INSTITUTO POLITÉCNICO NACIONAL

ESCUELA NACIONAL DE CIENCIAS BIOLÓGICAS

RESPUESTA ELECTROFISIOLÓGICA DE *Dendroctonus rhizophagus* (COLEOPTERA: CURCULIONIDAE: SCOLYTINAE) Y GENES CITOCROMO P450 EXPRESADOS ANTE COMPUESTOS KAIROMONALES

TESIS

QUE COMO UNO DE LOS REQUISITOS PARA

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PRESENTA:

M. en C. CLAUDIA CANO RAMÍREZ

MÉXICO, D. F., DICIEMBRE DE 2010

α-Pinene and myrcene induce ultrastructural changes in the midgut of the red turpentine beetle *Dendroctonus valens* (Coleoptera: Curculionidae: Scolytinae)

María Fernanda López[1], Caudia Cano-Ramírez[1], Mineko Shibayama,[2] and Gerardo Zúñiga[1]

[1]Escuela Nacional de Ciencias Biológicas-IPN. Laboratorio de Variación Biológica y Evolución. Departamento de Zoología. Carpio y Plan de Ayala s/n, Col. Santo Tomás, CP 11340 México DF., México.

[2]Centro de Investigación y Estudios Avanzados–IPN. Laboratorio 2 de Patología Experimental Departamento de Infectómica y Patogénesis Molecular. Avenida Instituto Politécnico Nacional 2508, Col. San Pedro Zacatenco, CP 07360 México DF., México

α-Pinene and myrcene induce ultrastructural changes in the midgut of the red turpentine beetle Dendroctonus valens (Coleoptera: Curculionidae: Scolytinae)
>
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> Review This MS will be acceptable with the changes listed below. Do note that some of the same issuess noted previously still need work.

1. L57 replace disorders with changes or abnormalities.

2. L 70 replace intensive activity of with "substantial".

3.Line 92 remove for food.

4. L 115 dilution curve.

5. L 121 replace "under" with "using".

6. L 166 significant.

7. L.183 .. 5 layers, an inner layer … Note : you call this muscle connective tissue in the figure. It is not… It is muscle--- myofibrils are evident.

8. L184. Using a pen draw a line around the nidus.

9. L 185 Remove sentence which begins The basal lamina develops… Figure 3 show a shallow infolding with basal lamina material and tracheoles and muscle tissue from below. The TEMS are very low contrast so for that reason you can not make too many conclusions from the morphology.

10. Line 211 … evident in throughout. Is the next sentence in contrast to this one?

11 Lines 212 and 213 Fig 8 and 9. What does discontinuous mean in this context? The figures are not the same magnification. Also, the line down the center of the SER shows a density but I can not see gold particles.

12. L228 biotransform.

13. L232 replace in despite of by "while" … amounts.

14. Line 235 :"simplifies".

15. Clarify point of lines 237 – 9.

16 L240 replace strong with "high" or "great".

17. L248 use "active" for "intense"

18. L250-252 shorten.

19. L257 SER is not a transitional organelle. Coated vesicles come from the RER. SER plays roles in detoxification and (along with mitochondria) in sterod biosynthesis.

20. L. 304 Use Scolytine beetles.

21. L558 Table. Include a short description. Maybe describe the data on the first line (pinene) so the table as a whole can be more easily understood.

22. Line 561 does > Figures and captions. In general, the figures I downloaded are of low contrast and often difficult to make out. Hopefully the images will reproduce better. Also, when making labels bring the tip of the arrows right up to, and touching, the structure being described. Fig. 1 Other than size I see no difference between VS and LS. Fig. 2 Here the LS has material inside it. I do not see what is being labeled as glycogen. Also compare the sizes of the microvilli in #1 and #2. The mag may be wrong. Fig. 3. The label CT is over a muscle cell. One BL appears to point to the plasmalemma of the epithelial cell while the other points to

something that is very light It appears to come from the tissue below the epithelial cell. Fig. 4 draw line around nidus. Fig. 5 3 LS point to what appears to be multivesiculate bodies; Do you know the function of VS? I see nothing in it. Fig. 6 Note the distinguishing features of VS, LP and LS in this caption as well as the evidence for cell stress in mitochondria and RER. Comparing with Fig. 7 why might the mitochondria look so much more normal in Fig. 7. Fig. 9. I presume the gold labeling is the light line down the center of the SER. This figure looks to be at 2X higher mag. than fig. 8. Also why choose a "control" where you do not identify SER? Fig 10 what is SER pointing to? Fig 11 L 627 high activity ? That may not be true. In the case of plasma cells the active RER have dilated cisternae. How do you justify your statement concerning the activity of the RER?
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>
> Sincerely,
>
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> Annals of the Entomological Society of America

El presente trabajo se llevó a cabo en los siguientes espacios:

El laboratorio de Variación Biológica y Evolución de la Escuela Nacional de Ciencias Biológicas del Instituto Politécnico Nacional bajo la dirección del Dr. Gerardo Zúñiga Bermúdez.

La estación USDA Forest Service Southern Research Station bajo la dirección del Dr. Brian T. Sullivan.

El laboratorio de Enemigos Naturales de el Colegio de la Frontera Sur en Tapachula, Chiapas bajo la dirección del Dr. Brian T. Sullivan y Dr. Jorge Macías Sámano.

La presente investigación formó parte del proyecto “Biología, ecología y manejo de *Dendroctonus rhizophagus* Thomas & Bright (Coleoptera: Curculionidae: Scolytinae) en el Estado de Chihuahua”, financiado por la Comisión Nacional Forestal-Consejo Nacional de Ciencia y Tecnología (CONAFOR-CONACyT, 69539) y Secretaría de Investigación y Posgrado-IPN (SIP-20090576).

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Lee Smolin

INTRODUCCIÓN GENERAL

Los escarabajos descortezadores (Coleoptera: Curculionidae: Scolytinae) del género *Dendroctonus* Erichson tienen una relación evolutiva antigua y estrecha con la familia *Pinaceae*. El papel ecológico principal de estos insectos es el actuar como un agente selectivo matando árboles viejos, dañados o susceptibles, lo cual favorece la sucesión natural en los bosques de coníferas. Una característica biológica de este grupo es que bajo condiciones de perturbación o desequilibrio ecológico de los bosques, pueden afectar grandes superficies de arbolado sano, producir importantes pérdidas económicas al sector forestal y provocar indirectamente, por los métodos que se aplican para su control, daños ecológicos irreversibles en los bosques.

La comunicación química es fundamental en el ciclo de vida de estos escolítidos, ya que les permite localizar, seleccionar y colonizar a sus árboles huéspedes y reproducirse con conespecíficos. Esta se lleva a cabo a partir de compuestos químicos (terpenoides hidroxilados) emitidos tanto por el huésped (kairomonas), así como por los producidos por el insecto (feromonas) que provocan una respuesta fisiológica sobre sus conespecíficos de atracción o repulsión.

Estructuras como las antenas y el canal alimentario han jugado un papel importante en la comunicación química y por consiguiente en la sobrevivencia de este grupo de insectos. A través de las antenas las especies del género *Dendroctonus* reconocen la composición y proporción exacta de las kairomonas y feromonas durante la fase de colonización, el canal alimentario es el sitio donde se producen las feromonas y se lleva a cabo parte del proceso de desintoxicación.

La participación de complejos enzimáticos en la desintoxicación y/o producción de feromonas ha sido decisivo en la sobrevivencia de estos insectos. Se conoce que los

complejos enzimáticos citocromo P450, las Glutatión *S*-Transferasas y las Esterasas juegan un papel clave en los procesos anteriormente mencionados.

Los citocromos P450 están presentes en la mayoría de los seres vivos, participan activamente en el metabolismo de compuestos endógenos y exógenos a través de reacciones de oxidación de los compuestos y se localizan en diferentes órganos o tejidos como son el intestino e hígado; en el caso de los insectos en el cuerpo graso, los túbulos de Malpighi y las antenas, entre otros. A nivel celular se localizan en membranas de organelos como el retículo endoplásmico y las mitocondrias, o bien pueden ser de tipo citoplasmático.

Desde un punto de vista evolutivo los genes *CYP* que codifican para estás proteínas en los insectos se han agrupado en cuatro clanes: CYP2, CYP3, CYP4 y CYP mitocondrial. Las funciones que se han asociado a los clanes CYP3, CYP4 y mitocondrial están relacionadas con el metabolismo de compuestos xenobióticos, producción de feromonas e hidroxilación de ácidos graso, las funciones del clan CYP2 están relacionadas con procesos de metamorfosis y muda de los insectos.

La evolución de los genes *CYP* se ha asociado con la respuesta de los insectos a una variedad de compuestos químicos, lo que ha permitido que estos organismos adquieran resistencia a xenobióticos naturales y sintéticos, entre los que destacan los aleloquímicos de las plantas e insecticidas del tipo de los piretroídes y organofosforados.

Además, la presión selectiva de estos genes ha desarrollado una diversidad en los patrones de expresión relacionados al estadio de desarrollo, sexo, tejido y dieta con el propósito de comprender el comportamiento de la maquinaria molecular durante el proceso de desintoxicación y/o producción de feromonas en el ciclo de vida del insecto.

Principalmente en genes de las familias *CYP4* y 6 se han encontrado patrones de expresión diferencial en insecto completo (Tomita & Scott 1994; Tarès *et al*., 2000), tejido específico (Davies *et al*., 2006), estadio de desarrollo (Helvig *et al*., 2004; David *et al*., 2006) y sexo (Bhaskara *et al*., 2006; David *et al*., 2006; Huber *et al*., 2007; Le Goff *et al*., 2006).

D. rhizophagus Thomas & Bright es una especie endémica de los bosques de pino de los estados de Sinaloa, Sonora, Chihuahua y Durango de la Sierra Madre Occidental (Zúñiga *et al*., 1999), estudios de distribución geográfica muestran que esta especie se desarrolla adecuadamente entre los 1200 y 2600 m de altitud, siendo sus principales huéspedes *Pinus engelmannii* Carr., *P. durangensis* Martínez, *P. arizonica* Engelm y *P. cooperi* Blanco (Salinas-Moreno *et al*., 2004). *D. rhizophagus* es una especie hermana derivada de *D. valens* Le Conte (Kelley & Farell, 1998), pero con diferencias biológicas y ecológicas muy marcadas. *D. rhizophagus* es un descortezador primario con un ciclo de vida anual sincrónico que parásita a los árboles de renuevo de 0.45 a 2.7 m de altura, mientras que *D. valens* es un descortezador secundario que prefiere árboles maduros colonizados previamente por otros descortezadores, tiene un ciclo de vida anual asincrónico y una amplia distribución geográfica (Cibrián *et al*., 1995).

El presente trabajo se dividió en dos capítulos. En el primero se estudio el sistema de comunicación química de *D. rhizophagus* a través de la respuesta electrofisiológica de las antenas a los compuestos de la resina y volátiles producidos por el insecto. Además, se analizó y cuantificó a los volátiles producidos por el escarabajo antes de emerger y después de emerger en diferentes estados de colonización tanto en condiciones de laboratorio como de campo. Por último, se evaluó mediante pruebas de campo el grado de atracción que tienen los diferentes monoterpenos oxigenados y no oxigenados sobre los insectos. En el segundo capítulo, se analizó la diversidad de genes *CYP* citocromo

P450 de los clanes 3 y 4 expresados en antenas y canal alimentario de *D. rhizophagus* ante el estímulo del α-pineno, el principal monoterpeno de la resina de los pinos, y los enantiómeros del α-pineno (*R*)-(+)-α-pineno y (*S*)-(-)-α-pineno y un enantiómero del β-pineno (*S*)-(-)-β-pineno a los cuales las antenas respondieron electrofisiológicamente. Se obtuvieron las secuencias completas de los genes expresados y se identificaron sus principales características físicas-químicas. Finalmente, se exploró a través de la reacción e cadena de la polimerasa (PCR) semicuantitativo el patrón de expresión de los genes *CYP* estimulados con los monortepenos referidos en antenas y las regiones del canal alimentario en tres diferentes tiempos de estimulación.

JUSTIFICACIÓN

Diferentes métodos han sido empleados en el control y manejo de los insectos descortezadores. Algunos de ellos han mostrado ser adecuados y eficientes, pero aquellos que han hecho uso de semioquímicos identificados y probados en los estudios de comunicación química han probado ser fundamentales para el monitoreo y manejo de estos insectos. A pesar de ello, los estudios de comunicación química en descortezadores mexicanos son prácticamente escasos, lo que ha traído como consecuencia que se traslade la información a nuestro país de las especies estudiadas en otras latitudes. Lo anterior no siempre funciona bien y probablemente sea la causa de se sigan utilizando métodos no preventivos como el derribo y abandono de los árboles, el derribo-descortezado-arropado o bien métodos de contención.

Dendroctonus rhizophagus es una especie primaria, endémica del noroeste de México que coloniza arboles de casi 11 especies de pino. Esta especie tiene un comportamiento atípico dentro del género, ya que ataca árboles de renuevo < 3m tanto en áreas de regeneración natural como de plantación comercial. Los cifras oficiales indican que tan solo en el periodo de 1977-1983 ocasionó la muerte de más de 2,000,000 de plántulas y hasta la fecha se estima que mantiene un daño anual constante de ≈12,500 ha de árboles jóvenes en toda su distribución geográfica. Por ello, es importante estudiar el sistema de comunicación química de este insecto con el propósito de desarrollar estrategias convenientes de manejo y control, así como analizar a través del estudio de los genes *CYP* uno de los mecanismos de desintoxicación que tienen estos insectos, que les ha permitido superar los mecanismos de defensa de los árboles a través de su coevolución con ellos.

OBJETIVO GENERAL

Determinar la respuesta electrofisiológica y de comportamiento de *Dendroctonus rhizophagus* a los semioquímicos de su huésped *P. arizonica* y sus conespecíficos, además de determinar la diversidad de genes *CYP* citocromo P450 expresados ante el estímulo de diferentes kairomonas y cuantificar los niveles de expresión de los genes *CYP*.

OBJETIVOS PARTICULARES

- Determinar la respuesta electrofisiológica del insecto ante los compuestos identificados en la resina de los huéspedes y en los volátiles producidos por el insecto mismo.
- Identificar y cuantificar los compuestos volátiles producidos por los conespecíficos de *D. rhizophagus*.
- Evaluar en campo la actividad comportamental de los compuestos electroantenográficamente activos.
- Determinar la diversidad de genes *CYP* citocromo P450 correspondientes a las familias 4, 6, 9 en las antenas y el tracto digestivo, expresados ante el estímulo del α-pineno los enantiómeros del α-pineno (*R*)-(+)-α-pineno y (*S*)-(-)-α-pineno y un enantiómero del β-pineno (*S*)-(-)-β-pineno.
- Analizar bioinformáticamente la secuencia completa de los genes *CYP*.
- Cuantificar los niveles de expresión de las familias de genes CYP4, CYP6 y CYP9 en las antenas, estomodeo, mesenterón y proctodeo de *D. rhizophagus* ante el estímulo del α-pineno, los enantiómeros del α-pineno (*R*)-(+)-α-pineno y (*S*)-(-)-α-pineno y un enantiómero del β-pineno (*S*)-(-)-β-pineno.
- Cuantificar los niveles de expresión de las familias de genes CYP4, CYP6 y CYP9 de *D. rhizophagus* a diferentes tiempos de estimulación ante el estímulo del α-pineno, los enantiómeros del α-pineno (*R*)-(+)-α-pineno y (*S*)-(-)-α-pineno y un enantiómero del β-pineno (*S*)-(-)-β-pineno.

