Agropecuaria, estación experimental Wilson Ferreira Aldunate, Ruta 48, km 10, Canelones, Uruguay

b Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), CCT La Plata Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) – Universidad Nacional de La Plata (UNLP), calles 60 y 120, 1900 La Plata, Argentina

1 authors with equal contribution

Abstract

The redbanded stink bug *Piezodorus guildinii* (Westwood) (Heteroptera: Pentatomidae) is the most economically important insect pest of soybean in South America, and tolerant to both chemical insecticides and natural infections by fungal entomopathogens. Recently, the virulence towards this pest of hypo- and hypervirulent strains (ILB299 and ILB308, respectively) of the entomopathogenic fungus *Beauveria bassiana*, as well as their virulence enhancement by a priming effect when grown in the epicuticular hydrocarbon *n*-pentadecane as the sole carbon source, were observed. In this study, we evaluated and compared these two *B. bassiana* strains regarding the differentially expressed genes (DEGs), gene ontology (GO) and KEGG pathways by RNAseq technology in response to two different cuticle extracts of *P. guildinii* and to synthetic *n*-pentadecane. We found a different expression profile of virulence related genes, as well as GO and KEGG parameters at 4 dpi, which could be accountable for their intrinsic virulence but also for the priming effect observed. Hypovirulent strain ILB299 showed predominantly overexpression of cuticle penetration genes, such as chitinases, proteases and cytochrome P450s (CYP), and had predominantly the GO terms 'heme binding' and 'monooxygenase activity' as well as de 'peroxisome' metabolic pathway enriched. Hypervirulent strain ILB308 showed overexpression of cell wall remodeling and cell cycle genes, and also cuticle adhesion and CYPs, together with the 'DNA-binding transcription factor activity' GO term and the 'Meiosis-yeast' and 'Cell Cycle' KEGG pathways enriched. These results revealed a 'delayed effect' observed in the hypovirulent strain ILB299 in comparison with the hypervirulent ILB308, which correlates with the lower virulence of the former strain.

37 Introduction

38 Pest resistance to chemical pesticides as well as the detrimental environmental impacts they
39 impose, generate novel research opportunities for the development of environment-friendly
40 crop management strategies. Biological control by entomopathogenic fungi seem a promising
41 alternative strategy for pest control, being they possess different modes of action as opposed
42 to synthetic insecticides. The insect cuticle is the first barrier against fungal pathogens and
43 its breaking by fungi is the precondition for a successful outcome of an entomopathogenic
44 infection (Pedrini et al., 2007). Briefly, the infection process initiates with the spore adhering
45 to the insect cuticle, the later spore germination and appressorium development culminates
46 with the penetration of the epicuticle and the breaching of the insects' hemocoel where the
47 blastospores proliferate leading to the host death (Roberts 1974; Boucias et al., 1988; St.
48 Leger et al., 1991). Insect cuticle composition varies among insect species (Andersen &
49 Roepstorff, 1978) and fungal cuticle degrading capability is vital in the early infection process.

50 The redbanded stink bug *Piezodorus guildinii* (Westwood) (Heteroptera: Pentatomidae) is
51 the most economically important insect pest of soybean *Glycine max* (L.) Merrill (Fabales:
52 Fabaceae) in the Americas, specially across the southern United States, Central and South
53 America and the Caribbean (Bundy et al., 2018; Musser et al., 2018). In Uruguay as well as
54 Argentina soybean is considered the main crop of rainfed agriculture, based on both
55 production and cultivated area, with 1.708.000 and 60.500.000 tons of production,
56 respectively, and a growing area over 908.000 and 13.809.328 ha in 2020/2021 harvest, in
57 Uruguay and Argentina respectively (MGAP 2021; SISA 2021). Currently, *P. guildinii* is the
58 most important stinkbug species attacking soybean crops in Argentina and Uruguay
59 (Cingolani et al., 2014) causing significant yield losses in soybean plantations (Zerbino,
60 2010). Chemical pesticides applied to reduce *P. guildinii* insect populations are not very
61 successful (Catchot et al., 2020; Baur et al., 2010). Moreover, the tolerance of *P. guildinii* to
62 pyrethroid insecticides was recently correlated with the epicuticular hydrocarbon amount and
63 pattern (Sessa et al., 2021).

64 Several studies have investigated the fungal infection of stink bugs, including *Nezara viridula*,
65 *Eushistus heros*, *Glaucias subpunctatus*, *Halyomorpha halys* and *Plautia crossota stali*, as
66 well as *Piezodorus guildinii*, by *Metarhizium anisopliae* and *Beauveria bassiana* under
67 laboratory conditions (Sosa-Gómez et al., 1997; Ihara, et al., 2008, Sessa et al., 2022) as
68 well as under field conditions (Sosa-Gómez & Moscardi, 1997; Parys & Portilla, 2020)
69 revealing their potential effectiveness as bio-controllers. It is reported that nutrition on
70 hydrocarbon enhances virulence in some *B. bassiana* strains, probably favored by the
71 augmented affinity to insect cuticle components (Crespo et al., 2002; Pedrini et al., 2009), as
72 a result of the induction of genes involved in cuticular hydrocarbon assimilation (Pedrini et

al., 2010, 2013; Zhang et al., 2012). The hydrocarbon composition of *Piezodorus guildinii* has recently been described (Sessa et al., 2021) and the virulence enhancement of *B. bassiana* strains towards this pest using a characteristic short-chain hydrocarbon (*n*-pentadecane) as the sole carbon source has been reported (Sessa et al., 2022).

Entomopathogen virulence genes related with early infection processes have been studied using RT-qPCR along with targeted gene knockouts of genes that code for hydrophobins (Zhang et al., 2011), cytochrome P450 monooxygenases (Pedrini et al., 2010, 2013, Zhang et al., 2012), chitinases (Fang et al., 2005; Zhang et al., 2009, 2010) and proteases (Fang et al., 2009). In addition, recent studies have focused on evaluating the gene expression profile of entomopathogenic fungi *in vitro* and *in vivo* by means of transcriptomic analyses applying the RNA-seq technology (Mantilla et al., 2012, Wang et al., 2017, Lai et al., 2017, Zhou et al., 2019; Zhang et al., 2019). The transcriptomics approach offers the possibility of finding novel virulence related genes or unknown metabolic routes but important for the infection process. *In vivo* studies evaluate the gene expression of the entomopathogenic fungi within the context of a host-pathogen interaction using dual RNA-seq (Chu et al., 2016, Lai et al., 2020; Zhou et al., 2019; Zhang et al., 2019; Kim et al., 2020). An advantage of this approach is the additional information regarding insect immune responses to fungal challenges as well as insect resistance and susceptibility genes (Pedrini 2018; Xing et al., 2015). *In vitro* studies often use cuticular components supplemented in the culture media, examples of which are the studies carried out by Mantilla et al. (2012), Wang et al., (2017) and Liu et al., (2021), who evaluated the gene expression of *B. bassiana* grown in cuticular extracts of the coffee berry borer, cuticle extracts of silkworm and cuticular extracts of several Lepidoptera insects, respectively.

Recently, we reported the expression of genes relevant to infection of hypovirulent (ILB299) and hypervirulent (ILB308) *B. bassiana* strains after growth on *n*-pentadecane as sole carbon source, and their association with the virulence enhancement observed when both strains were primed with *n*-pentadecane (Sessa et al., 2022). In this work, we continue to evaluate and compare these two *B. bassiana* strains regarding the differentially expressed genes (DEGs) by RNAseq technology in response to two different cuticle extracts of *P. guildinii* and to synthetic *n*-pentadecane, to help understand virulence and virulence enhancement processes at molecular level.

Materials and Methods

Fungal cultures

Beauveria bassiana strains ILB299 and ILB308 (hypo- and hypervirulent, respectively) were retrieved from the INIA Las Brujas fungal collection and were routinely grown on PDA plates for 14 days. Conidia were harvested, resuspended with Tween-saline solution (0.1% Tween 80 and 0.85% NaCl), filtered using autoclaved gauze and counted using a Neubauer chamber. Conidial suspensions were adjusted to a final concentration of 2.5×10^8 conidia/ml.

Growth on Piezodorus guildinii exuviae and n-pentadecane

Exuviae of 5th instar *P. guildinii* nymphs was collected from an indoor mass rearing, autoclaved, dried to constant weight in an oven at 40°C and utilized in two treatments. The first treatment -complete exuviae (CE)- consisted of grounded exuviae supplemented (0.04 g/cm²) to the surface of semi-solid minimal media (MM) plated in 90×15mm Petri plates before complete solidification (MM composition: 0.4g KH₂PO₄, 1.4g Na₂HPO₄, 0.6g MgSO₄·7H₂O, 1.0g KCl, 0.7g NH₄NO₃·7H₂O and 15g agarose in 1000ml of distilled water). The second treatment -lipids from exuviae (LE)- consisted of the lipidic exuviae components extracted with hexane (Cerkowniak et al., 2013) and supplemented to the surface of a solid MM 90×15mm Petri plate (2% final concentration). The third treatment -15 carbon hydrocarbon (H15)- consisted of commercial *n*-pentadecane (Acros Organics, Thermo Fisher Scientific) supplemented to the surface of a solid MM 90×15mm Petri plate (2% final concentration). All plates were covered with a cellophane membrane to avoid interference with RNA extraction and ultimately RNA quality (Pedrini et al., 2010) and 250µl of 2.5×10^8 conidia/ml suspension was inoculated over the membrane, obtaining a final concentration of 1×10^6 spores/cm². Plates were incubated at 26°C for 4 days. After incubation, mycelia were scraped from each plate with a sterile scalpel and placed on a DNase free 1.5ml eppendorf® tube with beads. All samples were stored on a -80°C freezer until further processing. The experiment was repeated three times and each one consisted of single repetitions.

RNA extraction, library construction and sequencing

Total RNA of all samples from both strains (ILB299 and ILB308) growing under the three culture media (CE, LE and H15) was extracted 4 days post inoculation (dpi) employing the RNeasy plant mini kit (Qiagen) according to the manufacturer's recommendations. RNA was quantified using Nanodrop (Thermo Fisher) and integrity and purity were evaluated on a 1% (w/v) agarose gel electrophoresis. Three biological independent samples (replicates) per strain under each condition were evaluated, except for ILB299 growing on *n*-pentadecane (H15) which comprised two replicates.

All RNA samples were stored at -80 °C until precipitation with 3M sodium acetate (pH 5.2) and 100% ethanol was performed. Precipitated samples were shipped to Macrogen where DNase treatment, RNA integrity, library construction and sequencing was completed. RNA integrity was assessed using an Agilent Technologies 2100 Bioanalyzer with an RNA Integrity Number (RIN) value greater than or equal to 7. The cDNA libraries of samples for Illumina RNA-seq were prepared using Illumina's kit according to the manufacturer's guide (TruSeq Stranded mRNA). The cDNA libraries were sequenced using Illumina NovaSeq6000 platform with pair-end (reads of 150pb) sequencing strategy at Macrogen Inc., Seoul, Korea.

RNA-seq mapping, quantitative gene expression and enrichment analysis

Reads were trimmed and adaptors were removed using Trimmomatic (Bolger et al., 2014) using MINLEN:75 and SLIDINGWINDOW:4:20. The clean reads were aligned to the high-quality chromosome scale *B. bassiana* ARSEF8028 reference genome (35.02Mb, Assembly GCA_001682635.1, released on 2016) with STAR (Dobin, et al., 2013) using the ENSEMBLE-FUNGI GFF3 file (https://ftp.ebi.ac.uk/ensemblgenomes/pub/release-55/fungi/gff3/beauveria_bassiana/) and RSEM (Li & Dewey, 2011). Statistical analysis of the expression data was performed using EdgeR (Robinson et al., 2010). For analysis of differentially expressed genes (DEG) removal of low expressed genes was done using expression limit of 1 CPM for 2 replicates and TMM method was used for normalization. ILB299 conditions were set as contrast condition and ILB308 as reference condition in all pairwise comparisons. The DEGs were identified using the following thresholds: log2FoldChange (FC) |1| and FDR ($p < 0.05$). Clustering analyses for correlation between samples were made using PtR script of the Trinity v2.11.0 package.

Gene ontology (GO) and KEGGs terms were detected as enriched ($FDR < 0.05$) in the up and downregulated gene sets from each comparison using the Fisher's exact test implemented in Omicsbox bioinformatic software (BioBam Bioinformatics). Each gene set was compared against the full set of genes in ARSEF8028 as background. GO enriched terms were reduced to most specific to remove more general GO terms with an adjusted p value of ($p < 0.05$).

Results

RNA-Seq sequencing data for *B. bassiana*

A total of 17 samples were sequenced and a total of 336.4 Gb of raw data was obtained (19.8 Gb per sample). After removing adaptors, unknown and low-quality sequences, average clean reads obtained per sample was 17.4 Gb (Supplementary Table S1). Reads mapping to *B. bassiana* ARSEF8028 genome are detailed in Supplementary Table 1.

A total of 16,905 genes were detected as expressed (TPM>1), 8,478 were expressed in hypovirulent strain ILB299 and 8,421 in hypervirulent strain ILB308. In hypovirulent strain 57, 54 and 340 genes were exclusively expressed in CE, LE and H15 culture media, while 58, 262 and 70 were exclusively expressed in hypervirulent strain, respectively (Supplementary Table S2). Both strains shared several culture medium specific genes (Supplementary Table S3). Four genes were expressed specifically in CE, 7 in LE and 22 in H15 culture medium. Genes expressed exclusively in H15 included genes with homology with cytochrome P450 monooxygenases CYP620D1, CYP570E2 and CYP5099A1 (*BB8028_0003g16510*, *BB8028_0002g05950* and *BB8028_0005g02520*, respectively), glutathione-S-transferase (GST), (*BB8028_0001g16860*), subtilisin-like protease PR1F (*BB8028_0004g01330*) and alpha/beta hydrolase superfamily protein (*BB8028_0007g00770*).

Differentially expressed genes (DEGs)

Comparisons between hypo and hyper virulent strains ILB299 and ILB308, respectively, were used to identify differentially expressed genes (DEGs) when growing under different nutrition sources. The pairwise comparisons between ILB299 vs. ILB308 set ILB299 as the contrast condition and ILB308 as the reference condition, where UP genes indicate up-regulated in the contrast and genes DOWN indicate up-regulated in the reference condition. The hierarchical cluster analysis heatmap of DEGs indicated that samples ILB299 and ILB308 could be distinguished based on the differentially expressed genes, showing a distinct expression pattern (Figure 1a).

A total of 2,632, 2,592 and 2,476 DEGs were obtained in CE, LE and H15 culture media, respectively. Of those, 1,512, 1,371 and 1,490 were upregulated, while 1,120, 1,221 and 986 were downregulated in CE, LE and H15 culture media, respectively (Figure 1b). Hypovirulent strain ILB299 had a higher number of differentially expressed genes in all three conditions, being more apparent when the carbon source was solely *n*-pentadecane (H15).