CAPÍTULO I

Respuesta electrofisiológica y de comportamiento de *Dendroctonus rhizophagus* (Coleoptera: Curculionidae: Scolytinae) a la resina y a los volátiles aislados de sus conespecíficos

Artículo en revisión para ser enviado a la revista *Journal Chemical Ecology*

Electrophysiological and Behavioral Responses of *Dendroctonus rhizophagus* (Coleoptera: Curculionidae; Scolytinae) to Pine Resin and Volatiles Isolated from Conspecifics

C. Cano-Ramírez[1], F. Armendariz-Toledano[1], J. E. Macías-Sámano[2] B. T. Sullivan[3] and G. Zúñiga[1].

[1]Departamento de Zoología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Prol. de Carpio y Plan de Ayala. Col. Sto. Tomas, México D. F., CP 11340, México. Telephone number 01 (52) 55-57296000 ext. 62419, E-mail address: capotezu@hotmail.com.

[2] El Colegio de la Frontera Sur ECOSUR, Carretera Antiguo Aeropuerto km 2.5, 30700 Tapachula, Chiapas, Mexico.

[3]USDA-Forest Service Southern Research Station, 2500 Shreveport Highway, Pineville LA USA 71360

ABSTRACT *Dendroctonus rhizophagus* Tomas & Bright is a primary and specialist specie of young pines and seedlings. The species is endemic to the northwestern Mexico and kills pines throughout all its range. Nothing is known about the chemical ecology of this bark beetle. Olfactory sensitivity of this species was assessed using coupled gas chromatography-electroantennographic detection, testing compounds isolated from: 1) resin of arizona pine *Pinus arizonica* Engelm.; 2) mid/hindgut of single gallery boring females and 3) from mid/hindgut of male paring in galleries. Our results show that the antenna of both consistently responded to the monoterpenes, α-pinene, β-pinene and 3-carene and to the oxygenated terpenes, fenchyl alcohol, myrtenal, *cis*-verbenol, *trans*-verbenol, verbenone and myrtenol, which all are frequently reported as pheromone components of *Dendroctonus* spp. The six oxygenated terpenes were quantified from pre-emerged and emerged individual of *D. rhizophagus* at different colonization phases on laboratory and field bioassays. In both bioassay myrtenol, *trans*-verbenol and *cis*-verbenol were the most abundant components, especially when males and females were separately feeding on bolts under laboratory conditions and when paired insects were in galleries in the field. Two trapping field experiments were performed to evaluate behavioral responses of *D. rhizophagus* to antennally active monoterpenes, three from the resin and six from the mid/hindgut. These compounds were deployed in modified multiple-funnel traps. (+)-3-carene was of the most significantly attractive, followed by the ternary (1:1:1) combination of (+)-α-pinene, (-)-β-pinene and (+) 3-carene. *Trans*-verbenol in combination with the indicated ternary mixture significantly increased beetles catches. Verbenone and all the oxygenated terpenes were less attractive compounds for *D. rhizophagus*. A significant difference response between the sexes was found in both field experiments. Our results suggest that *D. rhizophagus* may have a primary attraction to it host *P. arizonica*, mediated by (+)-3-carene. Secondary attraction was not apparent in *D. rhizophagus* as it is reported for aggressive species.

KEY WORDS: Kairomone, pheromone, scolytinae, semiochemical, chemical ecology

BARK BEETLES are important mortality agents of conifers in North and Central America, their outbreaks have both economic and ecological impact that affect forest resource management strategies (Wood 1982a). *Dendroctonus rhizophagus* Thomas & Bright (Curculionidae: Curculionidae: Scolytinae) is an endemic species from the northern portion of the Sierra Madre Occidental in the northwestern Mexico, which colonizes 11 pine species, especially Arizona pine (*Pinus arizonica* Engelm.), Apache pine (*Pinus engelmannii* Carr.), Chihuahua pine (*Pinus leiophylla* Schlecht and Cham.) and Durango pine (*Pinus durangensis* Martinez) (Mendoza et al. *in review*, Salinas-Moreno et al. 2004, 2010). In recent years, the impact of this bark beetle has reached important proportions. Between the years of 1977-1983 in Mesa del Huracán, Chihuahua (29° 38' N, 108°14' W) this bark beetle killed 2 million young trees, comprising an infested area of 12,000 ha (Estrada-Murrieta 1983). More recent reports indicate that *D. rhizophagus* affected, from 2003 to 2008, around 1200 ha in all its distribution range. In addition, conservative estimations indicate that the susceptible area to be attacked by this bark beetle is around of 1000 km^2 (SEMARNAT 2005).

D. rhizophagus is considered a primary pest, similar in appearance and phylogenetically closed to the red turpentine beetle, *D. valens* LeConte (Wood 1982a, Kelley and Farrell 1998). It only infests seedlings and young saplings (height < 3 m) from 11 pine species, whereas *D. valens* normally colonizes > 40 species of mature pines (height >20 m) throughout of its geographic range in North America (Wood 1982a). In addition, *D. rhizophagus* has an atypical behavior within genus, because regularly just one or two couples colonize and breed in a single pine tree, i.e. there is no mass attack (Estrada-Murrieta 1983, Cibrián et al. 1995). The couple creates a gallery at the root collar of the tree, the larval brood mine downward following the root system and latter excavate a weevil-like pupal chamber that deeply scores the wood.

To date, the responses of this beetle to host volatiles have not been investigated, and in contrast to its sibling species (Phillips et al. 1989, Hobson et al. 1993, Sun et al. 2004, Zhang et al. 2006, Erbilgin et al. 2007), its chemical ecology is unknown. To identify which host tree compounds are attractive to *D. rhizophagus* and those that the insects might produce, we run electrophysiological bioassays (GC-EAD) on *D. rhizophagus* individuals with host resin and with volatiles released by conspecifics. Then, we quantified oxygenated monoterpenes in female and male insects before and after emergence under different laboratory and of field conditions. Finally, we evaluated field behavioral response by this species to antennally active host monoterpenes and insect volatiles

Materials and Methods

Insects. Adult bark beetles (pre-emerged and emerged) used in the bioassays were collected from roots and root collar of naturally infested Arizona pine *Pinus arizonica* Engelm. in San Juanito, Bocoyna Municipality, Chihuahua State (27º 55' 54.9'' N and 107º 35' 54.6'' W; at 2452 m). Beetles were kept in plastic containers with moistened paper at 4°C up to 3 days before use. Insects were sexed using the shape of the seventh abdominal terguite (Hopkins 1909).

Resin. Resin from young *P. arizonica* pines (< 2 m of height) was collected following the procedure by Smith (2000), and after 24 h a 100 μL sample of the resin was taken with a Drummond capillary pipette, placed it into a vial with 900μL of hexane and stored at -20 °C.

Electrophysiological Assays. The response of *D. rhizophagus* antennae to volatile compounds present in the gut of recently emerged adults was studied with a GC-EAD set up. The general procedure was described by Sullivan (2005). For each assay, a glass pipette Ag/AgCl reference electrode (containing Beadle-Ephrussi saline and 0.5%

polyvinylpyrrolidone) was inserted into the foramen of a beetle's excised head. The tip of a similar recording electrode was cut to match the diameter of the antennal club, and it was laid flat against the electrode opening so that one entire side contact with the saline. The club's opposite side was positioned in an airstream receiving effluent from the GC. A resin dilution (1:10) in hexane was exposed to the antennal preparations of both sexes. One microliter was injected in GC-EAD in split mode and using a helium ionization detector (HID). The GC-EAD were made with no chiral phase column (HP-INNOWax 60 m x 0.25 mm x 0.25 μm film, Agilent Technologies, Wilmington, DE) and the oven program was 50°C for 1 min, 16°C/min at 80°C, then 7°C/min at 218°C and held for a final 5 min. Ten repetitions of each sex were made. Likewise, the posterior midgut and hindgut from 38 single females mining galleries and 38 males, with partner in the gallery, were directly extracted in the field, pooled by sexes and deposited separately in 1 mL of hexane. The extracts were concentrated with a nitrogen stream at 0.3 mL. Two microliter was injected in the GC-EAD in splitless mode following the conditions previously described. Female and male antennae were exposed to extracts from both sexes (8-10 replications of each combination). The identifications of GC peaks were confirmed by retention time matching known standards and by GC-EAD analyses of these standards with *D. rhizophagus* antennae.

Volatiles quantification of individual beetles at different colonization phases. Volatiles were quantified in extracts from individual males and females of *D. rhizophagus*, placed in two different bioassay settings (B1 and B2). For Bioassay B1, volatiles were collected in the laboratory from three different treatments of pre-emerged insects. Fed insects were obtained by introducing individuals into bolts of green young *P. arizonica* (30 cm of length x 6-10 cm of diameter) following Sullivan (2005). Treatments were as follows: T1) separated unfed females and males, T2) separated

females and males fed for 24 h and T3) females with males introduced 24 h later. These treatments simulated the sequence of females boring to construct nuptial chamber and males admitted into them. Each treatment had at least 10-15 repetitions for each sex and treatment.

Bioassay B2 was carried out entirely on the field, where adult insects were taken directly from trees undergoing colonization by *D. rhizophagus*, this implies that insects were collected as they were found in the trees, in such a manner that a sequence of the natural colonization of the tree was obtained, first single females, then females with males and then just females. These sets of insects conformed the treatments for this bioassay and they were: T1) females boring into the bark, T2) female and male together in the gallery without eggs laid, T3) female and male together with laid eggs and T7) females alone, with eggs and well developed galleries. Each treatment had 4-16 repetitions.

Volatiles extraction from each individual insect was done in conical vials containing ≈ 0.3 mg of clean Super Q adsorbent (80-100 mesh; Alltech, Deerfield, IL) following the procedure described by Sullivan (2005). In addition, the hindgut of each insect was dissected and immediately put into the conical vials with 50 μL of hexane spiked with 3.5 ng/μL of cycloheptanone, then the hindgut was macerated against the inside vial wall with the tip of the forceps, stored during 15 min at room temperature in a sealed. The extract was removed, and the vial contents were further rinsed with 50 μL of non spiked hexane that was subsequently combined with the original extract.

Three microliters of each extract were analyzed splitless by GC-MS with a non-chiral phase column (same used in the GC-EAD), the oven temperature was 40°C for 1 min, 16°C/min to 80°C, then 5°C/min to 240°C for a final 8 min. The compounds were quantified using response curve calculated from analyses of a dilution sequence of

known quantities of synthetic standards. The negative control extracts were those coming from vials without beetles.

Field Trials. Two trapping field experiments were performed to evaluate behavioral responses of *D. rhizophagus* to antennally active compounds: three from the host resin and six from the insect mid/hindgut. The experiments were conducted from June 26 to August 31, 2010, in a natural regeneration stand of *P. arizonica* at San Juanito, Bocoyna Municipality, Chihuahua. Both experiments followed a complete random block design. First experiment compared five treatments with six replicates each: T1) unbaited control, T2) (+)-α-pinene, T3) (-)-β-pinene, T4) (+)-3-carene and T5) a 1:1:1 mixture of treatments 2 to 4. The second experiment tested non-oxygenated (α-pinene, β-pinene and 3-carene) and oxygenated terpenes (myrtenal, myrtenol, *trans*-verbenol, *cis*-verbenol, verbenone, and fenchyl alcohol) conforming ten treatments with ten replications each: T1) unbaited control, T2) 1:1:1 mixture of (+) α-pinene, (-) β-pinene and (+) 3-carene, T3) T2 plus (-)-myrtenal, T4) T2 plus (-)-*trans*-verbenol, T5) T2 plus (-)-verbenone, T6) T2 plus (-)-myrtenol, T7) T2 plus (-)-*cis*-verbenol, T8) T2 plus (+)-fenchyl alcohol, T9) T2 plus all the oxygenated terpenes and T10) T2 plus all the oxygenated terpenes except (-) verbenone. Baits with non-oxygenated terpenes had a release rate of 100-1000 mg/d (Chemtica International S.A., San Jose, Costa Rica), while those with the oxygenated terpenes had a release rate of 1-10 mg/d (Chemtica International S.A.).

The traps used in this study were modified multiple-funnel traps attending some biological and ecological features of *D. rhizophagus*. Each trap was constituted by black six-unit multiple-funnels, and each funnel had a 10 (top) and 6 (bottom) cm in diameter and a length of 6.5 cm. The total length of the entire trap was ≈60 cm. The last funnel was placed at 10 cm from the collector cup which was buried in the ground. Traps were

suspended from a wooden stick and positioned >1 m from the closest young pine, and placed at a distance of > 5 m between treatment and at >10 m among line traps. Trap collection cups were filled with 2-isopropoxitenil metilcarbamate to prevent trapped insects from escaping and to preserve them. The baits were hung from the top and within the upper funnel to protect them from rain. Trapped *D. rhizophagus* individuals and Cleridae predators were collected, counted, and preserved in 70% ethanol. Only the bark beetles were sexed.

Statistical Analysis. Quantities of compounds collected from individual beetles were analyzed by two-way Kruskal-Wallis test, tacking as variation factors treatment and sex. Pairwise comparisons were carried out by Mann-Whitney test using Bonferroni correction (α= 0.05) (ZAR 1996). Compounds without values were eliminated from the analysis.

For both field experiments, the data were subjected to the Shapiro-Wilk (1965) test to assess the normal distribution assumption of the data. Raw trap catch numbers were normalized by log (X+1) and analyzed using one-way analysis of variance (ANOVA) (PAST v 1.95). Unbaited traps were omitted from statistical analyses. All pairwise comparisons of treatments were performed with a Tukey´s test (α= 0.05). Sex ratios for each treatment were analyzed using chi-square (χ^2) (α= 0.05).

Results

Electrophysiological Assays. The antenna of recently emerged females and males of *D. rhizophagus* consistently responded to α-pinene, β-pinene and 3-carene (Fig. 1) of the constitutive resin of *P. arizonica,* and to fenchyl alcohol, myrtenal, *cis*-verbenol, *trans*-verbenol, verbenone and myrtenol (Fig. 2) of mid and hindguts from *D. rhizophagus* females and males. GC-EAD tests with commercial standards of all these compounds confirmed their electrophysiological activity on both sexes.

Volatiles Analyses of Individual Beetles at different colonization phases. All the six antenally active oxygenated terpenes (myrtenal, myrtenol, *trans*-verbenol, *cis*-verbenol, verbenone, and fenchyl alcohol) were identified from volatiles of mid and hindgut of *D. rhizophagus*. *Trans*-pinocarveol, α-terpineol, *trans*-myrtanol, *cis*-myrtanol and 2-phenylethanol were also found in the mid and hindgut.