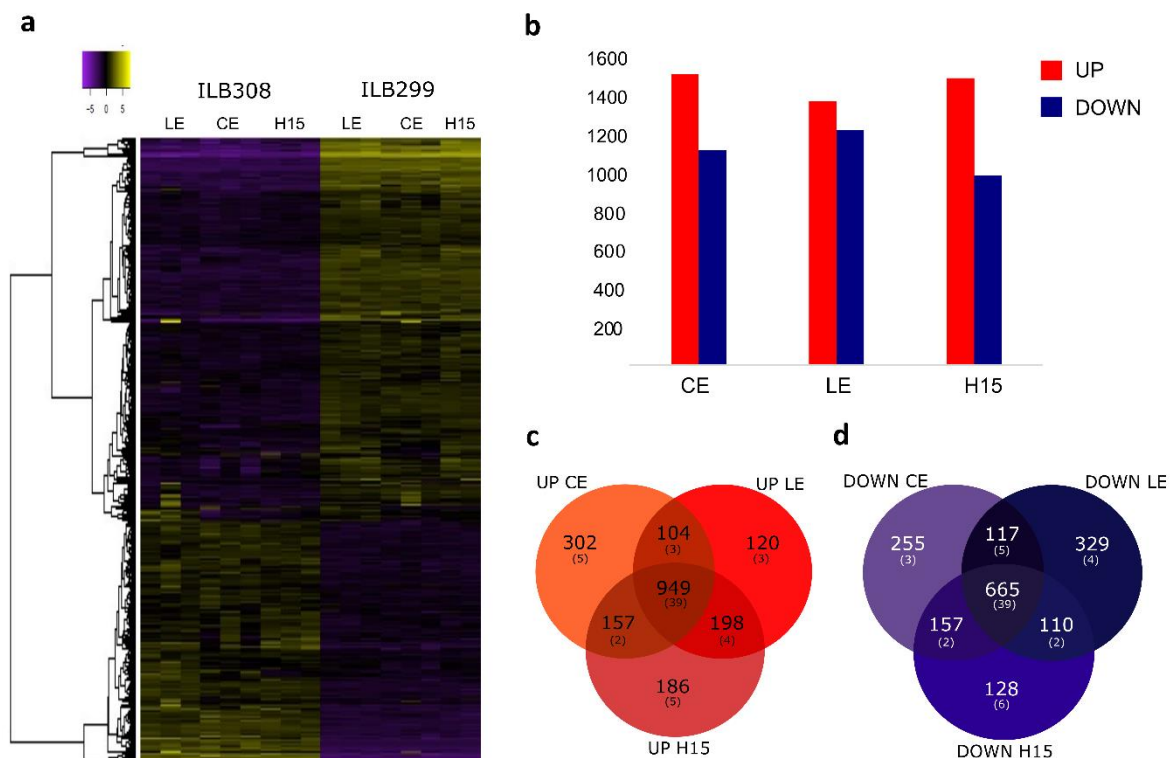


Figure 1. *Beauveria bassiana* ILB299 vs. ILB308 DEGs in CE, LE and H15 culture media at 4 dpi. a) Heat map of total DEGs expression in the different conditions. b) Summary of the DEGs expression. c) Venn diagram of up-regulated DEGs and d) Venn diagram of down-regulated DEGs. The numbers in brackets correspond to the 50 most highly expressed genes.

Venn diagrams of DEGs expressed on each culture media were constructed (Figure 1 c, d). Nine hundred and forty-nine up regulated and 665 down regulated DEGs were shared among the three culture media (Figure 1c, d). Up regulated DEGs exclusive to CE and H15 were higher than down regulated DEGs (302 and 186 vs. 255 and 128). However down-regulated DEGs exclusive to LE culture medium were higher than up-regulated DEGs (329 vs. 120, respectively) (Figure 1 c, d).

Of the top 50 up-regulated DEGs (up-regulated in hypovirulent strain ILB299), 39 were shared, 3 were common between CE and LE, 2 between CE and H15 and 4 between LE and H15 (Figure 1 c in brackets). The vast majority of these up-regulated shared DEGs had similar FC expression values, regardless of the nutritional source and merely a few genes were exclusive of a particular culture medium (Supplementary Table S4). Of the top 50 down-regulated DEGs (up-regulated in hypervirulent strain ILB308), 39 were shared, 5 were common between CE and LE and 4 were exclusive to LE culture medium (Figure 1 d in brackets). The vast majority of these down-regulated shared DEGs also had similar FC expression values, regardless of the nutritional source (Supplementary Table S5).

Virulence related genes (VRG)

A distinctive expression pattern was observed for several virulence related genes (VRG) in response to cuticle extracts and hydrocarbon nutrition (Table 1). Hypovirulent strain showed overexpression of several genes with homology to cuticle penetration, growth, and stress tolerance, including chitinases, proteases, cytochrome P450 monooxygenases (CYPs), and catalases; while hypervirulent strain showed mostly genes with homology to cuticle adhesion, cell wall remodeling and growth and cell cycle proteins, including hydrophobins, CFEM domain-containing proteins, 1,3-beta-glucanosyltransferase protein, Tudor domain-containing protein, and a cyclin dependent kinase, as well as two CYPs (Table 1).

Most of the genes in each function were found overexpressed regardless of the culture media (Table 1). Genes differentially overexpressed in hypovirulent strain ILB299 grown in H15/LE included beta-1,3-glucanase precursors (*BB8028_0003g11540* and *BB8028_0001g00880*), chitinase-like protein (*BB8028_0006g05320*) and chitinase III ChiA2 (*BB8028_0006g09420*), cuticle-degrading protease Bassiasin I precursor Cdep-1 (*BB8028_0002g13040*), subtilisin-like protease Pr1B (*BB8028_0001g00250*) and Cu/Zn superoxide dismutase BbSod1 (*BB8028_0005g07510*). In hypervirulent strain ILB308 grown in H15/LE chitin synthase activator (*BB8028_0002g10600*), mitogen-activated protein (BbHog1) (*BB8028_0001g11430*), cell wall protein (*BB8028_0003g09710*), GDP-mannose transporter (*BB8028_0004g05420*), Cyclin dependent kinase C Cdk (*BB8028_0003g03000*) and Mn-superoxide dismutase BbSod2 (*BB8028_0001g12420*) were detected as overexpressed (Table 1).

Interestingly, in ILB308 strain we observed only 1 gene of the 23 listed (4,3%) overexpressed exclusively in CE culture media, namely tudor domain-containing protein (BbTdp1), while ILB299 strain showed 5 genes of the 24 listed (20.8%) namely perilipin MPL1-like protein, chitinase II, squalene/oxidosqualene cyclase, glutathione S-transferase II (BbGST), and LysM domain-containing protein (Table 1).

Table 1 Proteins and VRG differentially expressed in *Beauveria bassiana* hypo- and hyper- virulent strains ILB299 and ILB308 grown in CE, LE and H15 culture media

Protein	Gene ID		Function	FC ILB299			FC ILB308			References
	ARSEF8028	ARSEF2860		CE	LE	H15	CE	LE	H15	
Hydrophobin (Hyd1)	BB8028_0007g04080	BBA_03015	Cuticle adhesion	1,25	1,4	1,42				Zhang et al. (2011)
Perilipin MPL1-like protein	BB8028_0001g16990	BBA_08759	Cuticle adhesion	1,19						Wang et al. (2007)
Hydrophobin (Hyd3)	BB8028_0002g12140	BBA_00530	Cuticle adhesion				1,57		3,42	Zhang et al. (2011)
hydrophobin-like protein (Hyd5)	BB8028_0007g04250	BBA_02999	Cuticle adhesion				9,16	9,43	7,11	He et al. (2016)
CFEM domain-containing protein	BB8028_0007g04960	BBA_08726	Cuticle adhesion				3,98	10,14	5,23	Wang et al. (2017)
CFEM domain-containing protein	BB8028_0001g16650	BBA_08726	Cuticle adhesion				1,02	1,58		Kulkarni et al. (2003)
CFEM domain-containing protein	BB8028_0006g00990	BBA_09339	Cuticle adhesion				1,42	1,03		Xiao et al. (2012)
CFEM domain-containing protein	BB8028_0007g00750	BBA_05711	Cuticle adhesion				1,74	1,21		This study
Fasciclin domain family protein	BB8028_0005g00660	BBA_07841	Cuticle adhesion				10,03	10,08	9,79	Santi et al. (2018)
Chitin synthase activator	BB8028_0002g10600	BBA_10281	Cell wall remodeling						1,15	This study
Class V chitin synthase (Chs5)	BB8028_0002g00770	BBA_06859	Cell wall remodeling				1,33	1,14	1	Qin et al. (2022)
1,3-beta-glucanosyltransferase (Gel)	BB8028_0004g10610	BBA_08600	Cell wall remodeling				1,29	1,41	1,8	Lai et al. (2017)
Tudor domain-containing protein (Tdp1)	BB8028_0005g07490	BBA_02313	Cell wall remodeling				1,05			Qiu et al. (2021)
beta-1,3-glucanase precursor	BB8028_0003g11540	BBA_05994	Cell wall remodeling		1,28	1,6				This study
beta-1,3-glucanase precursor	BB8028_0001g00880	BBA_03717	Cell wall remodeling			1,8				This study
CAMK family protein kinase	BB8028_0007g00310	BBA_10076	Signalling, cell cycle				6,53		6,61	Sun et al. (2022)
G-protein coupled receptor	BB8028_0003g04790	BBA_00828	Signalling				1,19	1,32		Lai et al. (2017)
Mitogen-activated protein kinase	BB8028_0001g11430	BBA_02280	Signaling					1,34	1	He et al. (2018)
G-protein coupled receptor	BB8028_0003g04380	BBA_00868	Signaling	3,01	3,57	3,21				Ying et al. (2013)
Chitinase II	BB8028_0001g11780	BBA_10303	Cuticle penetration	1,17						Fang et al. (2005)
Chitinase II	BB8028_0001g11790	BBA_10303	Cuticle penetration	2,89	3,5	3,54				This study
Chitinase III ChiA2	BB8028_0006g09420	BBA_05353	Cuticle penetration		3,83	4,47				Liu et al. (2021)
Chitinase-like protein	BB8028_0006g05320	BBA_06317	Cuticle penetration		2,18					Liu et al. (2021)
Cuticle-degrading serine protease	BB8028_0006g01520	BBA_09500	Cuticle penetration	1,52	1,08	2,22				Zhang et al. (2008)

Cuticle-degrading protease Bassiasin I precursor (Cdep-1)	BB8028_0002g13040	BBA_00443	Cuticle penetration	1,57				Zhang et al. (2008)
Trypsin-related protease	BB8028_0002g03570	BBA_05184	Cuticle penetration	8,97	7,61	7,82		Kim et al. (2020)
Subtilisin-like protease (Pr1B)	BB8028_0001g00250	BBA_03653	Cuticle penetration	2,15	2,34			Joshi et al. (1995)
Cytochrome P450 CYP5099A1	BB8028_0005g00330	BBA_07811	Cuticle penetration	3,8	2	2,95		Kim et al. (2020)
Cytochrome P450 CYP5099A1	BB8028_0005g02520	BBA_06472	Cuticle penetration	5,55	6,95	5,37		Kim et al. (2020)
Cytochrome P450 CYP655C1	BB8028_0004g01200	BBA_07335	Cuticle penetration				4,2 5,33 5,42	Pedrini et al. (2013)
Cytochrome P450 CYP625A1	BB8028_0005g08960	BBA_06726	Cuticle penetration				3,9 5,7	Pedrini et al. (2013)
Squalene/oxidosqualene cyclase	BB8028_0005g01760	BBA_06542	Growth	1,39				Wang et al. (2017)
Cyclophilin A	BB8028_0004g12690	BBA_05037	Growth	1,35	1,04			Viaud et al. (2003)
Glutathione S-transferase II (GST)	BB8028_0003g13230	BBA_04201	Growth	2,46				Oakley (2005)
LysM domain-containing protein	BB8028_0007g02820	BBA_03138	Growth	2,27				Mentlak (2012)
G-protein coupled receptor	BB8028_0003g04380	BBA_00868	Growth	3,01	3,57	3,21		Ying et al. (2013)
Conidial wall protein	BB8028_0008g00230	BBA_09998	Growth	1,26	1,56	1,43		Wang et al. (2017)
Cell wall protein	BB8028_0003g09710	BBA_05808	Growth				1,23	Wang et al. (2017)
Cell wall protein	BB8028_0005g11220	BBA_05808	Growth				1,93 2,29 2,97	Wang et al. (2017)
GDP-mannose transporter	BB8028_0004g05420	BBA_03233	Growth				1,3	Abe et al. (1999)
Siderophore iron transporter	BB8028_0003g09910	BBA_05827	Growth				2,26 2,99 2,95	Wang et al. (2017)
Fork head domain-containing protein (FKH)	BB8028_0003g14110	BBA_04116	Cell cycle				2,35 1,43 2,01	Wang et al. (2015)
Cyclin dependent kinase C (Cdk)	BB8028_0003g03000	BBA_07152	Cell cycle				1,01	Qiu et a. (2014)
Cu/Zn superoxide dismutase (Sod1)	BB8028_0005g07510	BBA_02311	Stress tolerance	1,92	1,68			Xie et al. (2012)
Catalase heme-dependent protein (CatB)	BB8028_0007g01900	BBA_05603	Stress tolerance	4,91	4,84	3,66		Guo et al. (2020)
Catalase heme-dependent protein (CatC)	BB8028_0004g13740	BBA_09109	Stress tolerance	1,34	1,07			Guo et al. (2020)
Mn-superoxide dismutase (Sod2)	BB8028_0001g12420	BBA_09706	Stress tolerance				1,79 1,03	Li et al. (2015)

In bold, genes among the top 50 up-reguated DEGs

Gene ontology (GO) annotation and enrichment analysis

To identify the functions of the DEGs during growth on each culture medium, GO functional analyses and classification were performed based on Fisher's exact test. Most specific enriched GOs were categorized into 12 GO terms belonging to: molecular function -MF- (8), biological process -BP- (3) and cellular component -CC- (1) (Figure 2).

Hypovirulent strain ILB299 showed enriched GOs when grown in all the evaluated culture media components, whereas ILB308 only showed enriched GOs when grown in LE medium (Figure 2). 'Heme binding' (GO:0020037) and 'monooxygenase activity' (GO:0004497) were the common annotation terms present exclusively in ILB299 strain (Figure 2 a-c). GO terms enriched when grown on CE included 'transmembrane transporter activity' (GO:0022857), 'aspartic-type endopeptidase activity' (GO:0004190) and 'serine-type carboxypeptidase activity' (GO:0004185) (Figure 2 a). In contrast, molecular function GOs over-enriched when grown in LE and H15 are solely 'heme binding' and 'monooxygenase activity' (Figure 2 b, c).

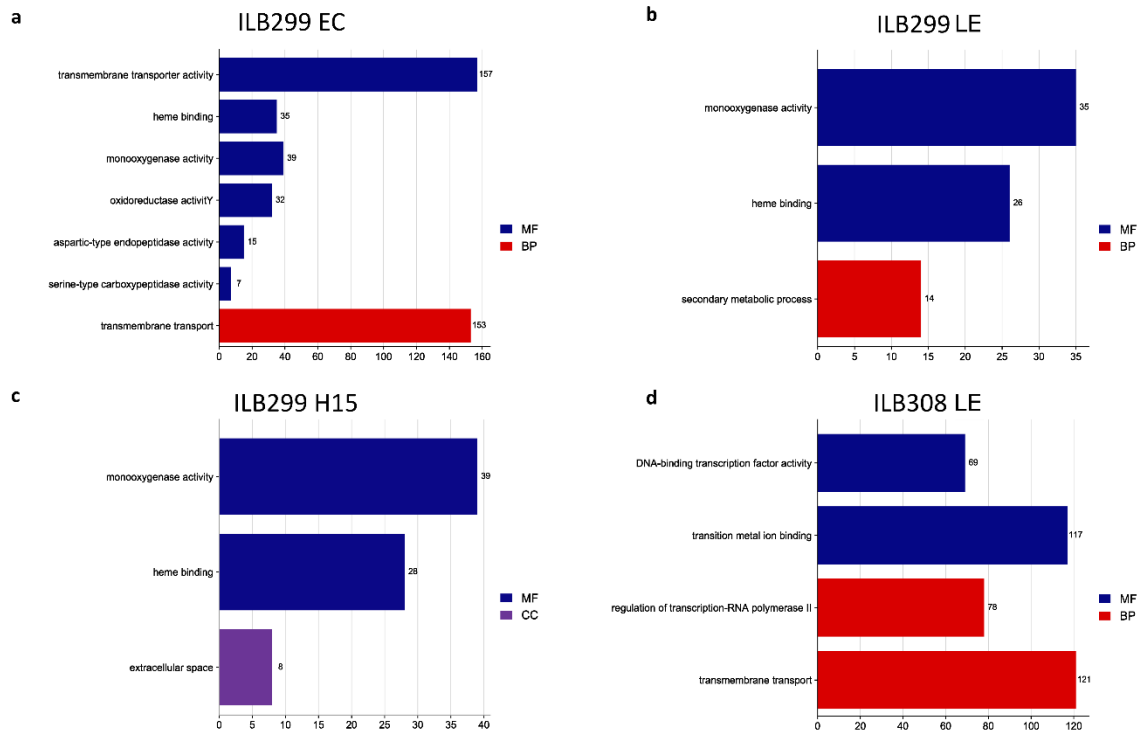


Figure 2. GO terms for *B. bassiana* DEGs in CE, LE and H15 culture media. ILB299 (a-c) and ILB308 (d). The DEGs were placed into the three main GO categories: molecular function (MF), biological process (BP), and cellular component (CC).