In Bioassay B1, pre-emerged unfed females and males (B1, T1) as well as paired insects (male and female) in a pine bolt for 48 h (B1, T3) showed a low or null production of the six oxygenated terpenes (Fig. 3). An increase in the production of those oxygenated terpenes (except for fenchyl alcohol) was recorded from males and females, separately feeding for 24 h (B1, T2), especially myrtenol, *trans*-verbenol and *cis*-verbenol, (Fig. 3). Fenchyl alcohol was only detected in unfed females (B1, T1). Two-way Kruskal-Wallis test on the different treatments revealed differences among them ($P \leq 0.05$), but these differences were only significant statistically in B1, T2 (α =0.05; Mann-Whitney test) for all compounds, except for fenchyl alcohol where B1, T1 was statistically significant (α =0.05; Mann-Whitney test). The Kruskal-Wallis test also showed differences between sexes ($P \leq 0.05$), but only for fenchyl alcohol.

In Bioassay B2, only five oxygenated terpenes were detected (fenchyl alcohol was not shown) in all the treatments (B2, 1-3) (Fig. 4) and their quantities, in compare with treatments in Bioassay B1, were higher. The larger concentrations of these compounds were observed in paired insects initiating colonization (B2, T2); being myrtenol and *trans*-verbenol the most abundant (Fig. 4). Two-way Kruskal-Wallis test revealed differences among treatments ($P \leq 0.05$), these differences were statistically significant for all the compounds in B2, T3 (α =0.05; Mann-Whitney test), with the exception of *trans*-verbenol that was not found in any treatment. No significant differences between sexes were found by Kruskal-Wallis test ($P \geq 0.05$).

Field Trials. In Experiment 1 (monoterpenes), both sexes of *D. rhizophagus* were trapped in all treatments (Fig. 5). Chi-square test showed significant differences in attraction by sex attraction (α=0.05). The one-way ANOVA test detected significant variation among treatments ($P \leq 0.05$). The (+)-3-carene was the most attractive terpene for both males and females (α=0.05; Tukey´s test), followed by the 1:1:1 mixture of (+)-α-pinene, (-)-β-pinene and (+) 3-carene that captured more males than females, and by the (+)-α-pinene and (-)-β-pinene which also caught more males and females, respectively.

In Experiment 2 (oxygenated terpenes), the Chi-square test showed significant differences between sexes (α=0.05). In all treatments more males than females were captured (Fig. 6). The one-way ANOVA test detected statistically variation among treatments ($P \leq 0.05$). Treatments T2 (1:1:1 mixture of (+) α-pinene, (-) β-pinene and (+) 3-carene), T3 (T2 plus (-) myrtenal) and T4 (T2 plus (-) *trans*-verbenol), were the most attractive for *D. rhizophagus* and only T4 showed significant differences (α=0.05; Tukey´s test). The treatments that captured the lowest number of insects were T5 (T2 plus (-) verbenone), T6 (T2 plus (-) myrtenol), T7 (T2 plus (-) *cis*-verbenol), T8 (T2 plus (+) fenchyl alcohol), T9 (T2 plus all the oxygenated terpenes) and T10 (T2 plus all the oxygenated terpenes except (-) verbenone), and only T5 and T9 showed significant differences (α=0.05; Tukey´s test).

Clerid beetles were caught in all the treatments of both field experiments including the control (Fig. 7). The one-way ANOVA tests for the monoterpenes and oxygenated terpenes showed significant differences among their treatments ($P \leq 0.05$). For the field experiment with monoterpenes the (+) 3-carene showed significant differences (α =0.05; Tukey´s test) (Fig. 7A). While for the experiment with oxygenated terpenes the

T3 (T2 plus (-) myrtenal) and T4 (T2 plus (-) *trans*-verbenol) (Fig. 7B) were statistically different (α =0.05; Tukey´s test) from the other treatments

Discussion

In a most apparent pattern of evolution in host use, *D. rhizophagus* a primary and specialist species, has a phyllogenetic relationship with two secondary and generalist species of bark beetles, *D. valens* and black turpentine beetle *D. terebrans* (Olivier) (Kelley and Farrell 1998). Nevertheless, these species present different behavioral elements; *D. rhizophagus* is endemic of the northwestern Mexico, while *D. terebrans* is found throughout the eastern United States and *D. valens* with a wide geographic range which overlaps both species. *D. rhizophagus* select young pine and seedling of at least 11 pine species, while the black turpentine beetle and red turpentine beetle colonize mature pines of >7 and >40 species respectively.

Our results demonstrate olfactory antennal sensitivity from both sexes of *D. rhizophagus*, towards (+)-α-pinene, (-)-β-pinene and (+)-3-carene, monoterpenes that are present in the resin of its host, *P. arizonica*. Compared to the other two active monoterpenes, the 3-carene was the one attracting the larger number of both sexes in the field. This compound elicit responses from antennae of various species of *Dendroctonus* (Seybold et al., 2006) and it is an important attractant for *D. valens* endemic populations in North America, (Hobson et al., 1993, White and Hobson 1993, Erbilgin et al., 2007) and for introduced populations of *D. valens* in China, (Sun et al., 2004, Erbilgin et al., 2007, Zhang et al., 2009).

In the field (+)-α-pinene caught more *D. rhizophagus* males than females and (-)-β-pinene more females. These two compounds caused low attraction to *D. valens*, however no reports of sex differential response are described (Erbilgin et al., 2007). In comparison, *D. terebrans* is antennally sensitive to α-pinene and β-pinene (Delorme

and Payne 1990) and in the field it is attracted to ethanol and (-)-α-pinene (Phillips et al. 1988, Miller and Rabaglia 2009).

The 1:1:1 monoterpenes mixture was the second most attractive bait for *D. rhizophagus*, catching more males than females. This combination, in different concentrations, is a commercial lure for *D. valens* (Phero Tech, Dalta, British), but no displayed sex differential attraction was displayed for this species (Sun et al., 2004, Erbilgin et al., 2007, Zhang et al., 2009).

The antennal activity elicited in both sexes of *D. rhizophagus* by c*is*-verbenol, *trans*-verbenol, verbenone, myrtenal and myrtenol and the higher concentration of these terpenoids observed in the mid and hindgut (fenchyl alcohol only in traces) suggest an important role of these compounds in the chemical ecology of *D. rhizophagus*. Our field tests would certainly validate this hypothesis, since all the oxygenated terpenes were attractive for individuals of *D. rhizophagus*. Electrophysiological response of the same terpenoids and their detection in hindgut were found for *D. valens* (Zhang and Sun 2006) and *D. terebrans* (Phillips et al., 1989) with the exception of fenchyl alcohol. *Trans*-pinocarveol, frontalin and *exo*-brevicomin were identified in hindguts of insects making galleries (Phillips et al., 1989). However the field tests with these compounds indicated no pheromonal activity for *D. valens* and *D. terebrans* (Phillips et al., 1989, Zhang and Sun 2006).

Oxygenated terpenes quantification in *D. rhizophagus* individuals taken directly from field infested trees, imply a close relationship between the production of these compounds and the progress of the different stages of tree colonization. There is an increase in oxygenated terpenes production among females during early colonization phase and in females paring with males and building galleries, which suggests that this increase might be a way of attracting and retaining males. This in turn, is supported in

part by the individual and collective attraction to these oxygenated terpenes in the field bioassays. The decrease in the production of these compounds observed in females after they have laid eggs apparently motivates males to leave the gallery. There were no statistical differences in the production of these oxygenated terpenes between males and females. However in the males, the production was low, and an increased in those individuals paired with females and where eggs were laid.

The production of these terpenes in males and females of *D. rhizophagus* under laboratory conditions was different from those observed in field bioassays. Males and females separately fed under laboratory conditions produced myrtenol (85.84±1.94 ng) and *trans*-verbenol (34.64±7.69 ng), in higher amounts than any other oxygenated terpene. *D. valens* females under similar conditions produced almost three times more of myrtenol 209.84 ng and *trans*-verbenol 110.29 ng (Shi & Sun 2010) than what we observed in *D. rhizophagus*. Furthermore, the production of these same terpenes in females and males of *D. terebrans* in the galleries within trees, accounted 8738.2 ng of myrtenol and 15,532 ng of *trans*-verbenol (Phillips et al., 1989), which are 30 times what it was found for *D. rhizophagus*.

Following the idea that in aggressive species the secondary attraction is carried out by aggregative and antiaggregative pheromones released by females and males respectively (Wood 1982b). Shi & Sun (2010) interpreted the high production of myrtenol and *trans*-verbenol in males and females of *D. valens*, a different way to attract conspecifics and to carry out massive tree colonization. The atypical behavior of *D. rhizophagus* makes it difficult to accept this hypothesis, given that no massive attack occurs; however both species produce high concentrations of myrtenol and *trans*-verbenol, suggesting that possibly these monoterpenes work synergistically to attract males.

However, there might be an antiaggregative effect for *D. rhizophagus*. Once the male is installed in the gallery along with the female, there is a high production of these oxygenated terpenes in both sexes and this may work as an antiaggregative mixture for other conspecifics. Results from our field tests partially support this possibility, because individual and combined oxygenated terpenes (except where the verbenone was present) attracted more males than females, and the *trans*-verbenol produced the best response. Further studies should confirm the validity of this hypothesis.

Phillips et al. (1989) proposed a similar hypothesis for *D. terebrans* where their field test suggested that males were attracted to female producing frontalin, and this response can be interrupted by the *exo*-brevicomin released by males that arrived earlier. Due to the significant responses only by males to frontalin and *exo*-brevicomin, the authors suggested to refer to these compounds as sex pheromones rather than as aggregation or antiaggregation pheromones.

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FIGURES LEGENDS

Fig. 1. Electrofisiological responses (GC-EAD) of antennae from emerged female (A) and male (B) of *D. rhizophagus* to compounds from *P. arizonica* resin. Antennal traces represent the combined responses from 8 individual insects (single EAD traces were digitized in a spreadsheet to produce a composite race). Compounds eliciting consistent antennal voltage spikes were α-pinene (1), β-pinene (2) and 3-carene (3).

Fig. 2. Electrofisiological responses of *D. rhizophagus* antennae to compounds in mid and hindgut extracts of 38 conspecifics emerged female (A) or male (B) as measured by GC-EAD. Antennal traces represent the combined responses from 8-10 individual insects (single EAD traces were digitized in a spreadsheet to produce a composite race). Compounds eliciting consistent antennal voltage spikes were fenchyl alcohol (1), myrtenal (2), *cis*-verbenol (3), *trans*-verbenol (4), verbenone (5) and myrtenol (6).

Fig. 3. Bioassay B1, Mean ± SEM quantities of compounds isolated from pre-emerged female and male *D. rhizophagus* (n = 10 and 15). Mean associated with different letter were significantly different (α =0.05; Mann-Whitney test). T1 = separated unfed females and males; T2 = males and females feeding separately for 24 h; T3 = paired male and female feeding for 48 h.

Fig. 4. Bioassay B2, Mean ± SEM quantities of compounds isolated from emerged female and male *D. rhizophagus* (n = 4 to16). Mean associated with different letter were significantly different (α =0.05; Mann-Whitney test). T1 = females boring into bark; T2 = female and male in a gallery; T3 = female and male in gallery with layed eggs; T4 = female alone with laid eggs and well developed galleries.

Fig. 5. Total number of caught *D. rhizophagus* insects ($\overline{X}$ ± SE) in funnel traps baited with the three antenally active monoterpenes and a mixture 1:1:1 of α-pinene, β-pinene,

3-carene. Treatments associated with different letter were significantly different (α = 0.05; Tukey's test). Catches in unbaited control traps were excluded from statistical analyses. Tests were replicated six times.

Fig. 6. Total number of caught *D. rhizophagus* insects ($\overline{X}$ ± SE) in funnel traps baited with a tertiary mixture 1:1:1 monoterpenes with six individual oxygenated monoterpene and a combination of the tertiary mixture and all six components. Treatments associated with different letter were significantly different (α = 0.05; Tukey's test). T1) an unbaited control, T2) 1:1:1 mixture of (+) α-pinene, (-) β-pinene and (+) 3-carene, T3) T2 plus (-) myrtenal, T4) T2 plus (-) *trans*-verbenol, T5) T2 plus (-) verbenone, T6) T2 plus (-) myrtenol, T7) T2 plus (-) *cis*-verbenol, T8) T2 plus (+) fenchyl alcohol, T9) T2 plus all the oxygenated terpenes and T10) T2 plus all the oxygenated terpenes except (-) verbenone. Catches in unbaited control traps were excluded from statistical analyses. Tests were replicated ten times.

Fig. 7. Total number of clerid beetles caught ($\overline{X}$ ± SE) in funnel traps baited with, monoterpenes (A) and oxygenated terpenes (B). Treatments associated with different letter were significantly different (α = 0.05; Tukey's test). T1) an unbaited control, T2) 1:1:1 mixture of (+) α-pinene, (-) β-pinene and (+) 3-carene, T3) T2 plus (-) myrtenal, T4) T2 plus (-) *trans*-verbenol, T5) T2 plus (-) verbenone, T6) T2 plus (-) myrtenol, T7) T2 plus (-) *cis*-verbenol, T8) T2 plus (+) fenchyl alcohol, T9) T2 plus all the oxygenated terpenes and T10) T2 plus all the oxygenated terpenes except (-) verbenone. Catches in unbaited control traps were excluded from statistical analyses. Tests were replicated six times for monoterpenes and ten times for oxygenated terpenes.

Fig. 1.

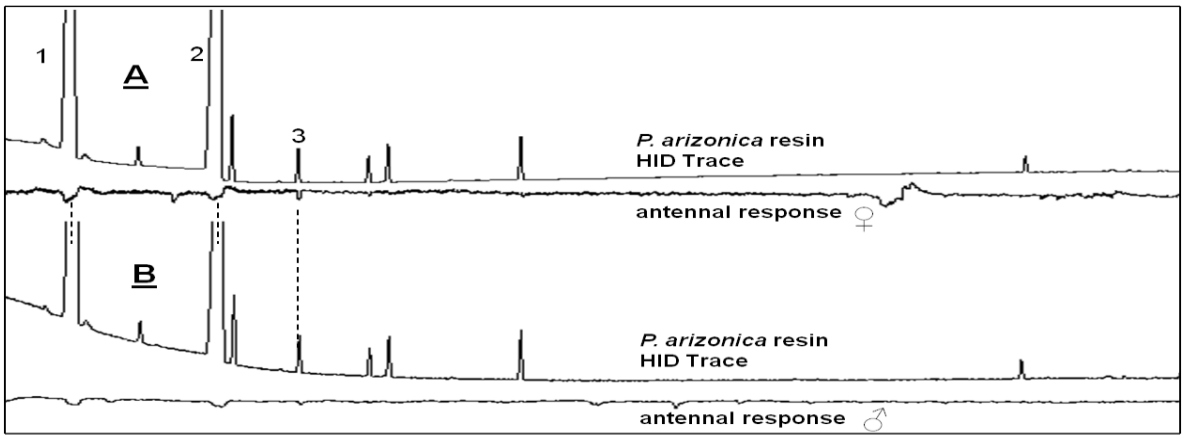

A
1
2
3
P. arizonica resin
HID Trace
antennal response ♀
B
P. arizonica resin
HID Trace
antennal response ♂

Fig. 2.