Most of the gene IDs within the 'monooxygenase activity' enriched GO term in ILB299 CE, LE and H15 correspond to Cytochrome P450 monooxygenases (CYP) (Table 2). A complete detailed list can be found in supplementary Table S6.

Table 2. Gene IDs with homology with Cytochrome P450 monooxygenases within ‘monooxygenase activity-’ (GO:0004497) and FC expression values found in ILB299 CE, LE and H15.

Gene ID ARSEF8028	CYP clan	ILB299		
		CE	LE	H15
BB8028_0003g13740 Cytochrome P450 CYP504B10	CYP504	1.14	-	-
BB8028_0005g00370 Cytochrome P450 CYP504B10	CYP504	2.83	-	2.22
BB8028_0007g06030 Cytochrome P450 CYP504E1	CYP504	1.19	-	-
BB8028_0003g09510 Cytochrome P450 CYP5060A1	nd	-	2.43	2.27
BB8028_0005g00330 Cytochrome P450 CYP5099A1	CYP56	3.80	2.00	2.95
BB8028_0005g02520 Cytochrome P450 CYP5099A1	CYP56	5.55	6.95	5.37
BB8028_0006g03190 Cytochrome P450 CYP5262A3	nd	3.58	3.23	2.65
BB8028_0009g01120 Cytochrome P450 CYP5293A2	nd	1.44	1.71	-
BB8028_0001g01990 Cytochrome P450 CYP540B16	CYP540	3.00	2.34	5.31
BB8028_0003g09600 Cytochrome P450 CYP541A2	CYP505	2.17	2.63	2.05
BB8028_0004g01830 Cytochrome P450 CYP561D2P	CYP65/53	3.74	2.79	3.40
BB8028_0007g04210 Cytochrome P450 CYP561D2P	CYP65/53	2.46	3.70	3.17
BB8028_0002g05950 Cytochrome P450 CYP570E2	CYP507/53	2.77	3.24	2.70
BB8028_0005g07270 Cytochrome P450 CYP584E2	CYP52	1.34	-	-
BB8028_0001g01960 Cytochrome P450 CYP6001C8	nd	2.58	1.58	1.73
BB8028_0001g10150 Cytochrome P450 CYP6003A1	nd	5.13	5.44	4.25
BB8028_0001g03360 Cytochrome P450 CYP6004A2	nd	1.89	2.13	1.18
BB8028_0002g00730 Cytochrome P450 CYP61A1	CYP61	1.22	-	-
BB8028_0003g05940 Cytochrome P450 CYP620C2	CYP533	2.35	1.52	2.17
BB8028_0003g16510 Cytochrome P450 CYP620D1	CYP533	3.26	2.99	2.72
BB8028_0002g14470 Cytochrome P450 CYP628A2	nd/CYP53	2.44	3.14	2.39
BB8028_0004g07860 Cytochrome P450 CYP682H1	CYP58/53	2.74	3.98	2.43
BB8028_0005g02510 Cytochrome P450 CYP684A2	CYP58/53	6.87	6.07	6.24

nd, not determined

-, no differentially expressed genes in the corresponding culture media

288

289

290 Interestingly, the three culture media shared 16 genes with homology to different CYP,
 291 including the genes with highest FC values that have homology with CYP5099A1
 292 (*BB8028_0005g02520*), CYP6003A1 (*BB8028_0001g10150*) and CYP684A2
 293 (*BB8028_0005g02510*). Only when grown in CE, specific CYP genes were observed. These
 294 included genes with homology with CYP504E1, CYP584E2 and CYP61A1 (genes
 295 *BB8028_0007g06030*, *BB8028_0005g07270* and *BB8028_0002g00730*, respectively) which
 296 correspond to CYP504, CYP52 and CYP61 clans.

The description of the gene IDs within the 'secondary metabolic process' (GO:0019748) in ILB299 grown in LE (Figure 2 b) was further investigated and all GOs corresponded to mycotoxin biosynthesis processes (Supplementary Table S7).

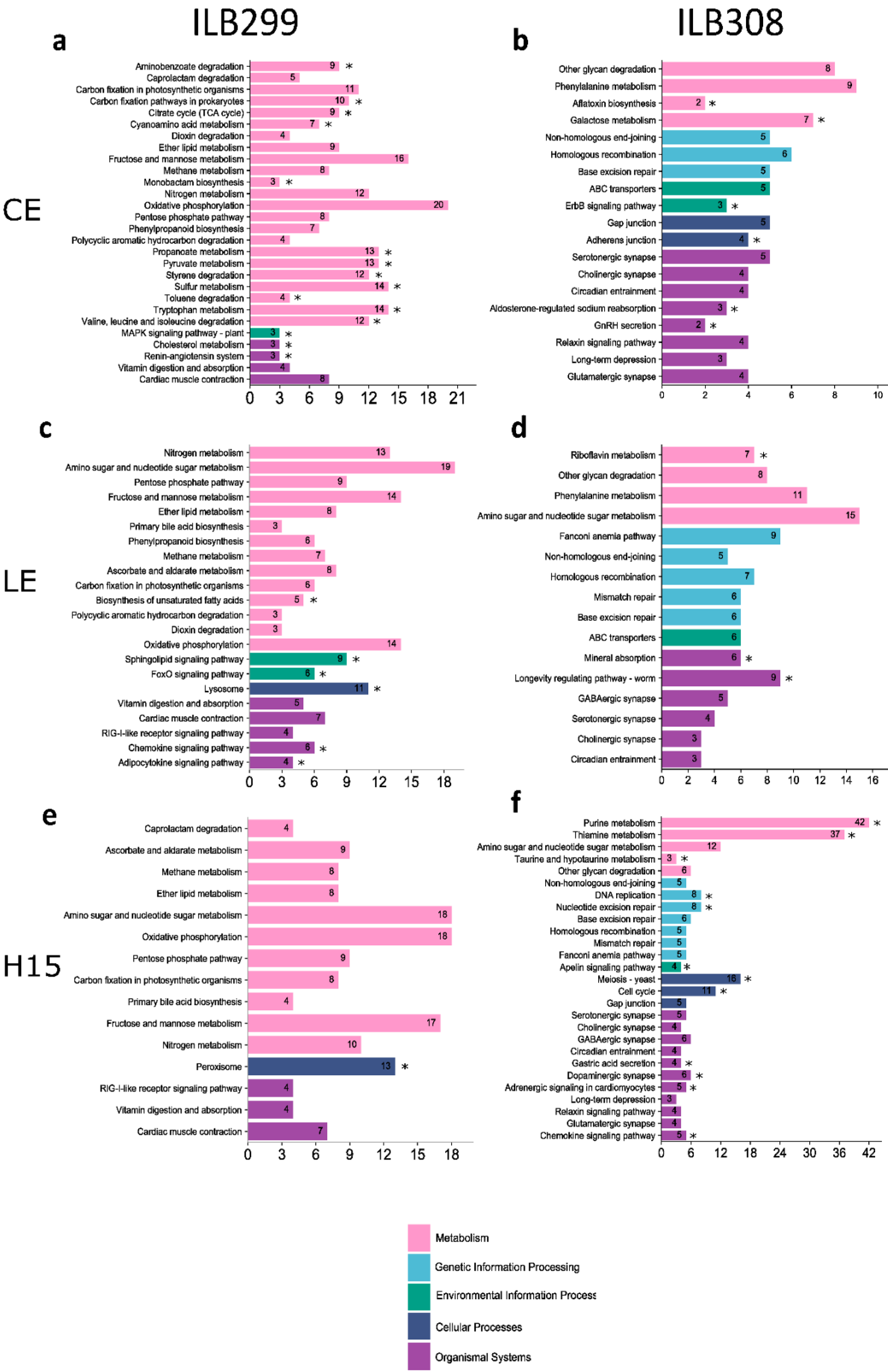
Notably, hypervirulent strain ILB308 only showed over enrichment of GOs when grown in LE medium (Supplementary Table S8), namely 'DNA-binding transcription factor activity' (GO:0000981) and 'transition metal binding' (GO:0046914) within the molecular function category and 'regulation of transcription-RNA pol II' (GO:0006357) and 'transmembrane transport' (GO:0055085) within the biological process category. Gene IDs within the 'DNA-binding transcription factor activity' enriched GO were mostly C6 finger domain protein, C6 zinc finger domain protein, C6 zinc finger domain-containing protein, C6 ion factor and Putative Zn(II)2Cys6ion factor, while genes ID within the 'transition metal binding' enriched GO were the same as mentioned above together with a set of Cytochrome P450 monooxygenases. Genes of the 'transmembrane transport' enriched GO included several ABC transporters, as well as oligopeptide transporters and siderophore iron transporters.

KEGG pathway enrichment analysis

To further assess the functions of the enriched DEGs, the KEGG database was consulted to analyze metabolic pathways. A total of 75 different metabolic pathways were identified. Hypovirulent strain ILB299 growing on CE, LE and H15 showed a total of 28, 22 and 15 enriched KEGGs, respectively (Figure 3 a-c). Interestingly, hypervirulent strain ILB308 growing under the same conditions showed 19, 16 and 27 enriched KEGGs, respectively (Figure 3 d-f), with the highest number of enriched pathways when grown on the least complex culture medium. The majority of the KEGGs in ILB299 strain belonged to 'metabolism' category, while ILB308 strain had a more diverse set of categories, including 'genetic information processing', which was absent in the hypovirulent strain. Of the total enriched KEGGs 15, 6 and 1 were specific to CE, LE and H15 in ILB299, respectively and 6, 3 and 12 were specific to ILB308 (Figure 3, asterisks), again showing the highest number of enriched pathways when grown in the least complex culture medium. IDs of the specific KEGGs are detailed in Supplementary Table S9 and S10.

There are no common enriched KEGGs between strains in CE culture medium, although there was a particular enriched KEGG shared among LE and H15 in both strains, namely 'amino sugar and nucleotide sugar metabolism' (ko00520) (Figure 4, asterisk). Comparison of both strains grown in *n*-pentadecane (H15) revealed hypovirulent strain ILB299 had the 'Peroxisome' (ko04146) pathway enriched within the 'cellular processes' category, while ILB308 showed 'Meiosis-yeast' (ko04113), 'Cell Cycle' (ko04110) and 'Gap junction'

332 (ko04540) within the same category (Figure 3 c, f). Also, ILB308 growing in CE and LE
 333 showed the ‘ABC transporters’ pathway enriched within the ‘environmental information
 334 processing’ category (Figure 3 b, d).



337 **Figure 3.** KEGG enrichment analysis of *B. bassiana* ILB299 (left) and ILB308 (right) strains in a, b)
 338 CE medium, c, d) LE medium and e, f) H15 medium. Asterisks indicate exclusive pathways.

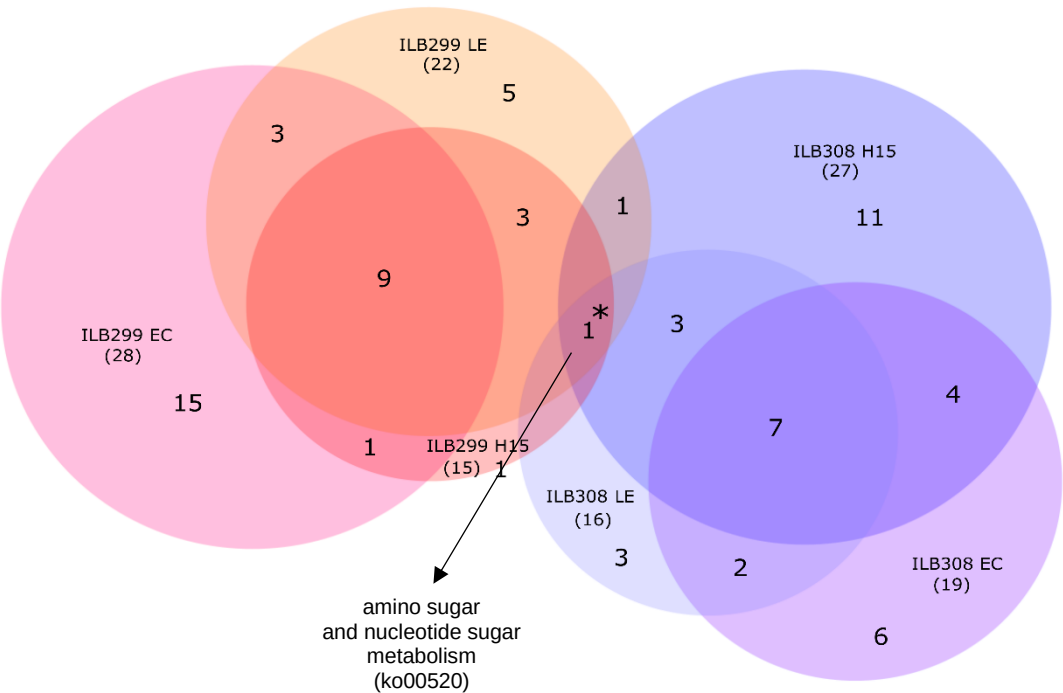


Figure 4. Venn Diagram of enriched KEGGs of *Beauveria bassiana* ILB299 and ILB308 in CE, LE and H15 culture media.

Discussion

In a previous study, we found virulence differences in *B. bassiana* strains against the redbanded stink bug *P. guildinii*, namely hypovirulent ILB299 and hypervirulent ILB308 strains (Sessa et al., 2022). Also, a *n*-pentadecane priming effect was evidenced on both strains regardless of their intrinsic basal virulence, and it was suggested that the activation of genes such as cytochromes P450 monooxygenases and hydrophobins, assessed by qPCR, were mainly responsible for the observed priming effect (Sessa et al., 2022). In the present study, we focused on the transcriptomic response of the same hypo- and hypervirulent *B. bassiana* strains to nutrition with *P. guildinii* cuticle extracts as well as synthetic *n*-pentadecane by RNA-seq sequencing on the fourth day post inoculation. The ultimate objective was to explore the expression of other virulence-related genes besides those activated in the very first steps of infection, their interaction with the insect cuticle and its lipids and hydrocarbon components, and to determine whether a set of genes and/or metabolic pathway/s related to their degradation might be strain specific.

For this, we obtained for both strains high quality sequencing data with up to 89% of total and 87% of uniquely mapped reads to the *B. bassiana* ARSEF8028 reference genome. We found that hypovirulent strain ILB299 showed higher total differentially expressed genes (DEGs) grown under the three culture media, as well as higher DEGs specific to CE and H15, while hypervirulent strain ILB308 showed higher DEGs specific to LE. Most of the genes belonging to the top 50 up-regulated DEGs were common between the three culture media and had similar FC values.

Differentially expressed virulence related genes (VRG) with medium-to-low FC were found, including cuticle adhesion, cuticle penetration, cell wall remodeling, signaling, growth, cell cycle, and stress tolerance genes. Interestingly, each strain revealed a different expression profile of these VRG DEGs which may be accountable for the intrinsic virulence differences observed between the two *B. bassiana* strains. Hypovirulent strain ILB299 showed predominantly overexpression of genes related with cuticle penetration, such as chitinases which serve to degrade and derive nutrients from chitin while also vital for growth (Hartl et al., 2012; Mondal et al., 2016), proteases including subtilisin-like protease (Pr1B) which is an extracellular cuticle degrading enzyme vital to penetrate the insect's exoskeleton (Gao et al., 2020) and cytochrome P450 monooxygenases (CYPs) implicated in insect hydrocarbon assimilation (Pedrini et al., 2013). Hypervirulent strain ILB308 showed overexpression of genes mostly related with cuticle adhesion, such as hydrophobin-like proteins that facilitate conidia adhesion to insect cuticles through formation of a hydrophobic coating (Holder & Keyhani, 2005), several CFEM domain-containing proteins that are reported to be involved in surface sensing and signaling, including conidial germination and appressorium formation (Kulkarni et al., 2003; Sabnam & Barman, 2017) and a fasciclin domain family protein which mediates cellular adhesion onto insect hosts (Miyazaki et al., 2007), as well as several CYPs and various cell wall remodeling proteins and cell cycle proteins. The cell wall remodeling proteins included Class V chitin synthase protein, reported to be involved in growth and development, conidial production and germination, stress response and penetration (Qin et al., 2022), the cell wall-remodeling enzyme implicated in stress response and cell wall integrity 1,3-beta-glucanosyltransferase, and Tudor domain-containing protein (Tdp1) that is reported to have a vital role in regulating conidia and blastospore yields and fungal morphological changes (Qiu et al., 2021). Cell cycle proteins overexpressed in ILB308 included the Fork head domain-containing protein (FKH) a transcription factor of the cell cycle, associated with fungal adaptation to host environment and morphogenesis (Wang et al., 2015), cyclin-dependent kinase (Cdk) that is involved in cell cycle G2/M transition (Qiu et al., 2014; Wang et al., 2015) and CAMK family protein kinase which is involved in the cAMP signaling pathway (Sun et al., 2022).

Moreover, the majority of these VRG were shown to be differentially overexpressed within each strain, with similar FC values regardless of the culture media, whether being cuticle, cuticular lipidic extracts or *n*-pentadecane, suggesting that growth in *n*-pentadecane as the sole carbon source might mimic exuviae and the hydrocarbon components, and therefore was sufficient to activate expression of these genes, which may help understand the priming effect previously observed in virulence towards *P. guildinii* (Sessa et al., 2022).

Hypovirulent strain ILB299 showed enriched GOs when grown in all the evaluated culture media and the diversity of the GO terms was greater in the more complex culture media (CE), whereas ILB308 only showed enriched GOs when grown in LE medium. Most of the genes within the common GO terms in ILB299 'Heme binding' and 'monooxygenase activity' had homology with cytochrome P450 monooxygenase genes (CYP), suggesting lipidic/hydrocarbon oxidation and assimilation. What is more, specific GOs found in ILB299 CE included 'aspartic-type endopeptidase activity' and 'serine-type carboxypeptidase activity' suggesting protein degradation processes as this medium is composed not only of lipids but also proteins and chitin. Also, the GO term 'transmembrane transporter activity' had the highest gene count, which may include ABC transporters that are reported to play an essential role in infection (Song et al., 2013). There were no specific 'molecular function' GOs on LE and H15, as the only carbon source was a blend of lipids and hydrocarbons or a specific hydrocarbon (*n*-pentadecane), respectively. Nonetheless, the identity of the 'biological process' GO 'secondary metabolic process' enriched in LE corresponded to mycotoxin biosynthesis genes. Hypervirulent strain ILB308 solely exhibited enriched GOs when grown in LE medium and most of the genes within the GO term 'DNA-binding transcription factor activity' had homology with Zinc finger proteins (transcription factors with the finger domain). Genes within the GO term 'transition metal binding' also had homology with a set of CYPs and genes within the GO term 'transmembrane transport' had homology with several ABC transporters, as well as oligopeptide transporters and siderophore iron transporters. These enriched terms and their associated functions suggest that ILB308 is experiencing transcriptional processes and are in concordance with the up-regulated DEGs with homology to VRG, such as CYPs (*BB8028_0004g01200* and *BB8028_0005g08960*), siderophore iron transporter (*BB8028_0003g09910*) and GDP-mannose transporter (*BB8028_0004g05420*). Comparison between hypo and hypervirulent strains' function categorization of GOs in LE medium (the only culture media where both strains showed enriched GOs) evidenced that they were experiencing different metabolic processes. As mentioned before ILB299 showed terms indicative of lipidic/hydrocarbon oxidation and assimilation processes whereas ILB308 GO terms were indicative of active transcriptional processes.

Focusing on the 'monooxygenase activity' GO:0004497 present in ILB299 strain on the 3 different culture media -but absent in ILB308- we found a set of genes with homology to different CYPs. The vast majority of these were common to the three culture media, including members of the CYP56 and CYP58/53 clans (CYP5099A1 and CYP684A2, respectively). Members of CYP56 clan are putatively involved in conidial wall maturation (Zhang et al., 2022). CYP684A2 has been reported to be involved in the defense mechanism of *B. bassiana* against Cd⁺² stress (Yu et al., 2021). Interestingly CYP52, CYP61 and two members of the CYP504 clan (CYP504B10 and CYP504E1) were only enriched in CE medium. The CYP52s and CYP61 are included in the group of CYP enzymes essential to primary metabolism and are responsible for the initial and rate-limiting step of hydroxylation of *n*-alkanes and fatty acids (Zhang et al., 2012), and for having a role as a Δ^{22} -desaturase in fungal membrane sterol synthesis as well as being reported as an aromatic hydrocarbon detoxification monooxygenase (Kelly et al., 1997; Lamb et al., 1999), respectively.

Examining the enriched KEGG pathways in both strains we found that ILB299 had almost exclusively enriched 'metabolism' pathways, while ILB308 had a broader spectrum of pathways including 'genetic' and 'environmental information processes'. The only KEGG shared among strains was 'amino sugar and nucleotide sugar metabolism' present in LE and H15; both culture media lacking proteins, chitin, and carbohydrates. In ILB299 this pathway was accompanied by 'ascorbate and aldarate metabolism' so it could be inferred that an abnormal sugar intake may be leading to a deficiency in the supply of specific sugars, leading to the overexpression of the amino sugar and nucleotide sugar metabolism. Comparing the KEGGs of both strains grown in H15 (*n*-pentadecane) it became apparent that hypovirulent strain ILB299 was undergoing hydrocarbon assimilation, as the 'peroxisome' -the fundamental organelle of hydrocarbon oxidation- (Pedrini et al., 2007), was enriched, whereas the hypervirulent strain ILB308 showed enriched, 'Meiosis-yeast' and 'Cell Cycle' KEGGs in the 'cellular processes' category. These results are in concordance with the GO enrichment analyses results, where both strains seemed at a different stage metabolically/biologically; hypovirulent strain ILB299 is at an earlier stage of response to cuticle components, seemingly undergoing active hydrocarbon oxidation and assimilation, whereas hypervirulent strain ILB308 seems to have already passed through this stage and now is seemingly undergoing active transcriptional processes related with cellular division. These differences reveal a 'delayed effect' in hypovirulent strain ILB299 in comparison with the hypervirulent strain ILB308 at 4dpi.

Novel VRG or metabolic pathways important to the infection process that were either strain specific or shared among hypo- and hyper- virulent strains ILB299 and ILB308 could not be determined in this work. As was mentioned, observed differences after 4 days of growth on cuticle or cuticle components seem to indicate that both strains are undergoing different

stages of a process. While ILB299 seems to be responding to the nutritional challenge posed by the cuticle, or the cuticle hydrocarbons or H15, ILB308 expresses a different cohort of genes and metabolic pathways, more diverse and not directly related to hydrocarbon assimilation.

Virulence related genes differentially expressed in hypovirulent strain ILB299, mostly chitinases, proteases and CYPs associated with cuticle degradation and penetration, correlated well with the function categorization of the enriched GOs as well as the enriched KEGGs, evidencing that hypovirulent strain ILB299 was undergoing metabolic processes associated with cuticle and cuticular hydrocarbon nutrition. On the other hand, the VRG differentially expressed in hypervirulent strain ILB308 were associated with cuticle adhesion, cell wall integrity and morphogenesis, and cell cycle events, while the function categorization of the enriched GOs as well as the enriched KEGGs were associated with active transcriptional processes related with cellular division. We presume that in our *in vitro* experiment the hypervirulent strain ILB308, at 4dpi, has successfully metabolized the cuticular and hydrocarbon components present in the culture media and is undergoing metabolic processes related to mycelial growth and sporulation.

On the other hand, because most of the VRG expressed in ILB308 are reported to be involved in conidial shift to blastopore and blastospore yield and germination, as well as host integument adhesion (Zhang et al., 2009; Zhang et al., 2011; Wang et al, 2015; Samalova et al., 2017; Qiu et al., 2021; Mascarín et al., 2021) and considering the *in vivo* virulence data obtained from previous bioassays (Sessa et al. 2022), we presume that hypervirulent strain could be mimicking the processes related with the last stages of the infection process, after the insect's death when blastospores in the hemocoel reach the cuticle from within to later sporulate on the cadaver (Zhang et al., 2010; Valero-Jiménez et al., 2016). Nonetheless, because our transcriptomic experiment was performed *in vitro*, we can only assume that the *P. guildinii* cuticle extracts evaluated were sufficient for ILB308 strain to activate these genes and mimic the aforementioned process. This hypothesis requires further investigation with a proper methodology, such as a dual-seq transcriptomic approach.

In summary, differentially expressed gene analyses, together with GO and metabolic pathway enrichment analyses allowed us to correlate the transcriptomic data with the intrinsic virulence and with the priming effect observed in virulence enhancement of *B. bassiana* strains ILB299 and ILB308 when grown in *n*-pentadecane. We found several strain-specific, -but not nutritional source specific- VRG with low-to-medium expression that could possibly be related with strain intrinsic virulence and with the priming effect previously observed. Enriched GOs together with KEGGs analyses revealed the 'delayed effect' observed in the

hypovirulent strain ILB299 with regards to ILB308, which correlates with the lower virulence of the former strain.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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Conclusiones Generales

La chinche del eucaliptus *Thaumastocoris peregrinus* y la chinche de la soja *Piezodorus guildinii* son dos hemípteros plaga de difícil control que provocan daños importantes en estos cultivos en nuestro país y otras regiones productoras. Ambos hemípteros presentan una diferencia fundamental en su tolerancia a diferentes agentes de control, siendo la chinche de la soja altamente tolerante a sufrir infecciones por hongos entomopatógenos (HEP), así como también a la aplicación de insecticidas químicos. Esta diferencia podría estar debida entre otras causas, a la composición de la capa más externa de la cutícula del insecto, la epicutícula, compuesta principalmente por hidrocarburos (CHC). En este trabajo se investigó la composición de CHC de individuos de *P. guildinii* colectados tanto en cultivos de soja tratados con insecticidas como sin tratamiento de insecticidas, lográndose identificar el perfil de hidrocarburos de este insecto. Encontramos un total de 72 compuestos, de los cuales los más abundantes fueron cadenas lineales saturadas de 27 y 29 átomos de carbono, y cadenas de mono- y di- metilo de 31 y 30 átomos de carbono. Además, se detectó un grupo de hidrocarburos no constitutivos, volátiles con *n*-C13 y *n*-C15 como compuestos predominantes. Pudimos encontrar diferencias en el patrón de CHC entre ninfas y adultos, así como dimorfismo sexual en chinches adultas y también se evidenció una alteración en el perfil de hidrocarburos de los insectos colectados en cultivos de soja tratados con insecticida, donde la cantidad total de hidrocarburos fue significativamente mayor en comparación con los insectos obtenidos de campos no tratados. En primer lugar, estos resultados sugieren que el uso de insecticidas de contacto como los piretroides produce un aumento de los CHC en los insectos sobrevivientes, tal vez por exposición a dosis sub-letal que ocurren en los niveles inferiores del follaje del cultivo, o a lo largo del tiempo tras la aplicación. Este fenómeno podría estar relacionado con la generación de resistencia a este insecticida, por lo que su utilización frecuente dentro del

manejo integrado de plagas en cultivos de soja debería de ser re-evaluada. Por otra parte, estos resultados nos permitieron reconocer las diferencias entre la composición de hidrocarburos en ambas chinches, ya que el perfil de CHC de *T. peregrinus* carece del grupo de hidrocarburos volátiles y los más abundantes son cadenas lineales saturadas más cortas. Estas diferencias en los perfiles de CHC entre ambas chinches podrían ayudar a explicar las diferencias en la susceptibilidad frente a la infección por HEP, ya que se ha reportado que algunos insectos varían su composición de hidrocarburos no constitutivos durante el proceso de infección (Sosa-Gómez et al., 1997). Considerando lo anterior seleccionamos el compuesto *n*-C15 como hidrocarburo “cebo o primer” para nuestros ensayos de aumento de virulencia en donde se evaluó la respuesta de diferentes cepas del hongo entomopatógeno *Beauveria bassiana* a su inclusión en el medio de cultivo como única fuente de carbono. Se caracterizó su respuesta en cuanto a la virulencia mediante bioensayos *in vivo* sobre los insectos y a nivel de la expresión de genes vinculados a la degradación de CHC y a la virulencia, mediante RT-qPCR. Pudimos evidenciar el efecto “priming o cebado”, al observar un aumento en la virulencia de *B. bassiana* frente a ambos insectos plaga, el cual pudimos explicar principalmente por un efecto de cebado producido por el *n*-C15 sobre la inducción de algunos genes relacionados con la asimilación de hidrocarburos como los genes del citocromo P450, junto con los genes de adhesión a la cutícula y de tolerancia al estrés. Finalmente, evaluamos la respuesta a nivel transcriptómico de dos cepas de *B. bassiana* de virulencia contrastante “ambas con efecto priming” a dos extractos de cutícula diferentes de *P. guildinii* y a *n*-C15. Se evaluaron y compararon los genes expresados diferencialmente (DEG), la ontología génica (GO) y las vías KEGG mediante tecnología RNAseq. Encontramos un perfil de expresión de genes relacionados con la virulencia diferente entre ambas cepas, así como los parámetros GO y KEGG a los 4 días post inoculación, lo que podría explicar su virulencia intrínseca pero también del efecto de aumento de la virulencia observado previamente. La cepa hipovirulenta ILB299 mostró sobreexpresión predominantemente de genes de penetración

de la cutícula, como quitinasas, proteasas y citocromo P450 monooxigenasas (CYP), y predominantemente términos GO y rutas metabólicas relacionadas a la degradación de hidrocarburos, mientras que la cepa hipervirulenta ILB308 mostró sobreexpresión de genes de remodelación de la pared celular y del ciclo celular, y también adhesión de la cutícula y CYP, junto con términos GO y vías KEGG relacionadas a la división celular y procesos transcripcionales. Estos resultados revelaron que ambas cepas se encontraron en diferentes etapas metabólicas en lo que respecta a su nutrición, evidenciando un "efecto de patogenicidad retardado" de la cepa hipovirulenta en comparación con la hipervirulenta, efecto que se correlaciona con su menor virulencia.