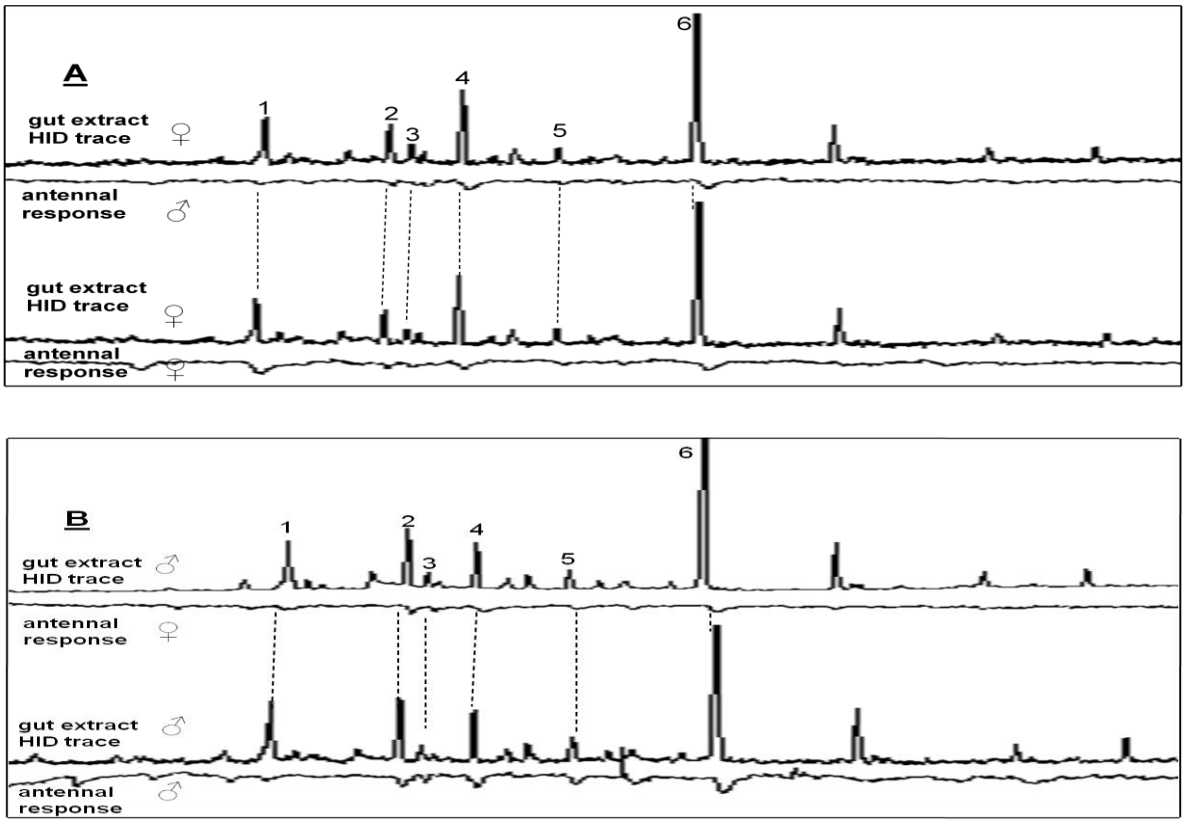

A
gut extract
HID trace ♀
1
2
3
4
5
6
antennal
response ♂
gut extract
HID trace ♀
antennal
response ♀
B
gut extract
HID trace ♂
1
2
3
4
5
6
antennal
response ♀
gut extract
HID trace ♂
antennal
response ♂

Fig. 3.

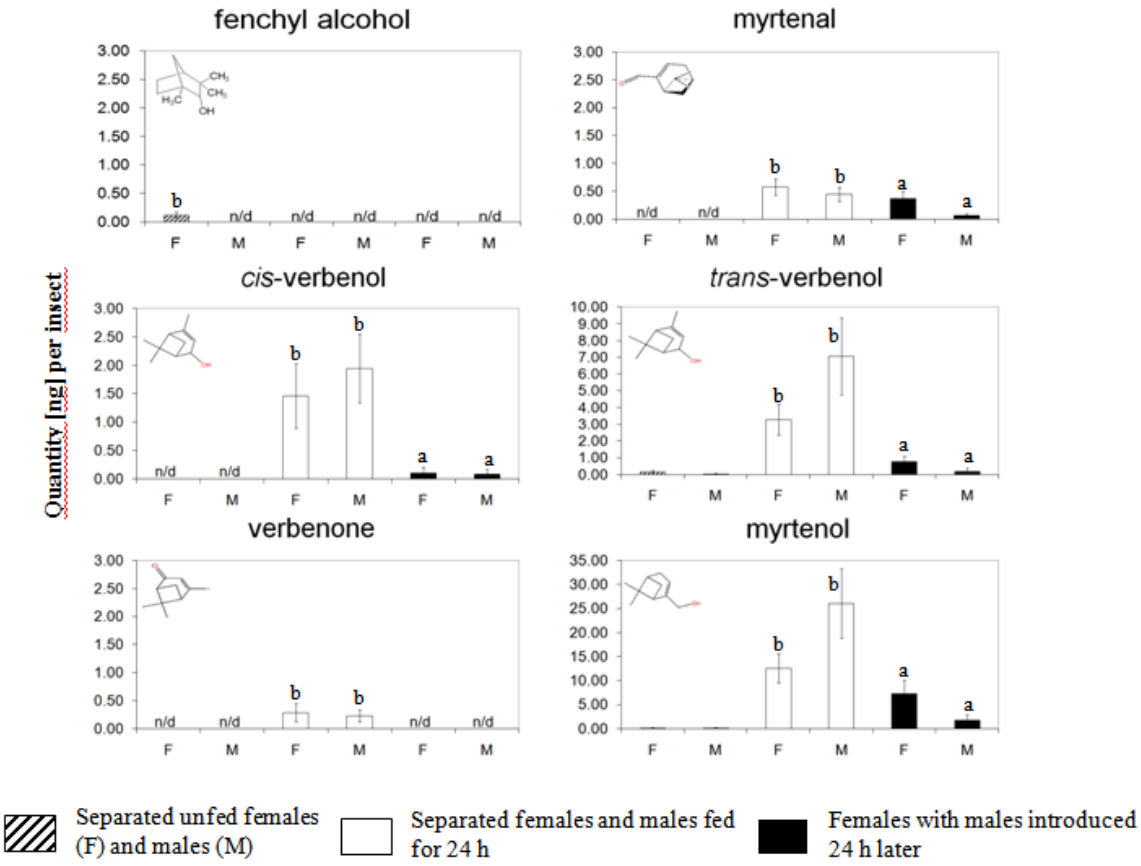

fenchyl alcohol
myrtenal
cis-verbenol
trans-verbenol
verbenone
myrtenol
Quantity [ng] per insect
F
M
n/d
a
b
Separated unfed females (F) and males (M)
Separated females and males fed for 24 h
Females with males introduced 24 h later

Fig. 4.

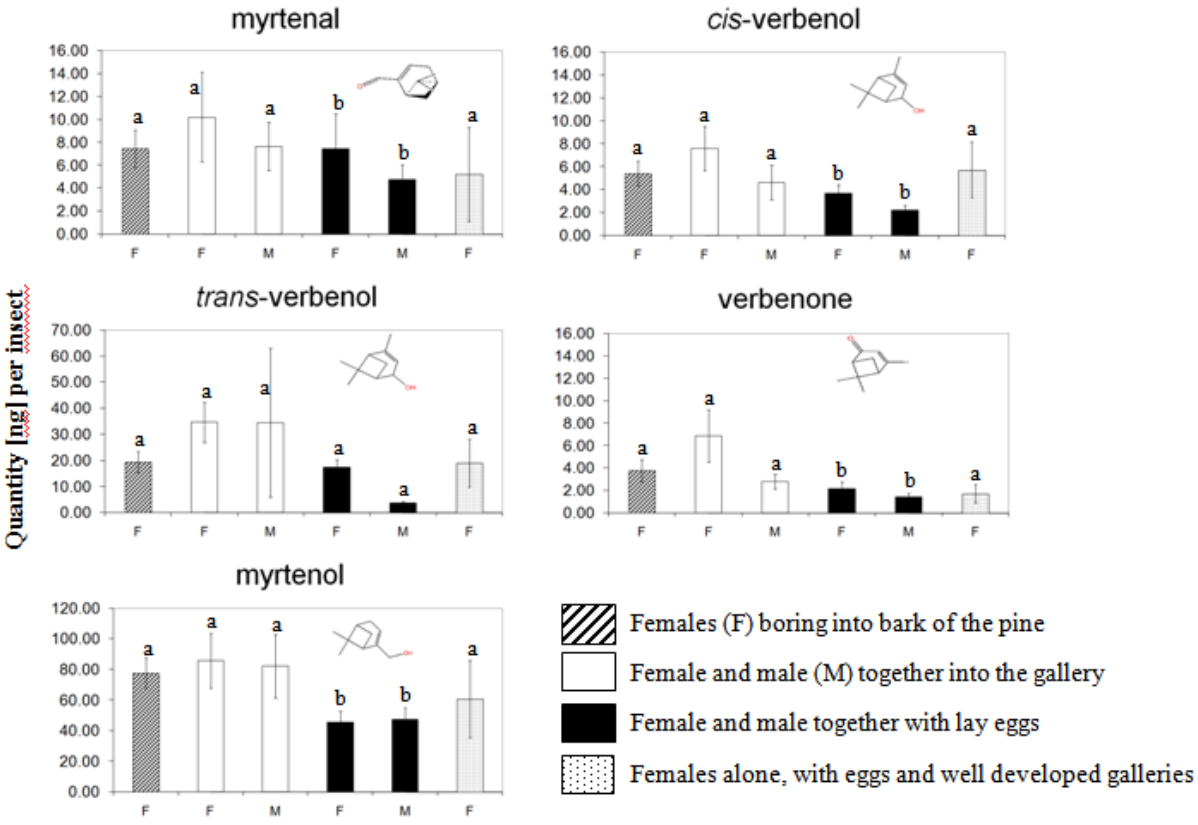

myrtenal
cis-verbenol
trans-verbenol
verbenone
myrtenol
Quantity [ng] per insect
F
M
Females (F) boring into bark of the pine
Female and male (M) together into the gallery
Female and male together with lay eggs
Females alone, with eggs and well developed galleries

Fig. 5.

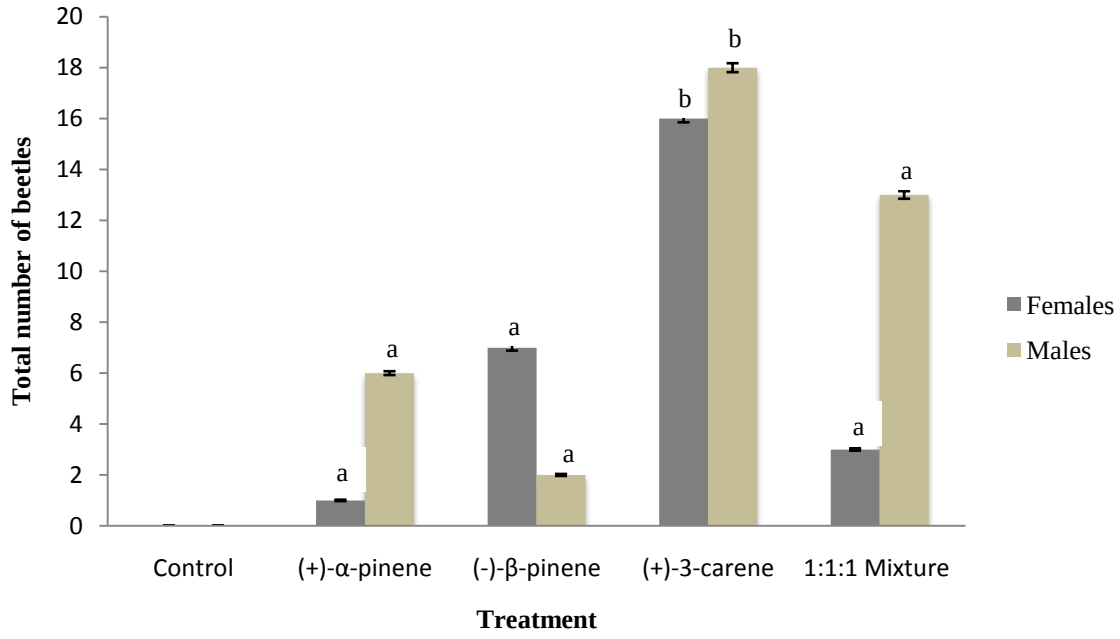

Total number of beetles
20
18
16
14
12
10
8
6
4
2
0
a
a
a
a
b
b
a
a
Control
(+)-α-pinene
(-)-β-pinene
(+)-3-carene
1:1:1 Mixture
Treatment
Females
Males

Fig. 6.