En síntesis, fue posible dilucidar la composición de hidrocarburos de la epicutícula de *P. guildinii*, evaluar cómo éstos son afectados por las condiciones de cultivo de soja y su interacción con la virulencia de cepas del hongo entomopatógeno *B. bassiana*.

Perspectivas

A partir de este trabajo se han obtenido dos publicaciones en revistas científicas internacionales arbitradas de alto impacto y un tercer manuscrito próximo a ser enviado para su publicación. Asimismo, como continuación de este trabajo se vienen desarrollando una tesis de Doctorado y una de Maestría que incluyen estudios funcionales con el objetivo de caracterizar cepas de *B. bassiana* mutantes (K.O) para diferentes genes candidatos relevantes para la virulencia, con el objetivo de determinar su función y obtener, mediante fusión de protoplastos, cepas con virulencia potenciada, así como también evaluaciones de patogenicidad y transcriptómica *in vivo* en insecto. La evaluación de la compatibilidad de las diferentes cepas frente a aceites minerales, insecticidas y fungicidas de uso común en el cultivo de la soja, así como evaluaciones de la patogenicidad de las cepas en insectos blanco, no blanco y blanco secundarios del cultivo de soja son también parte de los objetivos del trabajo de tesis de maestría.

Anexo

- Material suplementario Artículo

“Alkane-priming of *Beauveria bassiana* strains to improve biocontrol of the redbanded stink bug *Piezodorus guildinii* and the bronze bug *Thaumastocoris peregrinus*”

- Material suplementario Manuscrito

“Transcriptome analysis of *Beauveria bassiana* during assimilation of exuviae and cuticular lipids of the redbanded stink bug *Piezodorus guildinii* (Heteroptera: Pentatomidae)”

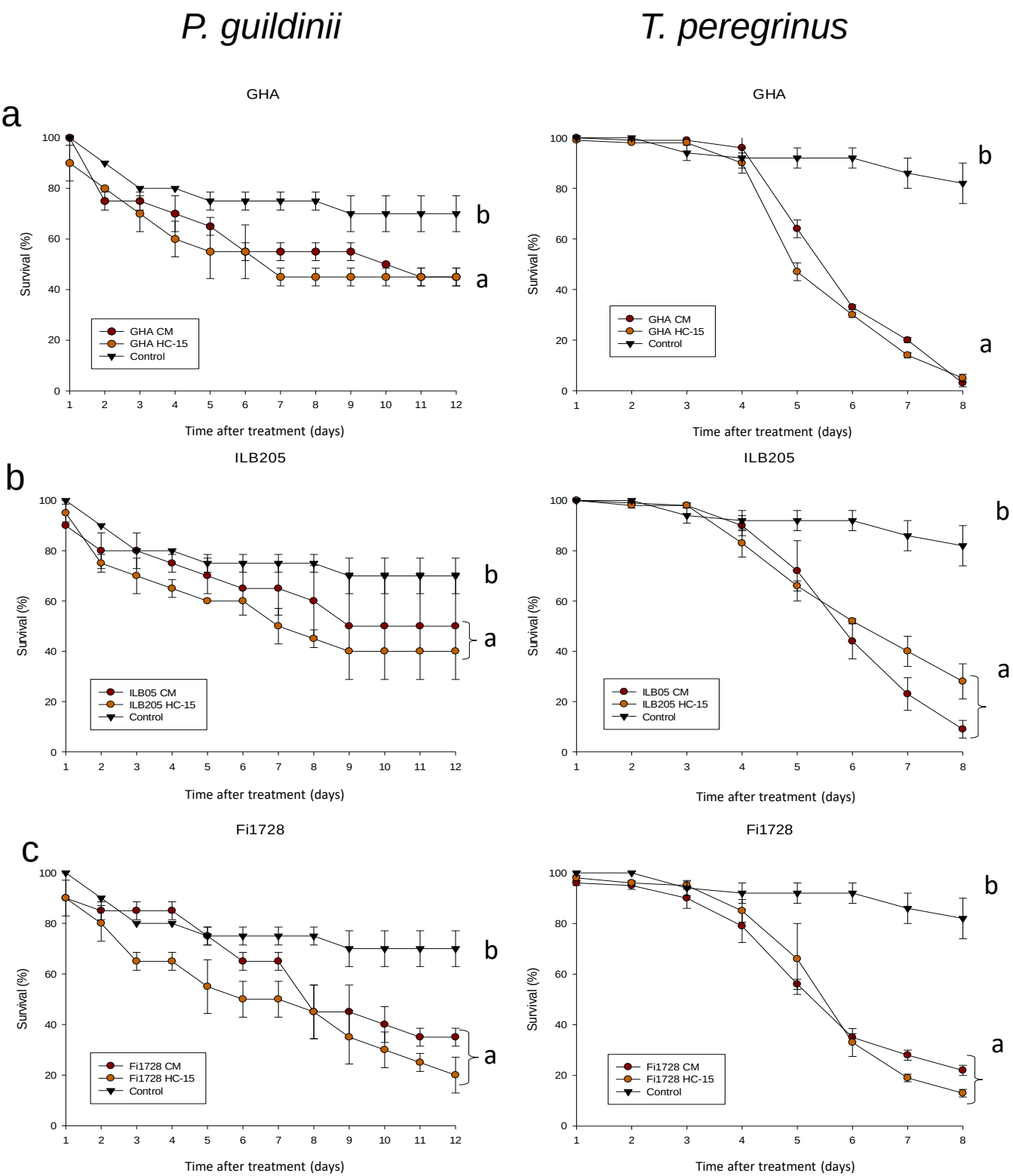


Figure S1 Survival of *P. guildinii* (left) and *T. peregrinus* (right) insects inoculated with a: GHA, b: ILB205 and c: Fi1728 isolates of *Beauveria bassiana* grown on *n*-pentadecane (HC-15) and CM. Different letters designate significant differences between survival curves (Log-rank test, $P < 0.05$).

1 **Supplementary material – Manuscrito**

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Table S1 Transcriptome sequencing and mapping of *B. bassiana* strains ILB308 and ILB299 grown in different carbon source culture media; cuticle extracts of *Piezodorus guildinii* (CE and LE) and *n*-pentadecane (H15).

Condition	Raw Data	Clean reads	Total Mapped	Uniquely Mapped	Multi-mapping	Unmapped
308LE1	19.566.070	17.504.639 / 89.4%	15.482.771 / 88.4%	15.027.485 / 85.8%	455.286 / 2.6%	2.021.786 / 11.5%
308LE2	19.206.496	17.083.573 / 88.9%	15.021.798 / 87.9%	14.589.181 / 85.3%	432.617 / 2.5%	2.061.987 / 12.0%
308LE3	22.943.654	20.621.249 / 89.8%	18.416.073 / 89.3%	17.828.158 / 86.4%	587.915 / 2.8%	2.204.412 / 10.6%
308CE1	23.890.124	20.871.852 / 87.3%	18.275.057 / 87.5%	17.626.178 / 84.4%	648.879 / 3.1%	2.596.458 / 12.4%
308CE2	18.744.917	14.922.014 / 79.6%	12.940.703 / 86.7%	12.491.787 / 83.7%	448.916 / 3.0%	1.980.151 / 13.2%
308CE4	18.380.087	16.386.564 / 89.1%	14.000.014 / 85.4%	13.606.086 / 83.0%	393.928 / 2.4%	2.385.884 / 14.5%
308152	20.760.970	18.892.621 / 91.0%	16.950.122 / 89.7%	16.469.036 / 87.1%	481.086 / 2.5%	1.942.161 / 10.2%
308153	27.117.557	24.434.192 / 90.1%	21.604.463 / 88.4%	20.911.478 / 85.5%	692.985 / 2.8%	2.829.479 / 11.5%
308154	17.949.322	16.185.153 / 90.1%	14.463.673 / 89.3%	14.050.410 / 86.8%	413.263 / 2.5%	1.720.482 / 10.6%
299LE1	17.693.084	14.893.541 / 84.1%	12.776.819 / 85.7%	12.373.739 / 83.0%	403.080 / 2.7%	2.114.883 / 14.2%
299LE2	17.035.614	14.929.015 / 87.6%	12.992.683 / 87.0%	12.580.000 / 84.2%	412.683 / 2.7%	1.936.293 / 12.9%
299LE3	23.933.005	21.220.427 / 88.6%	18.926.889 / 89.1%	18.348.769 / 86.4%	578.120 / 2.7%	2.293.928 / 10.8%
299CE1	22.337.077	19.924.181 / 89.1%	17.785.892 / 89.2%	17.227.720 / 86.4%	558.172 / 2.8%	2.137.865 / 10.7%
299CE2	15.361.739	13.592.479 / 88.4%	11.981.440 / 88.1%	11.655.086 / 85.7%	326.354 / 2.4%	1.610.709 / 11.8%
299CE3	19.535.569	17.221.525 / 88.1%	15.306.153 / 88.8%	14.848.409 / 86.2%	457.744 / 2.6%	1.915.034 / 11.1%
299151	15.706.762	14.282.983 / 90.9%	12.717.028 / 89.0%	12.309.538 / 86.1%	407.490 / 2.8%	1.565.415 / 10.9%
299152	16.309.212	13.932.969 / 85.4%	11.849.186 / 85.0%	11.469.858 / 82.3%	379.328 / 2.7%	2.082.979 / 14.9%

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Table S3. Culture media exclusively expressed genes in hypovirulent and hypervirulent strains ILB299 and ILB308

		Gene ID ARSEF8028	Description based in Blastp against ARSEF2860
<u>CE</u>	(4)	BB8028_0009g00230	NA
		BB8028_0009g00550	hypothetical protein
		BB8028_0001g06790	hypothetical protein
		BB8028_0003g05640	hypothetical protein
<u>LE</u>	(7)	BB8028_0006g00680	tat pathway signal sequence
		BB8028_0004g09930	dethiobiotin synthetase
		BB8028_0002g08220	hypothetical protein
		BB8028_0004g00120	prenyltransferase, UbiA family protein
		BB8028_0004g11280	hypothetical protein
		BB8028_0005g10830	---NA---
		BB8028_0003g10030	MFS transporter, putative
<u>H15</u>	(22)	BB8028_0004g03330	hypothetical protein
		BB8028_0006g06880	hypothetical protein
		BB8028_0004g11880	na
		BB8028_0003g16510	Cytochrome P450 CYP620D1
		BB8028_0008g00590	hypothetical protein
		BB8028_0001g01530	putative hydrolase
		BB8028_0004g01330	subtilisin-like protease PR1F
		BB8028_0003g15690	GCN5-related N-acetyltransferase
		BB8028_0007g01150	hypothetical protein
		BB8028_0002g05950	Cytochrome P450 CYP570E2
		BB8028_0001g09100	na
		BB8028_0006g01240	hypothetical protein
		BB8028_0001g12820	UDP-galactopyranose mutase
		BB8028_0004g01970	hypothetical protein
		BB8028_0004g09010	Peptidase S8/S53, subtilisin/kexin/sedolisin
		BB8028_0001g10590	neutral amino acid transporter
		BB8028_0001g16860	glutathione-S-transferase theta, GST
		BB8028_0005g10630	hypothetical protein
		BB8028_0004g05790	hypothetical protein
		BB8028_0007g00770	alpha/beta hydrolase superfamily protein
		BB8028_0005g02520	Cytochrome P450 CYP5099A1
		BB8028_0007g04170	tat pathway signal sequence

Table S4 Top 50 up-regulated DEGs in hypovirulent strain ILB299 in each culture media

Log2FC			Gene ID ARSEF8028	Description based in Blastp against ARSEF2860
<u>CE</u>	<u>LE</u>	<u>H15</u>	(39)	
19.2	16.2	19.4	BB8028_0004g04780	hypothetical protein
14.8	14.8	15.5	BB8028_0002g00040	cAMP-regulated phosphoprotein
14.4	13.3	14.8	BB8028_0007g01110	HHE domain-containing protein
14.3	12.9	14.7	BB8028_0006g10900	hypothetical protein
14.0	14.0	14.2	BB8028_0002g16910	fungal specific transcription factor
13.9	14.2	14.6	BB8028_0002g16890	MADS-box domain-containing protein
13.7	14.0	14.0	BB8028_0002g16880	cAMP-regulated phosphoprotein
13.5	13.6	13.7	BB8028_0008g00240	protein kinase domain-containing protein
13.2	13.5	13.6	BB8028_0005g02300	hypothetical protein
12.9	12.8	13.0	BB8028_0006g01180	hypothetical protein
12.8	13.4	13.6	BB8028_0002g00020	tubulin beta chain
12.4	12.3	12.3	BB8028_0005g09090	hypothetical protein
12.2	12.0	12.3	BB8028_0001g00810	serine/threonine-protein kinase
12.1	12.0	12.1	BB8028_0008g01150	hypothetical protein
12.0	12.0	12.3	BB8028_0004g13030	phosphotransferase enzyme
11.7	11.8	11.9	BB8028_0011g00020	fungal specific transcription
11.7	11.4	11.7	BB8028_0005g11390	hypothetical protein
11.7	12.2	12.4	BB8028_0002g16900	tubulin beta chain
11.4	11.1	11.3	BB8028_0005g06270	peptidase C14
11.4	11.1	11.5	BB8028_0006g11090	dynamain GTPase
11.4	11.3	11.4	BB8028_0006g06360	vegetative incompatibility protein
11.3	11.2	12.0	BB8028_0003g00460	---NA---
11.1	10.9	11.3	BB8028_0005g10322	---NA---
11.0	11.2	11.0	BB8028_0006g06780	hypothetical protein
11.0	11.4	11.2	BB8028_0010g00130	---NA---
11.0	11.3	11.3	BB8028_0006g10780	hypothetical protein
10.9	10.5	10.6	BB8028_0002g05750	ankyrin repeat protein
10.7	10.9	11.1	BB8028_0004g13690	LPS-induced tumor necrosis
10.7	10.5	10.6	BB8028_0010g00150	---NA---
10.6	10.8	10.3	BB8028_0004g13870	hypothetical protein
10.5	10.3	10.8	BB8028_0003g15890	vacuolar protein sorting-associated protein 62
10.2	10.6	11.1	BB8028_0005g10320	HMG box protein
10.1	9.9	10.1	BB8028_0009g01800	hypothetical protein
10.1	10.3	10.3	BB8028_0003g15970	interferon-induced GTP-binding protein Mx
9.9	11.2	11.6	BB8028_0001g06870	hypothetical protein
9.8	11.9	12.6	BB8028_0005g02500	cucumopine synthase
9.2	9.4	9.3	BB8028_0001g00350	---NA---
9.2	10.1	10.5	BB8028_0002g00010	fungal specific transcription
9.0	12.0	14.4	BB8028_0004g00860	peptidase S1 and S6.
<u>CE</u>	<u>LE</u>		(3)	
10.2	10.3		BB8028_0007g02270	hypothetical protein
9.4	9.2		BB8028_0004g01030	ribose 5-phosphate isomerase