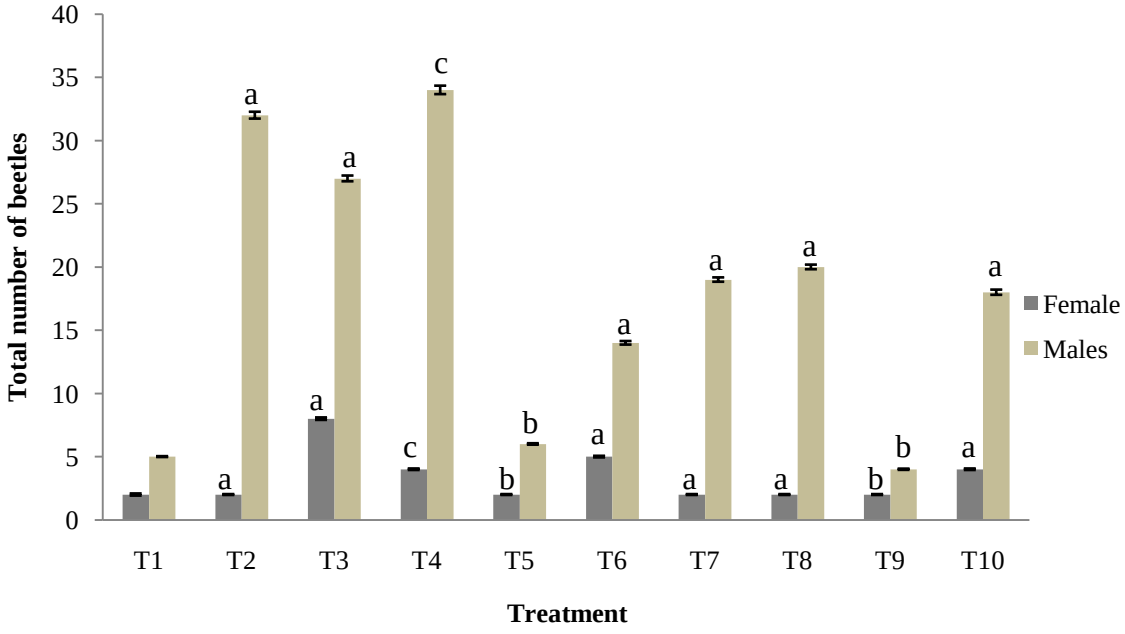
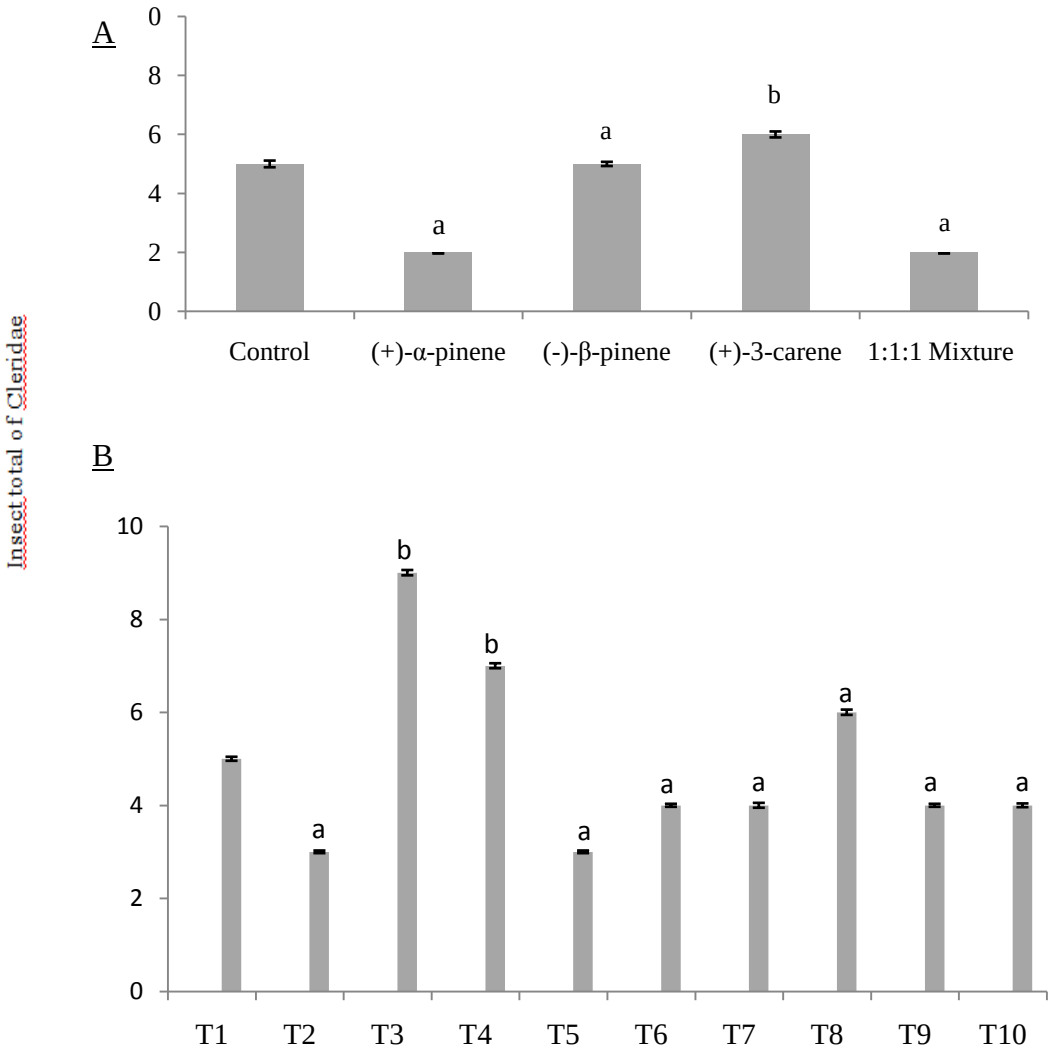

Total number of beetles
40
35
30
25
20
15
10
5
0
a
a
a
a
c
c
b
b
a
a
a
a
a
a
b
b
a
a
T1
T2
T3
T4
T5
T6
T7
T8
T9
T10
Treatment
Female
Males

Fig.7.


A
0
8
6
4
2
0
a
a
b
a
Control
(+)-α-pinene
(-)-β-pinene
(+)-3-carene
1:1:1 Mixture
B
Insect total of Cleridae
10
8
6
4
2
0
a
b
b
a
a
a
a
a
a
T1
T2
T3
T4
T5
T6
T7
T8
T9
T10

CAPÍTULO II

Aislamiento y expresión de los genes citocromo P450 en la antena y canal alimentario del escarabajo del pino *Dendroctonus rhizophagus* (Curculionidae: Scolytinae) después de su exposición a los monoterpenos del hospedero

Manuscrito enviado a la revista *Insect Molecular Ecology*

Isolation and expression of cytochrome P450 genes in the antennae and gut of pine beetle *Dendroctonus rhizophagus* (Curculionidae: Scolytinae) following exposure to host monoterpenes

C. Cano-Ramírez[1], A. K. Cesar-Ayala[1], V. Pineda-Martínez[1], B. T. Sullivan[2], and G. Zúñiga[1]

[1]Departamento de Zoología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional.

[2]USDA-Forest Service Southern Research Station.

[1]Prolongación de Carpio y Plan de Ayala. Col. Sto. Tomas, México D. F. CP 11340, México. Telephone number 01 (52) 55-57296000 ext. 62419, E-mail: capotezu@hotmail.com.

[2]2500 Shreveport Highway, Pineville LA USA71360

Abstract

Bark beetles oxidize the defensive monoterpenes of their host trees both to detoxify them and convert them into components of their pheromone system. This oxidation is catalyzed by cytochrome P450 enzymes and occurs in different tissues of the insect, including the gut (i.e., the site where the beetle's pheromones are produced and accumulated) and the antennae (i.e., the olfactory organs used for perception of airborne defensive monoterpenes as well as other host-associated compounds and pheromones). We identified ten new *CYP* genes in the pine beetle *Dendroctonus rhizophagus* in either antenna or gut tissue after stimulation with the vapours of major host monoterpenes α-pinene and β-pinene. Five genes belong to the CYP4 family, four to the CYP6 family and one to the CYP9 family. Differential expression of the *CYP* genes was observed among different sampled tissues (the fore-, mid- , and hindgut, and the antennae) as well as in response to the different stimuli. Only the genes *CYP6BW5v1* and *CYP9Z20v1* were expressed in all tissues with any of the stimuli. The expression level of *CYP4* genes was apparently reduced, whereas expression of *CYP6* and *CYP9* genes increased. Increased expression of these latter cytochrome P450 genes suggests they play a role in the detoxification of monoterpenes released by this insect's host trees.

Keywords: cytochrome P450, scolytines, monoterpenes, bark beetles, gene expression, detoxification.

Introduction

Several highly efficient enzyme systems (cytochrome P450, glutathione *S*-transferases and esterases) have evolved in herbivorous insects for metabolizing plant defensive chemicals to less toxic or more soluble compounds (Guengerich, 1990). Cytochrome P450 enzymes constitute an ancient multi-gene family that is highly variable, diverse, and widely distributed throughout the phylogenetic scale (Nelson & Strobel, 1987). Insect cytochrome P450 enzymes carry out aerobic oxidations on a diversity of organic molecules that are endogenous, exogenous, synthetic or natural. Cytochrome P450 enzymes are encoded by microsomal and mitochondrial *CYP* genes and each P450 is assigned to a family (designated by a number) and subfamily (designated by a letter) (Feyereisen, 1999).

For pine bark beetle species that infest living trees, survival and the reproductive success depends on their capacity to overcome the defense mechanisms of their hosts (Christiansen *et al*., 1987). Pines release both constitutive and induced oleoresins into the beetles' mines within the phloem tissue, and these can physically restrict beetle movement and thereby hinder colonization (Hanover, 1975). In addition, monoterpenes present in the resin, including α- pinene, β-pinene, myrcene, limonene and 3-carene, can injure or kill insects through their toxic effects (Smith, 1965). Pine bark beetles complete their life cycle entirely within the bark of host trees, and thus both adults and offspring are invariably exposed to high concentrations of these monoterpenes (Wood, 1982).

There is much direct and indirect evidence that bark beetles can oxidize a broad range of known or potentially toxic substrates with cytochrome P450 enzymes, and it is speculated that this capacity may represent an evolved response against host defensive chemicals (see Seybold *et al*., 2006 and citations therein). On the other hand, the

pheromone components of many bark beetle species are oxygenated monoterpenes possessing carbon backbones identical to monoterpenes of their host trees. Evidence suggests that P450 enzymes are involved in this pheromone synthesis through both the oxidation of host-derived monoterpene precursors and the transformation of intermediate compounds produced in endogenous metabolic routes (White *et al*., 1979; White *et al*., 1980; Vanderwell & Ochlschlager, 1987; Hunt & Smirle, 1988; Huber *et al*., 2007; Aw *et al*., 2010). Studies combining biochemical, molecular and functional genomics techniques recently demonstrated that cytochrome P450 enzymes encoded by orthologous genes *CYP9T1* and *CYP9T2* in the midgut of male *I. confusus* LeConte and *I. pini* (Say) are able to oxidize myrcene into (4*R*)-(-)-ipsdienol, an aggregation pheromone component produced by males of these species (Sandstrom *et al*., 2006, 2008). These results confirmed that these enzymes have a biochemical function associated with the *de novo* biosynthesis of monoterpenoid aggregation pheromone components within the midgut and possibly the fat body of bark beetles (Blomquist *et al*., 2010).

Dendroctonus rhizophagus Thomas & Bright is endemic to the northern portion of the Sierra Madre Occidental in northwestern Mexico (Salinas-Moreno *et al*., 2004), where it colonizes exclusively seedlings and young saplings (height < 3 m) of 11 pine species. This aggressive bark beetle infests both commercial plantations and areas of natural regeneration and has one generation per year. Typically, individual pine tree hosts are killed and colonized by merely one or a few beetle pairs, thus this species lacks the communal mass-attack behavior typical of this genus (Thomas & Bright 1970; Sánchez-Martínez & Wagner 2009). Electrophysiological assays have shown that the antennae of *D. rhizophagus* possess a high degree of olfactory sensitivity to host monoterpenes α-pinene, β-pinene and 3-carene (C. Cano-Ramírez, unpublished data). In addition,

oxygenated monoterpenes including *cis*-verbenol, *trans*-verbenol, verbenone, myrtenal and myrtenol have been identified in the midgut and hindgut of this species (C. Cano-Ramírez, unpublished data), and some or all of these compounds may function as pheromone components for this species much as they do in other members of this genus (Borden, 1982).

The purpose of our study was to identify and characterize the expression profile of cytochrome P450 genes in unfed *D. rhizophagus* individuals exposed to racemic α-pinene, (*R*)- (+)-α-pinene, (*S*)- (-)-α-pinene, and (*S*)- (-)-β-pinene. These compounds were chosen because they are the most abundant monoterpenes in the oleoresin of this beetle's principal hosts, *Pinus arizonica* Engelm. and *P. engelmannii* Carr., (Smith, 2000).

We investigated CYP expression specifically in the antennae because studies of other insect systems indicate that antennal P450s function as odorant degrading enzymes (ODEs) for pheromones (Wojtasek & Leal, 1999; Maïbèche-Coisne *et al*., 2004a) and host-plant volatiles (Dickens *et al*., 1992); these P450s presumably assure signal deactivation by eliminating odorants from the olfactory sensilla following stimulation. Bark beetles depend on their antennae to perceive a diversity of airborne chemicals that mediate host location and discrimination, mate selection and courtship, attack density regulation, and avoidance of interspecific competition (Byers, 1995). Hence antennal P450 enzymes may be necessary for bark beetles to perceive and respond appropriately to their olfactory environment. Further, we studied the gut because its tissues are involved in the detoxification processes of many insects (Kasai *et al*., 2000; Tartar *et al*., 2009), and the biosynthesis of pheromones in both *Ips* and *Dendroctonus* bark beetles has been demonstrated to occur in the anterior midgut (see Blomquist *et al*., 2010 and citations therein).

Results

Identification of cytochrome P450 genes

BLAST search analyses indicated that the *CYP* genes expressed in the antennae and gut regions of *D. rhizophagus* resemble members of the CYP4 (clade 4), CYP6, and CYP9 (clade 3) families in other insects (Supplementary Material, Table 1). Genes from the CYP4 family shared the highest amino acid identity with variants from the beetles *Ips paraconfusus* and *Tribolium castaneum* (Herbst); genes from the CYP6 family with variants from *Anopheles minimus* Theobald, *Hodotermopsis sjoestedti* Holmgren, *Leptinotarsa decemlineata* (Say) and *T. castaneum*; and the single gene from the CYP9 family with variants from *T.castaneum* and the bark beetles *I. confusus, I. paraconfusus*, and *I. pini*.

Gene induction differed among the sampled gut regions and antennae and also differed among the four stimuli assayed (Table 1). For example, the genes *CYP6BW5v1* and *CYP9Z20v1* were expressed to all stimuli in all portions of the gut and in the antennae, whereas the *CYP6DG1v1* gene was induced only in the antennae, and *CYP4G55v1* and *CYP4G56v1* were induced only in the midgut. The midgut region was the site where the greatest diversity of CYP genes was induced (7); followed by the hindgut (6-4), foregut (5-4), and antennae (4). The stimulus that induced the greatest diversity of genes was the racemic mixture of α-pinene (Table 1).

Ten CYP groups (five CYP4, four CYP6, and one CYP9) were found in the ML-phylogenetic analysis with putative cytochrome P450 partial gene sequences from 230 clones (Fig.1). The amino acid sequence identity (AAI) among sequences of the same group was high (> 85 %) and variable with respect to the five reported sequences (24 % <AAI < 81 %; higher match score in GeneBank, NCBI) (Supplementary material, Table 1). All groups had strong nodal support values (aLRT > 70 %), and in particular the

groups for *CYP4BQ1v1*, *CYP4BD5v1*, and *CYP4BG2v1* showed high diversity within the group, suggesting that serial duplication events had occurred (Fig. 1).

Full-length of CYP cDNA

The full-length of the cDNA genes varied from 1581 bp (*CYP4BD5v1*) to 1902 bp (*CYP9Z2Dv1*) with an ORF that varied between 1492 bp (*CYP4BQv1*) and 1689 bp (*CYP4G55v1*) (Table 2). The *CYP* genes were flanked by 5' and 3' UTRs, whose length varied from 27 to 91 bp and 42 to 241 bp, respectively. All ORFs encoded about 500 amino acids (Table 2). The complete amino acid sequences showed the typical conserved P450 domains, including the heme-binding region (FXXGXRXCXG) near the C-terminal end, the PERF domain (PXRX) and the K-helix (EXXR) (Figs. 2-4). The predicted molecular mass varied from 57 (*CYP4BQ1v1*) to 64 kDa (*CYP4G55v1*), and the isoelectric point varied from 6.75 (*CYP4G56v1*) to 9.2 (*CYP6DG1v1*) (Table 2). The alignment and comparison of the deduced amino acid sequences of *D. rhizophagus* with respect to the *CYP3A4* sequence from mammal, the *CYP4G27* sequence from *I. paraconfusus*, and the *CYP6BK17* and *CYP9Z4* sequences from *T. castaneum* allowed the identification of six putative substrate recognition sites (SRSs), which are located in regions with variable structural elements. Based on the *CYP3A4* secondary structure, 18-α helices and 9-β sheets were recognized (Figs. 2-4). With the exception of the P450 enzyme encoded by the *CYP4G55v1* gene, the predicted sub-cellular location of the P450 proteins showed that they putatively code for a typical microsomal signal peptide of approximately twenty hydrophobic residues that are likely membrane anchors in the endoplasmic reticulum. In contrast, the predicted sub-cellular location for the P450 enzyme encoded by the *CYP4G55v1* gene suggests a cytoplasmic location (Table 2). The amino acid sequence identities varied from 39.3% (*CYP4BD5v1*) to 79.7% (*CYP4G55v1*) with respect to the cytochrome P450 enzymes from *I. paraconfusus*, *T.*

castaneum, *L. decemlineata* and *Nasonia vitripennis* (Walker) (Supplementary Material, Table 2).

Semiquantitative expression of CYPs cDNAs

The expression levels of the *CYP4* genes in stimulated insects were generally lower than they were in non-stimulated insects for all analysed regions and compounds (Fig. 5). In contrast, the expression levels of the *CYP6* and *CYP9* genes were slightly higher with respect to the basal expression in non-stimulated insects (Figs. 6-7). In particular, the *CYP6* genes showed an increased expression in all sampled tissues 24 h after exposure to any of the stimuli. Likewise, the expression of the *CYP9* gene was increased in the midgut 24 h after exposure to (*S*)-(-)-β-pinene and (*S*)-(-)-α-pinene.

Discussion

In this study, we report ten new bark beetle cythochrome P450 cDNAs belonging to the families CYP4, CYP6 and CYP9, and we describe the first cloning and identification of *CYP* genes from antennae of a species of *Dendroctonus* (Table 1). The *CYP4* genes we isolated from *D. rhizophagus* exhibited amino acid identities of > 50% with four of 12 *CYP4* genes identified in a previous study of the bark beetle *Ips paraconfusus* (Huber *et al*., 2007), whereas *CYP6* and *CYP9* genes exhibited identities of >40% with CYPs from other species (Supplementary Material, Table 2). The difference in the number of genes recognized between both bark beetle studies is likely explained by the fact that Huber *et al*. (2007) analyzed whole insects rather than specific tissues; thus their samples included additional anatomical regions (such as the fat body and Malpighian tubules) where *CYP* genes have been reported previously. Furthermore, the insects had been feeding on the phloem of ponderosa pine, *Pinus ponderosa* Lanier and thus had presumably been exposed to a greater number of constituent host compounds than in the present study.

Another difference between the studies was the type of *CYP* genes identified. Twelve genes from the CYP4 family and one of the CYP9 family were reported in *I. paraconfusus*, whereas in *D. rhizophagus* we isolated five genes from CYP4, four from CYP6, and one from the CYP9 families. A recent study of functional genomics of the midgut and fat body of the mountain pine beetle *D. ponderosae* Hopkins identified representatives of tentative genes for *CYP4* and *CYP6* – but not *CYP9* – families in expressed sequence tags (ESTs) after insects were stimulated with juvenile hormone (Aw *et al*., 2010).

The identification of the genes *CYP4BD5v1*, *CYP6DJ1v1*, *CYP6DJ2v1*, *CYP6BW5v1*, *CYP6DG1v1*, and *CYP9Z20v1* in *D. rhizophagus* antennae suggests that cytochrome P450 enzymes might be induced by monoterpenes from the moment of host perception, regardless of whether these compounds, alone or blended, have an effect of attraction or repulsion on the beetles. In other insects, such as the fruit fly *Drosophila melanogaster* Meigen, the pale brown chafer *Phyllopertha diversa* Waterhouse, the tobacco hornworm *Manduca sexta* L, and the cabbage armyworm *Mamestra brassicae* L, the expression of genes in the antennae from the *CYP4*, *CYP6* and *CYP9* families has been associated with exposure to xenobiotics and odor molecules, and these CYPs have been proposed to play a role in the deactivation of pheromone molecules in the antennae (Wang *et al*., 1999; Wojtasek & Leal 1999; Maïbèche-Coisne *et al*., 2002, 2004a, 2005).

Because the different *CYP* genes appeared to be induced differentially in the presence of the alternative monoterpenes and their enantiomers (Table 1), our results suggest that P450 enzymes in antennae and gut of *D. rhizophagus* perform different functions depending on the identity and chirality of encountered monoterpenes. Presumably, this function is the oxidation of the specific monoterpene or enantiomer, as studies have demonstrated that bark beetle P450 enzymes can hydroxylyze monoterpenes. For

example, Sandstrom *et al*. (2006) showed that the P450 enzyme encoded by the *CYP9T2* gene and its orthologous *CYP9T1* gene in the midgut of *I. pini* and *I. confusus*, respectively, hydroxylate myrcene to the aggregation pheromone ipsdienol. Likewise, Aw *et al*. (2010) suggest that a putative cytochrome P450 (*CYP6CR1*) in addition to a dehydrogenase and an unknown protein could be involved in the synthesis of *exo*-brevicomin, a pheromone component produced by *D. ponderosae* males.

The theoretical analysis that was performed to infer the cellular localization of the deduced cytochrome P450 enzymes in the antennae and gut from *D. rhizophagus* suggests that all but one are anchored to the outer face of the endoplasmic reticulum. With regard to the antenna-localized cytochrome P450 enzymes, this implies that monoterpene odorants are actively transported from the sensilla lymph into support cells associated with the sensory neurons, possibly by odorant binding proteins (OBPs). This scenario has been proposed for other intracellular odor degrading enzymes (ODEs), such as GST and dehydrogenase reductase (SDR) (Wang *et al*., 1999). However, because odorants can be rapidly degraded by extracellular ODEs within the sensilla lumen such as esterases and aldehydes-oxidases (Maïbèche-Coisne *et al*., 2004b; Vogt, 2003), the role of antenna-localized, monoterpene-degrading P450 enzymes specifically on olfactory processes should be confirmed in future studies.

Because monoterpenes are volatile and, when exuded by damaged phloem, can coat the surface of mining beetles, these compounds may enter the bodies of bark beetles via the respiratory system and the cuticle as well as through the digestive tract (Prates *et al*., 1998). Thus one might expect distinct cytochrome P450 genes and expression patterns in different organs or anatomical systems of bark beetles. The induction of distinct *CYP* genes in the *D. rhizophagus* gut is consistent with this idea, but the differential induction seems to occur in response to the presence of different monoterpenes. This

differential response of the *CYP* genes explains why the midgut was the region where the highest number of genes was induced (from 7 to 3), followed by the hindgut (6-4) and foregut (5-4) (Table 1).

The large number of genes expressed in the midgut in response to monoterpenes and the high cellular diversity and secretory richness observed in this region in unfed *Dendroctonus* spp. (Díaz *et al*., 1998; Silva-Olivares *et al*., 2003) suggest that the CYP genes in this region fulfill a greater variety of functions than in the foregut and hindgut with respect to digestive processes, pheromone synthesis and the detoxification. In particular, the presence of a detoxification mechanism in the midgut is indirectly supported by the cellular damage that monoterpenes and their intermediaries produce in the midgut epithelial cells of these bark beetles (Silva-Olivares *et al*., 2003; F. López, unpublished data), the increase in the number of secretory vesicles carrying electron-dense material toward the gut lumen (Silva-Olivares *et al*., 2003; Nardi *et al*., 2002; Hall *et al*., 2002 a,b) and the differential induction of CYP genes observed in the midgut, foregut and hindgut in this study.

The expression patterns observed in the *CYP4*, *CYP6* and *CYP9* genes in the antennae and gut regions from *D. rhizophagus* suggest that each CYP family is induced in each site differently in response to the same monoterpenes (Figs. 5-7). In particular, CYP4 family genes were not induced or suppressed by any of the monoterpenes assayed, suggesting these are constitutively expressed. Expression patterns similar to those in unfed *D. rhizophagus* were observed in the majority of the *CYP4* genes that were identified in fed *I. paraconfusus* using quantitative real-time PCR (Huber *et al*., 2007). The similar expression patterns in both species suggest that the primary biochemical function of *CYP4* genes in bark beetles may not be the degradation or transformation of exogenous monoterpenes. Evidence from other insect systems suggests that *CYP4* genes

are involved in fatty acid hydroxylation (Fogleman & Danielson, 2001) and biosynthesis and metabolism of hormones (Tillman-Wall *et al*., 1992; Blomquist *et al*., 1994; Sutherland *et al*., 1998; Warren *et al*., 2002), including juvenile hormone and those related to the gonadotropic cycle (Davies *et al*., 2006).

In contrast, the increased levels of the *CYP6* and *CYP9* families' genes that occurred in response to monoterpene exposure (Figs. 6-7) suggest that these gene families play a role in the monoterpenes metabolism in antennae and gut of *D. rhizophagus*. The CYP6 family is unique to the class Insecta and its biochemical function has primarily been associated with the metabolism of plant chemicals in phytophagous insects (David *et al*., 2006); however, little is known about its function in bark beetles. Some members of this gene family have been identified in the midgut and fat body from *D. ponderosae*, and a cytochrome P450 enzyme expressed by the *CYP6BW1* gene can hydroxylate α-pinene to *trans*-verbenol, the major aggregation pheromone component of this species, (Aw *et al*., 2010). Likewise, the cytochrome P450 enzymes coded by *CYP9T1* and *CYPT92* genes from *I. confusus* and *I. pini* bark beetles can hydroxylate myrcene to ipsdienol, a component of the aggregation pheromone of these species (Sandstrom *et al*., 2006; 2008).

While there is increasing evidence that cytochrome P450 enzymes are involved in the *de novo* synthesis of hemiterpenoid and monoterpenoid pheromones via the mevalonate pathway or the cyclization of monounsaturated ketones (Seybold *et al*., 1995), our results are consistent with the hypothesis that *CYP* genes are involved in generalized metabolic processes triggered in bark beetles in response to host-produced toxins. Enhanced knowledge of the role of cytochrome P450 enzymes in the detoxification of exogenous compounds and pheromone synthesis is necessary for furthering understanding of the relationship between bark beetles and their host trees.

Experimental procedures

Insects and treatments

Pre-emergent, unfed adult *D. rhizophagus* were collected directly from naturally-infested Arizona pines (*Pinus arizonica* Engelm.) in the Predio La Laja I, San Juanito, Bocoyna Municipality, Chihuahua (27º 55' 54.9'' N and 107º 35' 54.6'' W; at 2452 m) in June 2008. The beetles were stored in plastic containers with moistened paper towel at 4°C, transported to the laboratory and starved for two weeks at 4 °C.

Four groups of 12 unfed beetles (1:1sex ratio) were placed in glass Petri dishes (100 mm x 15 mm) lined with dry filter paper and independently exposed to the vapours of racemic α-pinene (98 % chemical purity, 1:1 enantiomeric ratio), (*R*)-(+)-α-pinene (98%), (*S*)-(-)-α-pinene (98%), or (*S*)-(-)-β-pinene (99%) for 24 h at 8° C under conditions of darkness. All compounds were obtained from Sigma Aldrich, St. Louis, MO. Neat monoterpene (100 μL) was applied to the filter paper immediately prior to placement of beetles into the dish (not on the monoterpene application area), and then the dish was closed and sealed with Parafilm to minimize the diffusion of these compounds out of the dishes.

Immediately following terpene exposure, beetles (n = 48) were immersed in phosphate buffer solution (PBS) and dissected. The antennae were removed directly from the insects, and the gut was dorsally extracted after the elytra and wings were removed. The gut was separated from the fat body and the Malpighian tubules and sectioned into foregut, midgut and hindgut (after Díaz *et al*., 1998). The antennae and each gut section were separately placed into Eppendorf vials with 0.2 μL of Trizol® (Invitrogen Corp., Carlsbad, CA, USA) and macerated completely using a sterile pestle. Then each vial was topped with 1 mL Trizol and stored at -80 °C for posterior RNA extraction.

RNA isolation and cDNA synthesis

Total RNA isolation of the antennae and gut regions was conducted following the protocol provided in the Trizol® kit. The integrity of the total RNA was visually assessed on a denaturing formaldehyde 1% agarose gel that was stained with 10 μg/mL ethidium bromide (EtBr) at room temperature (Sambrook *et al;* 1989). The cDNA was synthesised from 1 μg of total RNA following the protocol provided with the M-MLV Reverse Transcriptase enzyme (Invitrogen) kit and using the Oligo (dT) 12-18 primer (GE Healthcare, Buckinghamshire, UK).

Degenerate polymerase chain reaction, cloning and sequence analyses

The synthesised cDNA from the antennae, foregut, midgut and hindgut from each monoterpene treatment was used as the template in all of the PCR. Pairs of degenerate primers (Supplementary Material, Table 3) were used to screen the putative P450 cDNA from the CYP4 (clade 4), CYP6 (clade 3) and CYP9 (clade 3) families, which are involved in detoxification processes (Feyereisen 2006). We did not look for *CYP2* genes because this clade is implicated in reactions during insect molting and metamorphosis (Namiky *et al*., 2005). The *CYP4* genes were amplified following Snyder *et al*. (1996), except for the T_m, which was 49 °C; the *CYP6* and *CYP9* genes were amplified following Dunkov *et al*. (1996). PCR was performed in a Biometra T Thermocycler (Biometra, Göttingen, Germany) using 30 μL reactions containing 2 μL cDNA, 1X Taq polymerase buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.33 μM of each primer, and 1.5 U of recombinant *Taq* polymerase (Invitrogen Life Technologies, Sao Paulo, Brazil). The reaction conditions were an initial 5-min step at 94 °C followed by 35 cycles of 60 s at 94 °C, 60 s at 50 °C and 60 s at 72 °C, and a final extension for 5 min at 72 °C. The PCR products were visualised on 1% agarose gels stained with 10 μg/mL EtBr and compared with a 100-bp DNA ladder (Gibco, BRL, Gaithersburg, MD, USA). The amplicons were then purified with the GFX PCR DNA kit (Amersham Biosciences,

UK) and/or Gel Band Purification kit (GE Healthcare), and each reaction product was cloned using the pDrive Cloning® Kit (Qiagen, Valencia, CA, USA).

The cloning reactions were transformed in chemically competent DH5α cells of *Escherichia coli* (Sambrook *et al*., 1989). The transformants (blue-white colonies) were selected on kanamycin/LB/X-gal/IPTG plates. Ten colonies of each treatment for each gene (n_{total} = 320) were grown overnight in kanamycin/LB broth and the plasmid DNA was extracted by the alkaline lysis method (Sambrook *et al.*, 1989). The insertion was verified by a simple restriction with the *Eco*R1 enzyme (Invitrogen) and visualized on 1 % agarose gels. The recombinant plasmids were purified for sequencing using the QIAquick® PCR Purification Kit (Qiagen). A total of 230 clones with the inserts were sequenced using a CEQ 8000 Genetic Analysis System and Quick Start Master Mix® 2X (Beckman Coulter, Fullerton, CA).