9.1	9.4	BB8028_0002g16780	---NA---
<u>CE</u>	<u>H15</u>	(2)	
9.7	10.9	BB8028_0003g00600	hypothetical protein
9.0	9.3	BB8028_0001g10900	hypothetical protein
<u>LE</u>	<u>H15</u>	(4)	
10.2	10.3	BB8028_0002g13890	hypothetical protein
10.0	10.3	BB8028_0006g00930	bacteriodes thetaiotaomicron
9.4	12.2	BB8028_0006g03520	bacteriodes thetaiotaomicron
9.1	10.1	BB8028_0003g08680	hypothetical protein
<u>CE</u>		(5)	
11.0		BB8028_0005g02490	adenylate-forming enzyme AfeA
9.7		BB8028_0003g00060	methyltransferase-like protein
9.3		BB8028_0005g10210	FAD dependent oxidoreductase
9.0		BB8028_0003g00070	monooxygenase-like protein
8.9		BB8028_0002g03570	Trypsin-related protease
<u>LE</u>		(3)	
9.2		BB8028_0005g02760	hypothetical protein
9.0		BB8028_0004g05580	hypothetical protein
8.9		BB8028_0002g00030	hypothetical protein
<u>H15</u>		(5)	
10.5		BB8028_0006g01200	hemolysin-III channel protein
10.4		BB8028_0006g09310	hypothetical protein
10.4		BB8028_0003g00030	hypothetical protein
10.1		BB8028_0004g13420	---NA---
9.9		BB8028_0005g04490	---NA---

In bold: logFC ≥2 between culture media

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Table S5 Top 50 up-regulated DEGs in hypovirulent strain ILB308 in each culture media

Log2FC			Gene ID ARSEF8028	Description based in Blastp against ARSEF2860
<u>CE</u>	<u>LE</u>	<u>H15</u>	(39)	
15.4	15.1	15.1	BB8028_0006g10970	hypothetical protein
13.6	13.3	13.8	BB8028_0007g00560	interferon-induced GTP-binding
13.3	15.5	10.7	BB8028_0006g11070	hypothetical protein
13.2	13.7	13.6	BB8028_0006g11040	interferon-induced GTP-binding protein Mx
12.9	12.9	10.4	BB8028_0007g00110	hypothetical protein
12.7	12.7	12.9	BB8028_0010g00410	reverse transcriptase
12.6	12.6	10.4	BB8028_0006g11060	arabinan endo-1.5-alpha-L-arabinosidase A
12.2	9.7	13.8	BB8028_0002g01660	multicopper oxidase
12.1	14.0	14.9	BB8028_0004g00350	antifungal protein
12.1	11.5	12.0	BB8028_0007g00570	hypothetical protein
12.0	14.0	13.9	BB8028_0012g00260	transposase-like protein
12.0	11.9	12.3	BB8028_0009g01840	metalloprotease-like protein
11.9	11.6	11.7	BB8028_0010g00050	hypothetical protein
11.3	11.5	11.4	BB8028_0002g03100	---NA---
11.2	10.9	10.9	BB8028_0007g00220	myb-like DNA-binding domain-containing protein
11.1	10.2	10.3	BB8028_0010g00370	hypothetical protein
11.0	11.6	11.3	BB8028_0007g00050	---NA---
11.0	10.8	11.0	BB8028_0005g11410	FluG domain-containing protein
10.0	9.9	9.6	BB8028_0002g14690	membrane transporter
10.9	11.2	11.5	BB8028_0006g00030	peptidase family M28
10.8	11.2	11.5	BB8028_0010g00420	dnaK protein
10.8	10.6	10.6	BB8028_0006g11030	hypothetical protein
10.5	10.0	10.2	BB8028_0004g11040	hypothetical protein
10.4	10.1	10.3	BB8028_0010g00490	
10.3	9.5	9.9	BB8028_0006g11110	TPR repeat protein
10.1	10.4	10.7	BB8028_0010g00480	---NA---
10.0	10.0	9.7	BB8028_0005g00660	fasciclin domain family protein
9.8	10.0	9.6	BB8028_0007g00120	---NA---
9.8	9.3	9.2	BB8028_0007g00230	hypothetical protein
9.7	9.6	9.8	BB8028_0004g13400	fic/DOC family protein
9.7	10.1	10.5	BB8028_0010g00350	aminotransferase class-III
9.6	9.8	9.6	BB8028_0010g00120	hypothetical protein
9.4	9.3	9.0	BB8028_0009g01930	transposase-like protein
9.3	9.7	9.9	BB8028_0004g08860	canalicular multispecific
9.3	9.3	9.1	BB8028_0001g00560	hypothetical protein
9.1	9.5	9.3	BB8028_0006g06500	Concanavalin A-like lectin/glucanase
9.1	11.4	11.5	BB8028_0002g16870	FluG domain-containing protein
8.9	9.2	9.0	BB8028_0005g01410	hypothetical protein
8.9	9.0	9.1	BB8028_0006g06470	Pectin lyase fold/virulence factor
<u>CE</u>	<u>LE</u>		(5)	

10.7	10.5	BB8028_0007g04250	hydrophobin-like protein
9.1	9.4	BB8028_0006g11080	hypothetical protein
9.7	10.1	BB8028_0003g00020	UPF0261 domain protein
9.6	10.0	BB8028_0003g00010	TIM-barrel enzyme family protein
9.5	9.4	BB8028_0004g11270	valiolone synthase ValA
<u>CE</u>	<u>H15</u>	(3)	
9.6	9.2	BB8028_0010g00110	Patatin-like serine hydrolase
9.0	9.0	BB8028_0001g04070	LMW6DL protein
9.7	9.9	BB8028_0002g00370	hypothetical protein
<u>LE</u>	<u>H15</u>	(2)	
10.1	9.0	BB8028_0004g01180	Alcohol dehydrogenase
11.1	10.0	BB8028_0004g04790	---NA---
<u>CE</u>		(3)	
9.5		BB8028_0001g03860	elongation factor-2 kinase
9.5		BB8028_0007g00800	putative membrane protein
9.0		BB8028_0010g00300	hypothetical protein
<u>LE</u>		(4)	
9.4		BB8028_0007g00130	hypothetical protein
9.3		BB8028_0007g06620	hypothetical protein
9.3		BB8028_0005g11310	polyprenyl synthetase. putative
10.1		BB8028_0007g04960	CFEM domain-containing protein
<u>H15</u>		(6)	
8.9		BB8028_0004g12400	polysaccharide lyase family 8 protein
8.9		BB8028_0004g00190	conidial pigment biosynthesis
8.8		BB8028_0006g02990	NACHT and WD domain protein
9.7		BB8028_0005g01380	ABC transporter
9.5		BB8028_0006g00350	cell surface protein
9.1		BB8028_0010g00470	

In bold: logFC ≥2 between culture media

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Table S6 Gene ID and Description of “monooxygenase activity” (GO:0004497)**CE ILB299**

Gene.ID.ARSEF8028	Description based in Blastp against ARSEF8028
BB8028_0002g04010	2-nitropropane dioxygenase
BB8028_0006g09820	2-nitropropane dioxygenase
BB8028_0003g13740	Cytochrome P450 CYP504B10
BB8028_0005g00370	Cytochrome P450 CYP504B10
BB8028_0007g06030	Cytochrome P450 CYP504E1
BB8028_0005g00330	Cytochrome P450 CYP5099A1
BB8028_0005g02520	Cytochrome P450 CYP5099A1
BB8028_0006g03190	Cytochrome P450 CYP5262A3
BB8028_0009g01120	Cytochrome P450 CYP5293A2
BB8028_0001g01990	Cytochrome P450 CYP540B16
BB8028_0003g09600	Cytochrome P450 CYP541A2
BB8028_0004g01830	Cytochrome P450 CYP561D2P
BB8028_0007g04210	Cytochrome P450 CYP561D2P
BB8028_0002g05950	Cytochrome P450 CYP570E2
BB8028_0005g07270	Cytochrome P450 CYP584E2
BB8028_0001g01960	Cytochrome P450 CYP6001C8
BB8028_0001g10150	Cytochrome P450 CYP6003A1
BB8028_0001g03360	Cytochrome P450 CYP6004A2
BB8028_0002g00730	Cytochrome P450 CYP61A1
BB8028_0003g05940	Cytochrome P450 CYP620C2
BB8028_0003g16510	Cytochrome P450 CYP620D1
BB8028_0002g14470	Cytochrome P450 CYP628A2
BB8028_0004g07860	Cytochrome P450 CYP682H1
BB8028_0005g02510	Cytochrome P450 CYP684A2
BB8028_0006g02290	dimethylaniline monooxygenase
BB8028_0006g03090	FAD binding domain protein
BB8028_0006g03100	FAD binding domain-containing protein
BB8028_0006g09980	FAD binding domain-containing protein
BB8028_0004g06170	fatty acid hydroxylase superfamily protein
BB8028_0005g09470	flavin-binding monooxygenase
BB8028_0009g00600	Flavin-binding monooxygenase-like family protein
BB8028_0002g14050	iron-sulfur cluster-binding protein
BB8028_0003g14050	MAK1-like monooxygenase
BB8028_0003g00070	monooxygenase-like protein
BB8028_0003g14600	monooxygenase-like protein
BB8028_0005g09460	NADH
BB8028_0002g15410	nitrilotriacetate monooxygenase component B
BB8028_0001g00370	oxidoreductase
BB8028_0004g01730	pristinamycin IIA synthase subunit A

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Gene.ID.ARSEF8028	Description based in Blastp against ARSEF2860
BB8028_0002g04010	2-nitropropane dioxygenase

BB8028_0003g10420	2-nitropropane dioxygenase
BB8028_0005g01610	2-nitropropane dioxygenase
BB8028_0006g09240	2-nitropropane dioxygenase
BB8028_0006g09820	2-nitropropane dioxygenase
BB8028_0003g09510	Cytochrome P450 CYP5060A1
BB8028_0005g00330	Cytochrome P450 CYP5099A1
BB8028_0005g02520	Cytochrome P450 CYP5099A1
BB8028_0006g03190	Cytochrome P450 CYP5262A3
BB8028_0009g01120	Cytochrome P450 CYP5293A2
BB8028_0001g01990	Cytochrome P450 CYP540B16
BB8028_0003g09600	Cytochrome P450 CYP541A2
BB8028_0004g01830	Cytochrome P450 CYP561D2P
BB8028_0007g04210	Cytochrome P450 CYP561D2P
BB8028_0002g05950	Cytochrome P450 CYP570E2
BB8028_0001g01960	Cytochrome P450 CYP6001C8
BB8028_0001g10150	Cytochrome P450 CYP6003A1
BB8028_0001g03360	Cytochrome P450 CYP6004A2
BB8028_0003g05940	Cytochrome P450 CYP620C2
BB8028_0003g16510	Cytochrome P450 CYP620D1
BB8028_0002g14470	Cytochrome P450 CYP628A2
BB8028_0004g07860	Cytochrome P450 CYP682H1
BB8028_0005g02510	Cytochrome P450 CYP684A2
BB8028_0006g09980	FAD binding domain-containing protein
BB8028_0004g06170	fatty acid hydroxylase superfamily protein
BB8028_0004g08040	fatty acid hydroxylase superfamily protein
BB8028_0005g09470	flavin-binding monooxygenase
BB8028_0006g05850	flavin-binding monooxygenase
BB8028_0009g00600	Flavin-binding monooxygenase-like family protein
BB8028_0004g01840	FMN-dependent dehydrogenase
BB8028_0004g02380	inositol oxygenase
BB8028_0002g14050	iron-sulfur cluster-binding protein
BB8028_0003g00070	monooxygenase-like protein
BB8028_0005g09460	NADH
BB8028_0004g01730	pristinamycin IIA synthase subunit A

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Gene.ID.ARSEF8028	Description based in Blastp against ARSEF2860
BB8028_0002g04010	2-nitropropane dioxygenase
BB8028_0003g10420	2-nitropropane dioxygenase
BB8028_0006g09240	2-nitropropane dioxygenase
BB8028_0006g09820	2-nitropropane dioxygenase
BB8028_0005g00370	Cytochrome P450 CYP504B10
BB8028_0003g09510	Cytochrome P450 CYP5060A1
BB8028_0005g00330	Cytochrome P450 CYP5099A1
BB8028_0005g02520	Cytochrome P450 CYP5099A1

BB8028_0006g03190	Cytochrome P450 CYP5262A3
BB8028_0001g01990	Cytochrome P450 CYP540B16
BB8028_0003g09600	Cytochrome P450 CYP541A2
BB8028_0004g01830	Cytochrome P450 CYP561D2P
BB8028_0007g04210	Cytochrome P450 CYP561D2P
BB8028_0002g05950	Cytochrome P450 CYP570E2
BB8028_0001g01960	Cytochrome P450 CYP6001C8
BB8028_0001g10150	Cytochrome P450 CYP6003A1
BB8028_0001g03360	Cytochrome P450 CYP6004A2
BB8028_0003g05940	Cytochrome P450 CYP620C2
BB8028_0003g16510	Cytochrome P450 CYP620D1
BB8028_0002g14470	Cytochrome P450 CYP628A2
BB8028_0004g07860	Cytochrome P450 CYP682H1
BB8028_0005g02510	Cytochrome P450 CYP684A2
BB8028_0006g09980	FAD binding domain-containing protein
BB8028_0004g06170	fatty acid hydroxylase superfamily protein
BB8028_0004g08040	fatty acid hydroxylase superfamily protein
BB8028_0005g09470	flavin-binding monooxygenase, putative
BB8028_0006g05850	flavin-binding monooxygenase, putative
BB8028_0009g00620	flavin-binding monooxygenase, putative
BB8028_0009g00600	Flavin-binding monooxygenase-like family protein
BB8028_0004g01840	FMN-dependent dehydrogenase
BB8028_0004g02380	inositol oxygenase, putative
BB8028_0002g14050	iron-sulfur cluster-binding protein
BB8028_0003g15780	monooxygenase, FAD-binding protein
BB8028_0003g00070	monooxygenase-like protein
BB8028_0005g09460	NADH
BB8028_0002g15410	nitrilotriacetate monooxygenase component B
BB8028_0001g00370	oxidoreductase, 2-nitropropane dioxygenase family, putative
BB8028_0004g01730	pristinamycin IIA synthase subunit A
BB8028_0002g02460	thiol-specific monooxygenase

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Table S7 Gene ID and Description of “secondary metabolic process” (GO:0019748)**LE ILB299**

Gene.ID.ARSEF8028	Description based in Blastp against ARSEF2860
BB8028_0001g17410	metallo-beta-lactamase superfamily protein
BB8028_0002g10440	metallo-beta-lactamase superfamily protein
BB8028_0003g00080	hypothetical protein
BB8028_0003g00190	3-octaprenyl-4-hydroxybenzoate carboxy-lyase
BB8028_0004g13190	SnoaL-like polyketide cyclase
BB8028_0005g00960	conidial pigment biosynthesis scytalone
BB8028_0005g02400	dehydratase Arp1
BB8028_0006g00800	Lactamase B
BB8028_0006g00820	tat pathway signal sequence
BB8028_0006g00830	tat pathway signal sequence
BB8028_0007g04170	tat pathway signal sequence
BB8028_0007g04800	tat pathway signal sequence
BB8028_0008g00590	hypothetical protein

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Table S8 Gene ID and Description of enriched GOs ILB308 LE