Phylogenetic analysis and identification of CYP variants

Fragments of ≈ 400 to 500 bp were subjected to Blastx searches against the NCBI database. Putative P450 gene sequences were manually edited with SEAVIEW v 4.2.5 (Gouy *et al*., 2010) to achieve positional homology with the variants that matched. The deduced amino acid sequences were obtained with the ExPasy Translate Tool (http://www.expasy.org/tools/dna.html) and were then once more subjected to a BLASTp search (Altschul *et al*., 1990) against the NCBI database. A multiple sequence alignment of the P450 proteins was conducted with CLUSTAL X v 2.0.10 (Thompson *et al.*, 1997) using default parameters. To recognize the different CYP variants that were expressed in the antennae and gut tissue, a phylogenetic reconstruction analysis was performed using maximum likelihood (ML) with LRT-PHYML (Guindon & Gascuel, 2003). The CYP topology was used to identify groups, but not to establish phylogenetic relationships. Before the ML analysis, we determined an appropriate model of protein

evolution and model parameters using both the Akaike Information Criterion Corrected (AICc = 3965.92, -lnL = 1678.1, K = 8) and the Bayesian Information Criterion (BIC = 5678.75, -lnL = 1678.10, K =8) tests, implemented in PROTEST v 2.4 (Abascal *et al*., 2005). Both tests supported the WAG model (Whelan & Goldman, 2001) with an estimated proportion of invariable sites (I = 0.104). We chose to optimize the topology of the tree rather than the branch length. To estimate the support for each node, the approximate likelihood ratio test (aLRT, Anisimova & Gascuel, 2006) with the Shimodaira-Hasegawa-like procedure option was used. As outgroup sequences for this analysis, we used *T. castaneum* (CYP4: accession no. AAF70496), *I. paraconfusus* (CYP4: accession no. ABF06544, ABF06546, ABF06550, ABF06553), *Helicoverpa armigera* (Hübner) (CYP4: accession no. AA033078), *I. paraconfusus* (CYP9: accession no. EU915209), and *An. minimus* (CYP6: accession no. AY129952). To determine whether the sequences that were included within the main groups output from the phylogenetic analysis actually belonged to the same CYP family, the identity percentages among the amino acid sequences were estimated using MatGAT v. 2.01 (Campanella *et al*., 2003). Our cut-off for considering one sequence within the same family was > 75 %, an upper limit similar to those established by other authors (Feyereisen, 1999; Scott & Wen, 2001).

End sequence determination and cloning of full-length cDNAs.

The complete sequences of CYP genes identified in the phylogenetic analysis were achieved with the RNA ligase-mediated rapid amplification of the 5' and 3' cDNA ends (RLM-RACE) kit (Invitrogen). The total RNA of both the antennae and the gut of unfed bark beetles stimulated for 24 h with racemic α-pinene was obtained, following the protocol described in the RiboPure® Kit (Ambion, Inc. Austin, TX, USA); its integrity was checked on denaturing formaldehyde 1% agarose gels. Partial sequences were used

in the primer design and PCR was carried out following the protocol described in the RLM-RACE kit. The amplicons were purified, cloned and sequenced as previously described. The complete sequences were compared using a BLASTp search with those deposited in GenBank (Altschul *et al*., 1990).

To avoid chimera sequences, we designed specific primers (Supplementary Material, Table 3) based on the complete sequences obtained for each CYP gene with RACE; these specific primers, were used to amplify the complete DNA for each gene. Amplification reactions were carried out in 25 μL volumes containing: 2 μL cDNA from a 1:10 dilution, 1X high fidelity PCR buffer, 2.0 mM $MgSO_4$, 0.2 mM dNTPs, 0.33 μM of each primer (Invitrogen), and 1.25 U of Platinum® *Taq* DNA polymerase high fidelity (Invitrogen). Touchdown PCR was performed as follows: 2 min at 94 °C, 5 cycles of 30 s at 94 °C, 2 min at 72 °C, 5 cycles of 30 s at 94 °C, 2 min at 70 °C, 35 cycles of 60 s at 94 °C, 60 s at 68 °C, 60 s at 68 °C, and then a final extension of 10 min at 68 °C. PCR products of ≈1500 bp were visualised on 1% agarose gels, purified, cloned and sequenced in both strands as previously described. The deduced amino acid sequences were submitted to the P450 nomenclature committee and names assigned based upon their criteria for the classification of *CYP* genes (D. Nelson Department of Molecular Sciences, University of Tennessee, personal communication). All sequences were deposited in GenBank (Accession no.HQ113133 - HQ113142).

Analysis of full length cytochrome P450 sequences

The molecular mass (kDa) and isoelectric point (PI) of each sequence were determined using the ProtParam program (Gasteiger *et al*., 2005). The ten fully sequence *CYP* genes were grouped in five alignments. For each alignment, the sequences from *I. paraconfusus* (*CYP4G27*: accession no. ABF06553), *T. castaneum* (*CYP6BK17*, XP969813 and *CYP9Z4*, NP001164248) were selected as a representative set from

GenBank. Crystal structure data for the mammalian P450 CYP3A4 (PDB code 1TQN) protein were downloaded from the RCSB (Brookhaven protein data bank) website (http://www.rcsb.org/) and used as the template for the secondary structure. The program ESPript (Gouet *et al*., 2003) was used for the assignment of secondary structure elements onto the corresponding aligned sequences, and the substrate recognition sites were manually indicated based on the CYP3A4 enzyme information. All putatively functional *D. rhizophagus* P450 proteins were checked for likely sub-cellular localization using the TargetP program (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson *et al*., 2000) and WoLF PSORT (http://wolfsport.org/) (Horton *et al*., 2007) with the default parameters.

Semiquantitative expression

Pre-emerged insects were collected as described above. Five groups each consisting of 10 unfed beetles were separately placed in glass Petri dishes and stimulated for 8 and 24 h with 100 μL (1 M) of α-pinene (1:1 racemic mix), (*R*)-(+)- α-pinene, (*S*)-(-)- α-pinene, and (*S*)- (-)- β-pinene. Non-stimulated insects were used as the time zero controls.

The antennae and gut of the insects were removed and processed as previously described. The total RNA isolation of the antennae and gut sections, the cDNA synthesis and amplification of the *CYP4*, *CYP6* and *CYP9* genes were also performed as described earlier. The expression levels of these genes were normalized to that of *CYP4G55v1*, an orthologue to the *CYP4G27* gene (Huber *et al*., 2007), because its expression was more stable than that of other genes such as cytoplasmic β-actin. The *CYP4G27* gene was amplified with primers HSK-F/ HSK-R (Supplementary Material, Table 3). The reaction mix and the programmed temperature were identical to the amplification conditions for the *CYP4* gene described above, except that T_m was 46 °C.

The band intensities were measured on a densitometer Multimage® Light Cabinet (Alpha Innotech Corporation®). The semiquantitative expression of each family was replicated twice.

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Table 1. *CYP* genes expressed in antennae and gut of *Dendroctonus rhizophagus* under different stimuli

Stimuli	α-pinene				(*R*)-(+)-α-pinene				(*S*)-(-)-α-pinene				(*S*)-(-)-β-pinene			
Gene	A	Fg	Mg	Hg	A	Fg	Mg	Hg	A	Fg	Mg	Hg	A	Fg	Mg	Hg
CYP4BQ1v1				x		x	x	x		x	x			x	x	x
CYP4BD5v1	x		x		x				x		x	x	x	x	x	x
CYP4BG2v1		x	x			x				x				x		x
CYP4G55v1			x													
CYP4G56v1			x													
CYP6DJ1v1			x		x	x		x		x		x	x		x	x
CYP6DJ2v1	x	x		x							x					
CYP6BW5v1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
CYP6DG1v1									x							
CYP9Z20v1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Total	4	4	7	4	4	5	3	4	4	5	5	4	4	5	5	6

A = Antennae, F = Foregut, M = Midgut, H = Hindgut

Table 2. Characteristics of cytochromes P450 cDNAs isolated of antennae and gut samples from *Dendroctonus rhizophagus*

Full length *D.rhizophagus* CYP name	ORF size (bp)	Protein flanked in 5' 3'(bp)	Molecular mass[a] (kDa)	PI[a]	Signal Peptide Prediction[bc]
CYP4BQ1v1	1488	48 – 91	57352.6	8.51	SP 0.952 mTP 0.133 others 0.011
CYP4BD5v1	1512	45 – 24	57648.7	9.05	SP 0.954 mTP 0.027 others 0.067
CYP4BG2v1	1503	52 – 101	57836.1	6.99	SP 0.944 mTP 0.37 others 0.042
CYP4G55v1	1686	42 – 128	64434.4	8.28	SP 0.403 mTP 0.034 others 0.627
CYP4G56v1	1650	42 – 51	63639.3	6.75	SP 0.715 mTP 0.22 others 0.542
CYP6DJ1v1	1500	27 – 67	57433.6	9.05	SP 0.985 mTP 0.11 others 0.053
CYP6DJ2v1	1521	57 – 225	58344.5	8.82	SP 0.980 mTP 0.17 others 0.047
CYP6BW5v1	1515	37-159	57683.8	8.43	SP 0.887 mTP 0.83 others 0.095
CYP6DG1v1	1521	24 – 72	58765.0	9.2	SP 0.965 mTP 0.017 others 0.053
CYP9Z20v1	1596	90 – 216	61387.2	8.65	SP 0.914 mTP 0.069 others 0.068

[a]As predicted by ProtParam (Gasteiger *et al*., 2005). [b]As predicted by TargetP (Emanuelsson *et al*., 2000) and [c]WoLF PSORT (Horton *et al*., 2007)

FIGURES LEGENDS

Figure 1. Maximum-likelihood tree of cytochrome P450 genes from *D. rhizophagus* based 230 amino acid sequences using the WAG model (Whelan & Goldman, 2001) with an estimated proportion of invariable sites (*I* = 0.104). *T. castaneum* (*CYP4Q7*), *I. paraconfusus* (*CYP4AY1*, *CYP5BD1*, *CYP4BG1*, *CYP4G27*, *CYP9T1*), *H. armigera* (*CYP4G9*) and *An. minimus* (*CYP6AA3*) were used as the reference sequences. The accession numbers of GenBank sequences are shown in brackets. The (n) indicates the number of clones obtained for each *CYP* gene, and the branch support and aLRT values are shown at the nodes.

Figure 2. Multiple sequence alignment and secondary structure elements assignment. The alignment included *CYP4BQ1v1* (accession no. HQ113133), *CYP4BG2v1* (HQ113135), *CYP4BD5v1* (HQ113134), *CYP4G55v1* (HQ113136) and *CYP4G56v1* (HQ113137) from *D. rhizophagus*, the predicted *CYP4G27* from *I. paraconfusus* (accession no. ABF06553) and the mammalian CYP3A4 protein sequence. Substrate recognition sites (SRSs) 1-6 were manually determined. The heme-binding region (FXXGXRXCXG), PERF domain (PXRX) and K-helix (EXXR) are indicated with arrows. The alpha helices are marked as alpha or beta, based on the automatic assignment according to the template of the CYP3A4 protein structure in the program ESPript.

Figure 3. Multiple sequence alignment and secondary structure elements assignment. The alignment included *CYP6DJ1v1* (accession no. HQ113138), *CYP6DJ2v1* (HQ113138), *CYP6DG1v1* (HQ113141), *CYP6BW5v1* (HQ113140) from *D. rhizophagus*, the predicted *CYP6BK17* from *T. Castaneum*(accession no.XP969813) and the mammalian CYP3A4 protein sequence. Substrate recognition sites (SRSs) 1-6 were manually determined. The heme-binding region (FXXGXRXCXG), PERF domain

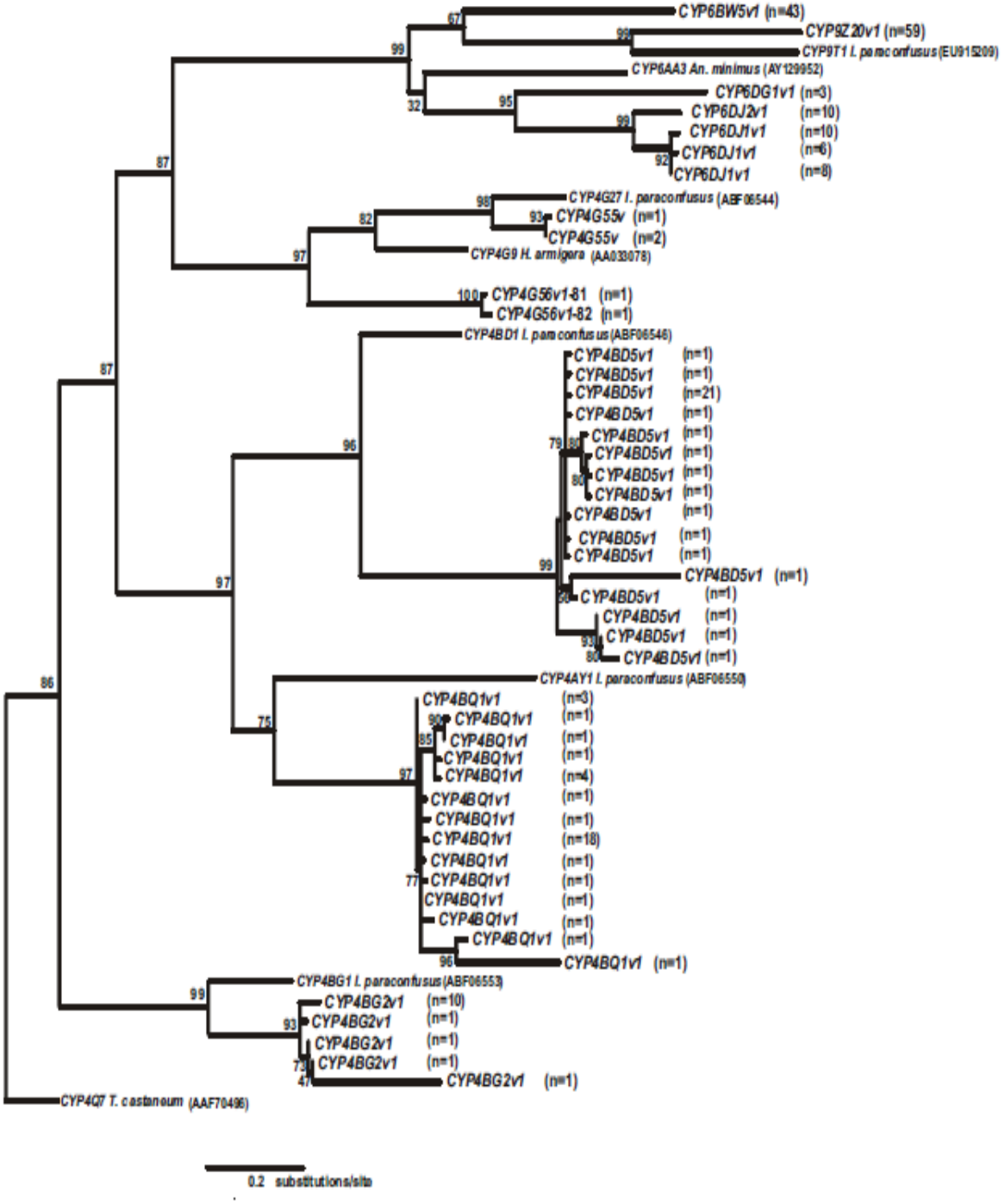
(PXRX) and K-helix (EXXR) are indicated with arrows. The alpha helices are marked as alpha or beta based on the automatic assignment according to the template of the CYP3A4 protein structure in the program ESPript.

Figure 4. Multiple sequence alignment and secondary structure elements assignment. The alignment included *CYP9Z20v1* (accession no. HQ113142) from *D. rhizophagus*, the predicted *CYP9Z4* from *T. castaneum* (accession no. NP001164248) and the mammalian CYP3A4 protein sequence. Substrate recognition sites (SRSs) 1-6 were manually determined. The heme-binding region (FXXGXRXCXG), PERF domain (PXRX) and K-helix (EXXR) are indicated with arrows. The alpha helices are marked as alpha or beta based on the automatic assignment according to the template of the CYP3A4 protein structure in the program ESPript.