DNA-binding transcription factor activity GO:0000981	
Gene.ID.ARSEF8028	Description based in Blastp against ARSEF2860
BB8028_0006g09760	bacterial transferase hexapeptide
BB8028_0002g03630	C6 finger domain protein
BB8028_0002g03840	C6 finger domain protein
BB8028_0002g07200	C6 finger domain protein
BB8028_0004g07740	C6 finger domain protein
BB8028_0003g00320	C6 finger domain protein, putative
BB8028_0001g02510	C6 finger domain-containing protein
BB8028_0004g07600	C6 finger domain-containing protein
BB8028_0003g05360	C6 zinc finger domain protein
BB8028_0003g10200	C6 zinc finger domain protein
BB8028_0005g09450	C6 zinc finger domain protein
BB8028_0008g02870	C6 zinc finger domain protein
BB8028_0001g05250	C6 zinc finger domain-containing protein
BB8028_0001g12750	C6 zinc finger domain-containing protein
BB8028_0001g13060	C6 zinc finger domain-containing protein
BB8028_0003g07170	C6 zinc finger domain-containing protein
BB8028_0004g01210	C6 zinc finger domain-containing protein
BB8028_0004g03610	C6 zinc finger domain-containing protein
BB8028_0004g07100	C6 zinc finger domain-containing protein
BB8028_0001g10620	C6ion factor
BB8028_0001g16940	C6ion factor
BB8028_0002g15830	C6ion factor
BB8028_0003g00540	C6ion factor
BB8028_0003g04450	C6ion factor
BB8028_0003g14030	C6ion factor
BB8028_0004g01530	C6ion factor
BB8028_0004g09680	C6ion factor
BB8028_0004g13920	C6ion factor
BB8028_0003g09840	C6ion factor, putative
BB8028_0004g01670	C6ion factor, putative
BB8028_0002g02160	Cutinaseion factor 1 alpha
BB8028_0005g04080	cutinaseion factor 1 beta
BB8028_0001g10890	fungus specific ion factor
BB8028_0001g13970	fungus specific ion factor
BB8028_0001g14890	fungus specific ion factor
BB8028_0002g01980	fungus specific ion factor
BB8028_0002g02270	fungus specific ion factor
BB8028_0002g02470	fungus specific ion factor
BB8028_0003g04640	fungus specific ion factor
BB8028_0003g11040	fungus specific ion factor
BB8028_0003g13410	fungus specific ion factor
BB8028_0004g12440	fungus specific ion factor
BB8028_0005g01220	fungus specific ion factor

BB8028_0006g01460	fungal specifiction factor
BB8028_0006g08930	fungal specifiction factor
BB8028_0007g02410	fungal specifiction factor
BB8028_0005g11720	fungal specifiction factor domain protein
BB8028_0003g12070	Fungal specifiction factor, putative
BB8028_0003g01900	general repressor ofion
BB8028_0005g11370	glucoamylase-like protein
BB8028_0005g03040	hypothetical protein
BB8028_0007g00130	hypothetical protein
BB8028_0003g16470	nitrate assimilation regulatory protein nirA
BB8028_0004g09580	nitrate assimilation regulatory protein nirA
BB8028_0005g01440	nitrate assimilation regulatory protein nirA
BB8028_0003g14870	Putative C2H2ion factor
BB8028_0011g00180	Putative C2H2ion factor
BB8028_0009g00680	putative C6-zinc finger TF, regulator of conidiation
BB8028_0002g04200	putative MLH1 protein
BB8028_0001g03200	Putative Zn(II)2Cys6ion factor
BB8028_0001g16660	Putative Zn(II)2Cys6ion factor
BB8028_0002g01600	Putative Zn(II)2Cys6ion factor
BB8028_0002g13800	Putative Zn(II)2Cys6ion factor
BB8028_0003g01090	Putative Zn(II)2Cys6ion factor
BB8028_0004g00210	Putative Zn(II)2Cys6ion factor
BB8028_0004g01160	Putative Zn(II)2Cys6ion factor
BB8028_0005g06580	Putative Zn(II)2Cys6ion factor
BB8028_0001g08940	zinc finger protein 1
BB8028_0004g06370	Zn(II)2Cys6ion factor

transition metal binding (GO:0046914)

Gene.ID.ARSEF8028	Description based in Blastp against ARSEF2860
BB8028_0003g06430	3-hydroxyanthranilic acid dioxygenase
BB8028_0005g07170	4-aminobutyrate aminotransferase
BB8028_0003g15460	alcohol dehydrogenase I
BB8028_0007g04630	AN1-like Zinc finger protein
BB8028_0006g09760	bacterial transferase hexapeptide
BB8028_0002g03630	C6 finger domain protein
BB8028_0002g03840	C6 finger domain protein
BB8028_0002g07200	C6 finger domain protein
BB8028_0004g07740	C6 finger domain protein
BB8028_0003g00320	C6 finger domain protein, putative
BB8028_0001g02510	C6 finger domain-containing protein
BB8028_0004g07600	C6 finger domain-containing protein
BB8028_0003g05360	C6 zinc finger domain protein
BB8028_0003g10200	C6 zinc finger domain protein
BB8028_0005g09450	C6 zinc finger domain protein
BB8028_0008g02870	C6 zinc finger domain protein
BB8028_0001g05250	C6 zinc finger domain-containing protein

BB8028_0001g12750	C6 zinc finger domain-containing protein
BB8028_0001g13060	C6 zinc finger domain-containing protein
BB8028_0003g07170	C6 zinc finger domain-containing protein
BB8028_0004g01210	C6 zinc finger domain-containing protein
BB8028_0004g03610	C6 zinc finger domain-containing protein
BB8028_0004g07100	C6 zinc finger domain-containing protein
BB8028_0001g10620	C6ion factor
BB8028_0001g16940	C6ion factor
BB8028_0002g15830	C6ion factor
BB8028_0003g00540	C6ion factor
BB8028_0003g04450	C6ion factor
BB8028_0003g14030	C6ion factor
BB8028_0004g01530	C6ion factor
BB8028_0004g09680	C6ion factor
BB8028_0004g13920	C6ion factor
BB8028_0003g09840	C6ion factor, putative
BB8028_0004g01670	C6ion factor, putative
BB8028_0002g15340	CLAP1-like protein
BB8028_0001g09630	copper amine oxidase
BB8028_0001g09640	copper amine oxidase
BB8028_0002g02160	Cutinaseion factor 1 alpha
BB8028_0005g04080	cutinaseion factor 1 beta
BB8028_0003g13280	Cytochrome P450 CYP504A6
BB8028_0001g11380	Cytochrome P450 CYP5202A1
BB8028_0006g08610	Cytochrome P450 CYP539B1
BB8028_0002g07190	Cytochrome P450 CYP548A5
BB8028_0005g11300	Cytochrome P450 CYP551C1
BB8028_0008g00320	Cytochrome P450 CYP586B1
BB8028_0004g01190	Cytochrome P450 CYP623C1
BB8028_0003g05850	Cytochrome P450 CYP645A1
BB8028_0004g01200	Cytochrome P450 CYP655C1
BB8028_0001g01780	Cytochrome P450 CYP682H1
BB8028_0005g05950	DNA repair protein
BB8028_0003g02860	extracellular dioxygenase
BB8028_0007g00990	extracellular dioxygenase
BB8028_0003g13580	Extradiol ring-cleavage dioxygenase class III protein subunit B
BB8028_0001g12610	fatty acid hydroxylase superfamily protein
BB8028_0002g00480	fatty acid hydroxylase superfamily protein
BB8028_0003g16480	fatty acid hydroxylase superfamily protein
BB8028_0005g04760	fatty acid hydroxylase superfamily protein
BB8028_0006g03170	fatty acid hydroxylase superfamily protein
BB8028_0004g01420	ferro-O2-oxidoreductase
BB8028_0002g16870	FluG domain-containing protein
BB8028_0001g10890	fungal specificion factor
BB8028_0001g13970	fungal specificion factor

BB8028_0001g14890	fungaI specifiCion factor
BB8028_0002g01980	fungaI specifiCion factor
BB8028_0002g02270	fungaI specifiCion factor
BB8028_0002g02470	fungaI specifiCion factor
BB8028_0003g04640	fungaI specifiCion factor
BB8028_0003g11040	fungaI specifiCion factor
BB8028_0003g13410	fungaI specifiCion factor
BB8028_0004g04710	fungaI specifiCion factor
BB8028_0004g12440	fungaI specifiCion factor
BB8028_0005g01220	fungaI specifiCion factor
BB8028_0006g01460	fungaI specifiCion factor
BB8028_0006g08930	fungaI specifiCion factor
BB8028_0007g02410	fungaI specifiCion factor
BB8028_0007g03200	fungaI specifiCion factor
BB8028_0005g11720	fungaI specifiCion factor domain protein
BB8028_0003g12070	FungaI specifiCion factor, putative
BB8028_0001g10330	galactose-1-phosphate uridylyltransferase
BB8028_0003g01900	general repressor of ion
BB8028_0005g11370	glucoamylase-like protein
BB8028_0002g07440	GRF zinc finger domain-containing protein
BB8028_0001g04550	hypothetical protein
BB8028_0005g03040	hypothetical protein
BB8028_0007g00130	hypothetical protein
BB8028_0006g05200	iron sulfur cluster assembly protein 1
BB8028_0001g04800	mlo2 protein
BB8028_0001g03960	multicopper oxidase
BB8028_0002g01660	multicopper oxidase
BB8028_0004g01550	Multicopper oxidase family protein
BB8028_0003g16470	nitrate assimilation regulatory protein nirA
BB8028_0004g09580	nitrate assimilation regulatory protein nirA
BB8028_0005g01440	nitrate assimilation regulatory protein nirA
BB8028_0001g10600	nitrogen assimilation factor nirA
BB8028_0006g01360	nmrA family iron regulator
BB8028_0003g14870	Putative C2H2ion factor
BB8028_0011g00180	Putative C2H2ion factor
BB8028_0009g00680	putative C6-zinc finger TF, regulator of conidiation
BB8028_0002g04200	putative MLH1 protein
BB8028_0001g03200	Putative Zn(II)2Cys6ion factor
BB8028_0001g16660	Putative Zn(II)2Cys6ion factor
BB8028_0002g01600	Putative Zn(II)2Cys6ion factor
BB8028_0002g13800	Putative Zn(II)2Cys6ion factor
BB8028_0003g01090	Putative Zn(II)2Cys6ion factor
BB8028_0004g00210	Putative Zn(II)2Cys6ion factor
BB8028_0004g01160	Putative Zn(II)2Cys6ion factor
BB8028_0005g06580	Putative Zn(II)2Cys6ion factor
BB8028_0006g02750	quinone oxidoreductase

BB8028_0002g07430	riboflavin-specific deaminase domain-containing protein
BB8028_0005g09890	sorbitol dehydrogenase
BB8028_0009g00220	sterol desaturase
BB8028_0009g00810	SWIM zinc finger protein
BB8028_0004g04640	urea carboxylase
BB8028_0001g08940	zinc finger protein 1
BB8028_0003g10270	zinc finger protein Yan
BB8028_0004g06370	Zn(II)2Cys6ion factor
BB8028_0004g04290	ZZ type zinc finger domain-containing protein

regulation of transcription-RNA pol II' (GO:0006357)

Gene.ID.ARSEF8028	Description based in Blastp against ARSEF2860
BB8028_0006g09760	bacterial transferase hexapeptide
BB8028_0002g03630	C6 finger domain protein
BB8028_0002g03840	C6 finger domain protein
BB8028_0002g07200	C6 finger domain protein
BB8028_0004g07740	C6 finger domain protein
BB8028_0003g00320	C6 finger domain protein, putative
BB8028_0001g02510	C6 finger domain-containing protein
BB8028_0004g07600	C6 finger domain-containing protein
BB8028_0003g05360	C6 zinc finger domain protein
BB8028_0003g10200	C6 zinc finger domain protein
BB8028_0005g09450	C6 zinc finger domain protein
BB8028_0008g02870	C6 zinc finger domain protein
BB8028_0001g05250	C6 zinc finger domain-containing protein
BB8028_0001g12750	C6 zinc finger domain-containing protein
BB8028_0001g13060	C6 zinc finger domain-containing protein
BB8028_0003g07170	C6 zinc finger domain-containing protein
BB8028_0004g01210	C6 zinc finger domain-containing protein
BB8028_0004g03610	C6 zinc finger domain-containing protein
BB8028_0004g07100	C6 zinc finger domain-containing protein
BB8028_0001g10620	C6ion factor
BB8028_0001g16940	C6ion factor
BB8028_0002g15830	C6ion factor
BB8028_0003g00540	C6ion factor
BB8028_0003g04450	C6ion factor
BB8028_0003g14030	C6ion factor
BB8028_0004g01530	C6ion factor
BB8028_0004g09680	C6ion factor
BB8028_0004g13920	C6ion factor
BB8028_0003g09840	C6ion factor, putative
BB8028_0004g01670	C6ion factor, putative
BB8028_0002g09580	CP2ion factor
BB8028_0002g02160	Cutinaseion factor 1 alpha
BB8028_0005g04080	cutinaseion factor 1 beta
BB8028_0003g14110	fork head domain-containing protein

BB8028_0001g10890	fungus specific factor
BB8028_0001g13970	fungus specific factor
BB8028_0001g14890	fungus specific factor
BB8028_0002g01980	fungus specific factor
BB8028_0002g02270	fungus specific factor
BB8028_0002g02470	fungus specific factor
BB8028_0003g04640	fungus specific factor
BB8028_0003g11040	fungus specific factor
BB8028_0003g13410	fungus specific factor
BB8028_0004g12440	fungus specific factor
BB8028_0005g01220	fungus specific factor
BB8028_0006g01460	fungus specific factor
BB8028_0006g08930	fungus specific factor
BB8028_0007g02410	fungus specific factor
BB8028_0005g11720	fungus specific factor domain protein
BB8028_0003g12070	Fungus specific factor, putative
BB8028_0003g01900	general repressor of iron
BB8028_0005g11370	glucoamylase-like protein
BB8028_0001g06700	HECT-domain-containing protein
BB8028_0007g01550	helix-loop-helix DNA-binding domain-containing protein
BB8028_0005g03040	hypothetical protein
BB8028_0007g00130	hypothetical protein
BB8028_0003g16470	nitrate assimilation regulatory protein nirA
BB8028_0004g09580	nitrate assimilation regulatory protein nirA
BB8028_0005g01440	nitrate assimilation regulatory protein nirA
BB8028_0001g03070	PAK kinase
BB8028_0003g14870	Putative C2H2 zinc finger factor
BB8028_0011g00180	Putative C2H2 zinc finger factor
BB8028_0009g00680	putative C6-zinc finger TF, regulator of conidiation
BB8028_0002g04200	putative MLH1 protein
BB8028_0001g03200	Putative Zn(II)2Cys6 zinc finger factor
BB8028_0001g16660	Putative Zn(II)2Cys6 zinc finger factor
BB8028_0002g01600	Putative Zn(II)2Cys6 zinc finger factor
BB8028_0002g13800	Putative Zn(II)2Cys6 zinc finger factor
BB8028_0003g01090	Putative Zn(II)2Cys6 zinc finger factor
BB8028_0004g00210	Putative Zn(II)2Cys6 zinc finger factor
BB8028_0004g01160	Putative Zn(II)2Cys6 zinc finger factor
BB8028_0005g06580	Putative Zn(II)2Cys6 zinc finger factor
BB8028_0004g08570	serine/threonine-protein phosphatase 2A activator 1
BB8028_0003g07840	SNF2 family DNA-dependent ATPase domain-containing protein
BB8028_0006g07910	Subunit of the RNA polymerase II mediator complex
BB8028_0001g12960	transcriptional Coactivator p15
BB8028_0001g08940	zinc finger protein 1
BB8028_0004g06370	Zn(II)2Cys6 zinc finger factor

transmembrane transport' (GO:0055085)

Gene.ID.ARSEF8028	Description based in Blastp against ARSEF2860
BB8028_0001g09280	ABC multidrug transporter, putative
BB8028_0002g08400	ABC multidrug transporter, putative
BB8028_0001g08700	ABC transporter
BB8028_0004g08960	ABC transporter
BB8028_0005g01380	ABC transporter
BB8028_0005g01540	ABC transporter
BB8028_0008g00380	ABC transporter
BB8028_0008g01210	ABC transporter
BB8028_0008g01570	ABC transporter
BB8028_0004g10990	ABC transporter, putative
BB8028_0003g02630	aldo/keto reductase
BB8028_0005g11830	amidohydrolase-like protein
BB8028_0001g03330	amino acid permease
BB8028_0003g08260	amino acid permease
BB8028_0003g14940	amino acid permease
BB8028_0005g06330	amino acid permease
BB8028_0005g07190	amino acid permease
BB8028_0003g09470	Amino acid/polyamine transporter I
BB8028_0006g00480	Amino acid/polyamine transporter I
BB8028_0005g01290	arsenite efflux transporter ArsB, putative
BB8028_0002g10020	augmenter of liver regeneration
BB8028_0001g09660	autophagy protein Atg22
BB8028_0002g09030	bicyclomycin resistance protein
BB8028_0001g02420	calcium/proton exchanger
BB8028_0005g00080	calcium-translocating P-type ATPase
BB8028_0001g14230	canalicular multispecific organic anion transporter 1
BB8028_0004g08860	canalicular multispecific organic anion transporter 1
BB8028_0002g09170	carrier protein YMC1, mitochondrial
BB8028_0002g15470	cation diffusion facilitator family transporter
BB8028_0002g03190	cation efflux family protein
BB8028_0006g08300	chaperonin GroL
BB8028_0003g09940	CHCH domain-containing protein
BB8028_0002g15340	CLAP1-like protein
BB8028_0003g06440	ctr copper transporter
BB8028_0004g02230	drug resistance protein
BB8028_0004g03400	fucose permease
BB8028_0004g07550	GABA-specific permease
BB8028_0006g05760	general amino acid permease AGP2
BB8028_0004g04920	glucokinase regulator family protein, putative
BB8028_0007g05620	heavy metal translocating P-type ATPase
BB8028_0002g10730	hexose transporter
BB8028_0002g13500	hexose transporter
BB8028_0003g09750	hexose transporter
BB8028_0007g03790	hexose transporter
BB8028_0008g02780	hexose transporter

BB8028_0001g00550	hypothetical protein
BB8028_0001g00560	hypothetical protein
BB8028_0004g10300	integral membrane protein
BB8028_0001g08890	iron permease FTR1 family protein
BB8028_0003g10490	magnesium-translocating P-type ATPase family protein
BB8028_0004g01050	Major Facilitator Superfamily protein
BB8028_0001g08080	major facilitator superfamily transporter
BB8028_0002g01110	major facilitator superfamily transporter
BB8028_0002g02450	major facilitator superfamily transporter
BB8028_0002g04290	major facilitator superfamily transporter
BB8028_0002g05150	major facilitator superfamily transporter
BB8028_0002g08580	major facilitator superfamily transporter
BB8028_0002g09590	major facilitator superfamily transporter
BB8028_0002g12790	major facilitator superfamily transporter
BB8028_0003g02890	major facilitator superfamily transporter
BB8028_0003g08200	major facilitator superfamily transporter
BB8028_0003g09440	major facilitator superfamily transporter
BB8028_0003g13430	major facilitator superfamily transporter
BB8028_0004g06830	major facilitator superfamily transporter
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BB8028_0004g09660	major facilitator superfamily transporter
BB8028_0004g12410	major facilitator superfamily transporter
BB8028_0005g00380	major facilitator superfamily transporter
BB8028_0005g04610	major facilitator superfamily transporter
BB8028_0006g00080	major facilitator superfamily transporter
BB8028_0006g00630	major facilitator superfamily transporter
BB8028_0006g05090	major facilitator superfamily transporter
BB8028_0006g06710	major facilitator superfamily transporter
BB8028_0007g02940	mechanosensitive ion channel
BB8028_0002g14690	membrane transporter
BB8028_0001g16130	MFS allantoin transporter, putative
BB8028_0001g01770	MFS gliotoxin efflux transporter GliA
BB8028_0003g14840	MFS monocarboxylate transporter
BB8028_0001g13460	MFS multidrug transporter
BB8028_0005g04550	MFS multidrug transporter
BB8028_0006g00960	MFS quinate transporter, putative
BB8028_0005g11280	MFS sugar transporter, putative
BB8028_0009g01110	MFS toxin efflux pump (AflT)
BB8028_0001g00420	MFS transporter, putative
BB8028_0002g12950	MFS transporter, putative
BB8028_0003g10030	MFS transporter, putative
BB8028_0004g12830	MIP family channel protein
BB8028_0005g09500	mitochondrial carrier protein RIM2
BB8028_0004g01150	monocarboxylate permease-like protein
BB8028_0004g04480	monocarboxylate transporter
BB8028_0005g11350	multidrug resistance ABC transporter, putative

BB8028_0002g11950	multidrug resistance protein
BB8028_0003g16470	nitrate assimilation regulatory protein nirA
BB8028_0001g04930	oligopeptide transporter
BB8028_0003g13330	oligopeptide transporter
BB8028_0001g02680	OPT oligopeptide transporter
BB8028_0001g05770	OPT oligopeptide transporter
BB8028_0006g08670	pantothenate transporter liz1
BB8028_0005g02900	permease-like protein
BB8028_0004g05220	phthalate transporter
BB8028_0008g00290	polyamine transporter TPO5
BB8028_0004g11860	potassium/sodium efflux P-type ATPase
BB8028_0007g06010	proline transporter
BB8028_0004g13600	protein kinase domain protein
BB8028_0002g10910	purine-cytosine permease FCY21
BB8028_0001g15240	putative calcium P-type ATPase
BB8028_0006g03390	putative peptide transporter
BB8028_0003g09910	siderophore iron transporter
BB8028_0008g00790	siderophore iron transporter 1
BB8028_0001g16150	siderophore iron transporter mirA
BB8028_0004g00760	siderophore iron transporter mirB
BB8028_0003g04560	small oligopeptide transporter, OPT family
BB8028_0003g09820	Sodium
BB8028_0008g02790	sodium/hydrogen exchanger family protein
BB8028_0005g02620	solute carrier family 12 member 3
BB8028_0005g09790	sugar transporter
BB8028_0007g06660	sugar transporter
BB8028_0003g13400	tat pathway signal sequence
BB8028_0004g01070	vitamin H transporter
BB8028_0001g11530	V-type ATPase
BB8028_0003g06020	ZIP Zinc transporter

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Table S9 KEGGs specific to CE, LE and H15 of hypovirulent strain ILB299

	KEGG Name	G count	pvalue
CE (15)			103
	ko00620 Pyruvate metabolism	13	2.46 $\times 10^{-2}$
	ko00020 Citrate cycle (TCA cycle)	9	2.09 $\times 10^{-2}$
	ko00261 Monobactam biosynthesis	3	2.94 $\times 10^{-2}$
	ko00627 Aminobenzoate degradation	9	1.72 $\times 10^{-2}$
	ko00623 Toluene degradation	4	3.94 $\times 10^{-2}$
	ko04974 Cholesterol metabolism	3	2.94 $\times 10^{-2}$
	ko00920 Sulfur metabolism	14	6.95 $\times 10^{-4}$
	ko00280 Valine, leucine and isoleucine degradation	12	1.31 $\times 10^{-2}$
	ko00720 Carbon fixation pathways in prokaryotes	11	5.85 $\times 10^{-5}$
	ko00460 Cyanoamino acid metabolism	7	4.43 $\times 10^{-2}$
	ko04614 Renin-angiotensin system	3	1.62 $\times 10^{-2}$
	ko00380 Tryptophan metabolism	14	3.15 $\times 10^{-2}$
	ko04016 MAPK signaling pathway - plant	3	2.94 $\times 10^{-2}$
	ko00643 Styrene degradation	12	2.78 $\times 10^{-4}$
	ko00640 Propanoate metabolism	13	3.29 $\times 10^{-4}$
LE (6)			
	ko01040 Biosynthesis of unsaturated fatty acids	5	4.75 $\times 10^{-2}$
	ko04068 FoxO signaling pathway	6	3.56 $\times 10^{-2}$
	ko04062 Chemokine signaling pathway	6	3.56 $\times 10^{-2}$
	ko04071 Sphingolipid signaling pathway	9	2.72 $\times 10^{-2}$
	ko04142 Lysosome	11	2.83 $\times 10^{-2}$
	ko04920 Adipocytokine signaling pathway	4	4.07 $\times 10^{-2}$
H15 (1)			
	Ko04146 Peroxisome	13	1.30 $\times 10^{-2}$

Table S10 KEGGs specific to CE, LE and H15 of hypervirulent strain ILB308

	KEGG Name	G count	pvalue
CE (6)			
	ko04520 Adherens junction	4	3.51 x10 ⁻²
	ko00052 Galactose metabolism	7	3.01 x10 ⁻²
	ko04929 GnRH secretion	2	1.85 x10 ⁻²
	ko00254 Aflatoxin biosynthesis	2	3.51 x10 ⁻²
	ko04012 ErbB signaling pathway	3	2.16 x10 ⁻²
	ko04960 Aldosterone-regulated sodium reabsorption	3	1.44 x10 ⁻²
LE (3)			
	ko04978 Mineral absorption	6	1.71 x10 ⁻⁵
	ko04212 Longevity regulating pathway - worm	9	1.53 x10 ⁻²
	ko00740 Riboflavin metabolism	7	1.66 x10 ⁻²
H15 (12)			
	ko04110 Cell cycle	11	4.27 x10 ⁻³
	ko04371 Apelin signaling pathway	4	3.68 x10 ⁻²
	ko00430 Taurine and hypotaurine metabolism	3	3.09 x10 ⁻²
	ko03420 Nucleotide excision repair	8	7.98 x10 ⁻³
	ko04261 Adrenergic signaling in cardiomyocytes	5	1.21 x10 ⁻²
	ko04062 Chemokine signaling pathway	5	3.60 x10 ⁻²
	ko00230 Purine metabolism	42	3.22 x10 ⁻³
	ko03030 DNA replication	8	3.46 x10 ⁻³
	ko04113 Meiosis – yeast	16	1.63 x10 ⁻⁴
	ko04971 Gastric acid secretion	4	5.93 x10 ⁻³
	ko00730 Thiamine metabolism	37	3.64 x10 ⁻³
	ko04728 Dopaminergic synapse	6	7.49 x10 ⁻³

Table S2 Hypovirulent strain ILB299 and hypervirulent strain ILB308 expressed genes (ARSEF8028) in each culture medium

ILB299			ILB308		
CE (57)	LE (54)	H15 (340)	CE (58)	LE (262)	H15 (15)
BB8028_0006g01880	BB8028_0003g07450	BB8028_0005g01320	BB8028_0006g01880	BB8028_3003g13390	BB8028_0006g01240
BB8028_0005g10830	BB8028_0001g06790	BB8028_0006g11090	BB8028_0004g07800	BB8028_0006g00350	BB8028_0001g03470
BB8028_0001g08000	BB8028_0009g00550	BB8028_0003g01800	BB8028_0002g14710	BB8028_0004g01320	BB8028_0002g16340
BB8028_0001g09860	BB8028_0003g06530	BB8028_0006g06780	BB8028_0004g01830	BB8028_0010g00300	BB8028_0005g06360
BB8028_0001g06890	BB8028_0004g10350	BB8028_0007g01900	BB8028_0001g06790	BB8028_0003g00020	BB8028_0005g07350
BB8028_0005g00370	BB8028_0005g01470	BB8028_0002g00110	BB8028_0007g00780	BB8028_0005g10830	BB8028_0001g15690
BB8028_0002g04950	BB8028_0002g15630	BB8028_0001g00350	BB8028_0005g08220	BB8028_0012g00070	BB8028_0001g01530
BB8028_0002g13610	BB8028_0002g13420	BB8028_0001g07050	BB8028_0009g00550	BB8028_0005g01430	BB8028_0005g02520
BB8028_0006g07740	BB8028_0003g05640	BB8028_0002g14710	BB8028_0006g00520	BB8028_0006g00960	BB8028_0006g00970
BB8028_0004g00770	BB8028_0003g05930	BB8028_0004g13870	BB8028_0005g06370	BB8028_0003g12510	BB8028_0006g02590
BB8028_0001g09470	BB8028_0002g02440	BB8028_0006g04490	BB8028_0002g02480	BB8028_0007g00110	BB8028_0001g12920
BB8028_0009g00970	BB8028_0005g00710	BB8028_0004g01830	BB8028_0008g01280	BB8028_0010g00430	BB8028_0005g10630
BB8028_0003g10030	BB8028_0009g00230	BB8028_0006g01240	BB8028_0001g04060	BB8028_0003g04730	BB8028_0003g15690
BB8028_0008g01280	BB8028_0002g02260	BB8028_0006g03520	BB8028_0003g10640	BB8028_0003g07450	BB8028_0003g08230
BB8028_0004g11280	BB8028_0003g12970	BB8028_0006g04020	BB8028_0001g02770	BB8028_0004g13570	BB8028_0004g01330
BB8028_0001g13030	BB8028_0005g04600	BB8028_0003g09800	BB8028_0007g05200	BB8028_0005g01410	BB8028_0005g05570
BB8028_0005g04230	BB8028_0004g13480	BB8028_0006g09410	BB8028_0005g00280	BB8028_0002g13880	BB8028_0004g11880
BB8028_0001g10110	BB8028_0005g08440	BB8028_0001g13860	BB8028_0002g03490	BB8028_0004g08310	BB8028_0006g06880
BB8028_0002g08220	BB8028_0001g03830	BB8028_0011g00200	BB8028_0006g07880	BB8028_0009g01580	BB8028_0003g00070
BB8028_0006g03200	BB8028_0001g09790	BB8028_0004g08940	BB8028_0003g05640	BB8028_0010g00490	BB8028_0007g01150
BB8028_0005g00280	BB8028_0001g00500	BB8028_0004g09200	BB8028_0006g05660	BB8028_0001g00550	BB8028_0002g10050
BB8028_0004g00120	BB8028_0004g01680	BB8028_0005g01920	BB8028_0002g15590	BB8028_0008g00480	BB8028_0003g13240
BB8028_0006g05670	BB8028_0006g04640	BB8028_0005g05050	BB8028_0009g00230	BB8028_0001g13860	BB8028_0004g01080
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BB8028_0004g01190	BB8028_0008g00220	BB8028_0002g03570	BB8028_0001g02520	BB8028_0003g15750	BB8028_0004g01970
BB8028_0002g13550	BB8028_0002g11950	BB8028_0005g04970	BB8028_0004g00270	BB8028_0010g00410	BB8028_0009g01300
BB8028_0001g13080	BB8028_0007g06010	BB8028_0001g12130	BB8028_0002g00120	BB8028_0004g13400	BB8028_0003g09720
BB8028_0004g13080	BB8028_0002g09250	BB8028_0007g02270	BB8028_0007g04770	BB8028_0006g09280	BB8028_0004g05790
BB8028_0004g08540	BB8028_0007g03790	BB8028_0004g05270	BB8028_0003g08910	BB8028_0004g11340	BB8028_0003g03770
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BB8028_0005g09810	BB8028_0001g17250	BB8028_0001g08910	BB8028_0001g00250	BB8028_0008g01250	BB8028_0001g11880
BB8028_0004g01270	BB8028_0005g01350	BB8028_0006g00520	BB8028_0005g08680	BB8028_0005g01030	BB8028_0001g06960
BB8028_0001g00250	BB8028_0001g00910	BB8028_0003g09130	BB8028_0007g04160	BB8028_0001g01510	BB8028_0005g00340
BB8028_0003g01920	BB8028_0006g07180	BB8028_0001g08330	BB8028_0001g01930	BB8028_0002g08450	BB8028_0003g01920
BB8028_0006g00680	BB8028_0001g01520	BB8028_0002g05750	BB8028_0002g02820	BB8028_0003g06530	BB8028_0001g13290
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BB8028_0003g16070	BB8028_0001g00150	BB8028_0005g11290	BB8028_0003g09380	BB8028_0007g00230	BB8028_0004g03330
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