Figure 5. Relative expression of *CYP4* genes in antennae and gut after stimulating with vapours of α-pinene (1:1 racemic mixture), (*R*)-(+)-α-pinene, (*S*)-(-)-α-pinene, and (*S*)-(-)-β-pinene in *D. rhizophagus* at times 0, 8 and 24 h.

Figure 6. Relative expression of *CYP6* genes in antennae and gut after stimulating with vapours of α-pinene (1:1 racemic mixture), (*R*)-(+)-α-pinene, (*S*)-(-)-α-pinene, and (*S*)-(-)-β-pinene in *D. rhizophagus* at times 0, 8 and 24 h.

Figure 7. Relative expression of *CYP9* genes in antennae and gut after stimulating with vapours of α-pinene (1:1 racemic mixture), (*R*)-(+)-α-pinene, (*S*)-(-)-α-pinene, and (*S*)-(-)-β-pinene in *D. rhizophagus* at times 0, 8 and 24 h.


CYP6BW5v1 (n=43)
CYP9Z20v1 (n=59)
CYP9T1 I. paraconfusus (EU915209)
CYP6AA3 An. minimus (AY129952)
CYP6DG1v1 (n=3)
CYP6DJ2v1 (n=10)
CYP6DJ1v1 (n=10)
CYP6DJ1v1 (n=6)
CYP6DJ1v1 (n=8)
CYP4G27 I. paraconfusus (ABF06544)
CYP4G55v (n=1)
CYP4G55v (n=2)
CYP4G9 H. armigera (AA033078)
CYP4G56v1-81 (n=1)
CYP4G56v1-82 (n=1)
CYP4BD1 I. paraconfusus (ABF06546)
CYP4BD5v1 (n=1)
CYP4BD5v1 (n=1)
CYP4BD5v1 (n=21)
CYP4BD5v1 (n=1)
CYP4BD5v1 (n=1)
CYP4BD5v1 (n=1)
CYP4BD5v1 (n=1)
CYP4BD5v1 (n=1)
CYP4BD5v1 (n=1)
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CYP4BD5v1 (n=1)
CYP4BD5v1 (n=1)
CYP4BD5v1 (n=1)
CYP4BD5v1 (n=1)
CYP4BD5v1 (n=1)
CYP4AY1 I. paraconfusus (ABF06550)
CYP4BQ1v1 (n=3)
CYP4BQ1v1 (n=1)
CYP4BQ1v1 (n=1)
CYP4BQ1v1 (n=1)
CYP4BQ1v1 (n=4)
CYP4BQ1v1 (n=1)
CYP4BQ1v1 (n=1)
CYP4BQ1v1 (n=18)
CYP4BQ1v1 (n=1)
CYP4BQ1v1 (n=1)
CYP4BQ1v1 (n=1)
CYP4BQ1v1 (n=1)
CYP4BQ1v1 (n=1)
CYP4BQ1v1 (n=1)
CYP4BG1 I. paraconfusus (ABF06553)
CYP4BG2v1 (n=10)
CYP4BG2v1 (n=1)
CYP4BG2v1 (n=1)
CYP4BG2v1 (n=1)
CYP4BG2v1 (n=1)
CYP4Q7 T. castaneum (AAF70496)
0.2 substitutions/site

Figure 1.

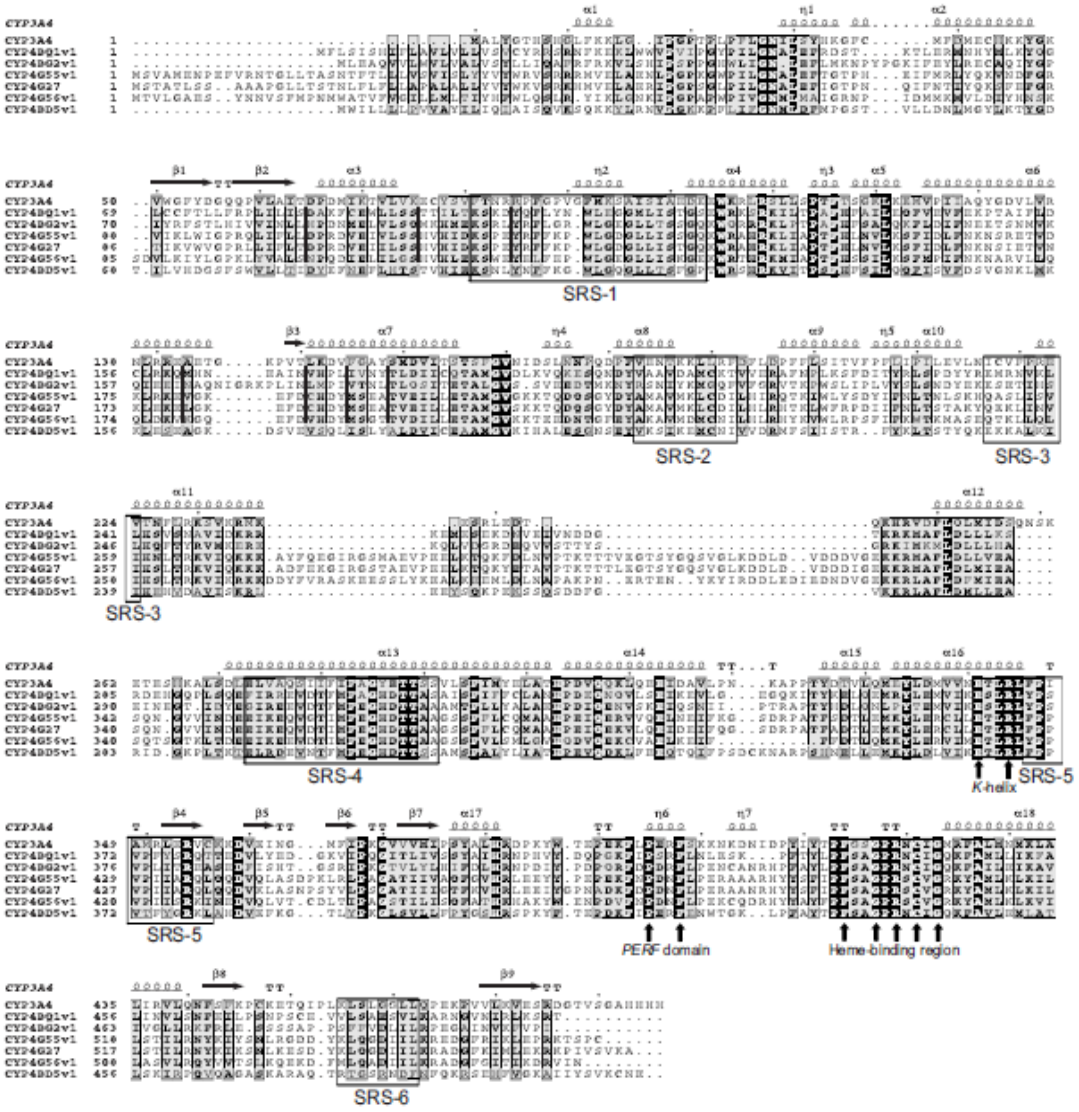

SRS-1
SRS-2
SRS-3
SRS-3
SRS-4
SRS-5
K-helix
SRS-5
PERF domain
Heme-binding region
SRS-6

Figure 2.

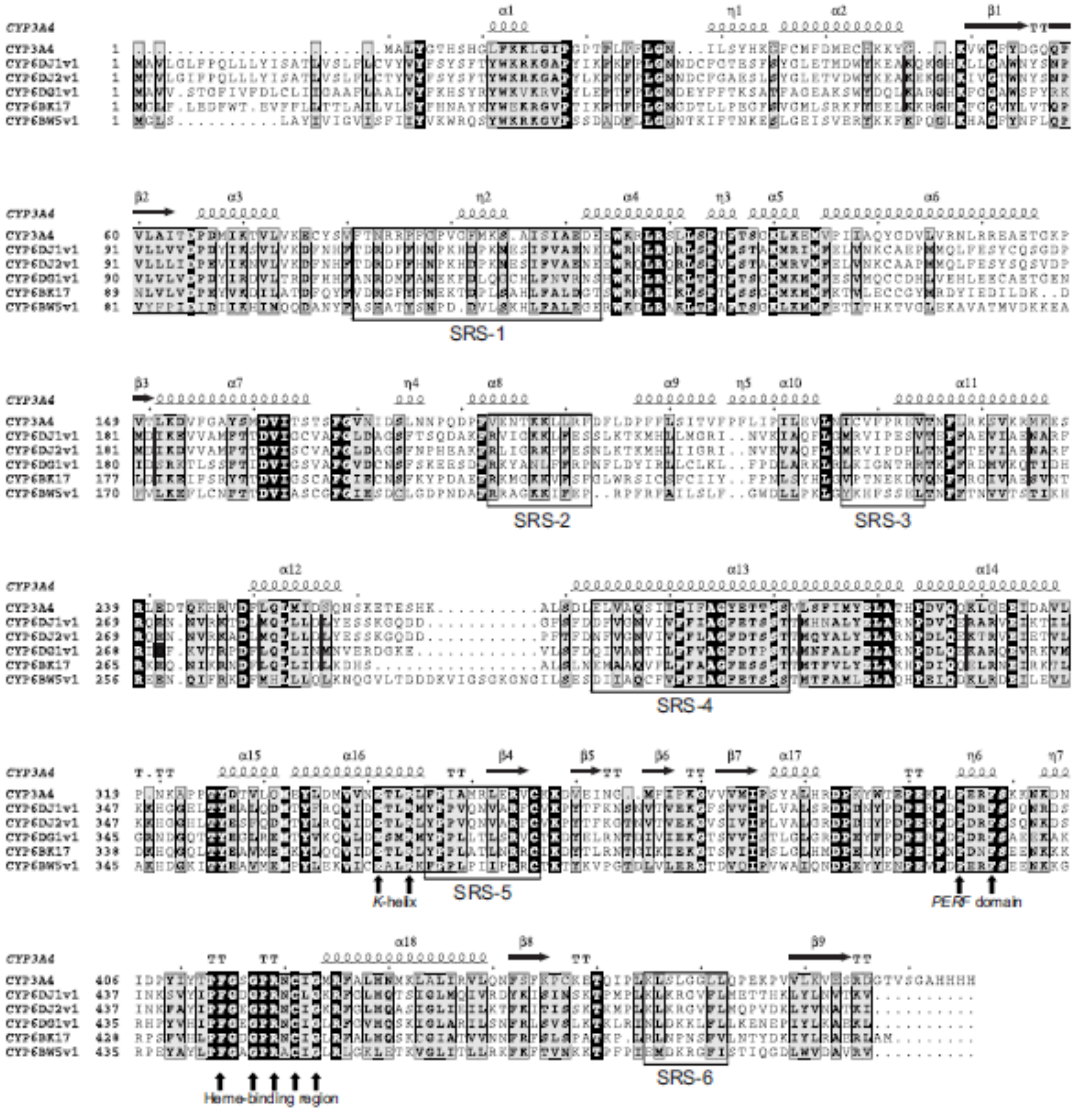
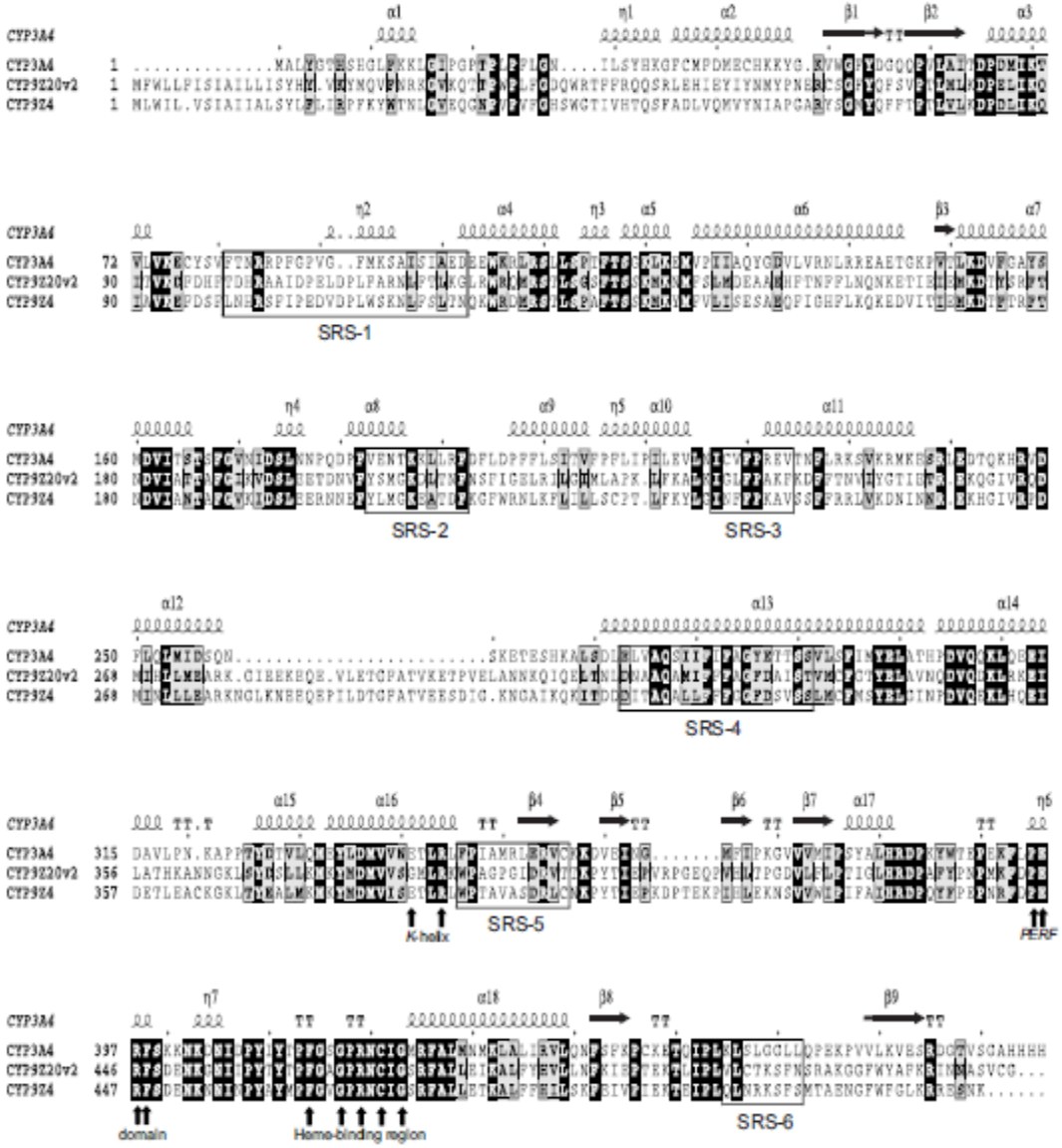

SRS-1
SRS-2
SRS-3
SRS-4
SRS-5
SRS-6
K-helix
PERF domain
Heme-binding region

Figure 3.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SRS-2
SRS-3
SRS-4
SRS-5
SRS-6
K-helix
PERF
domain
Heme-binding region

Figure 4.

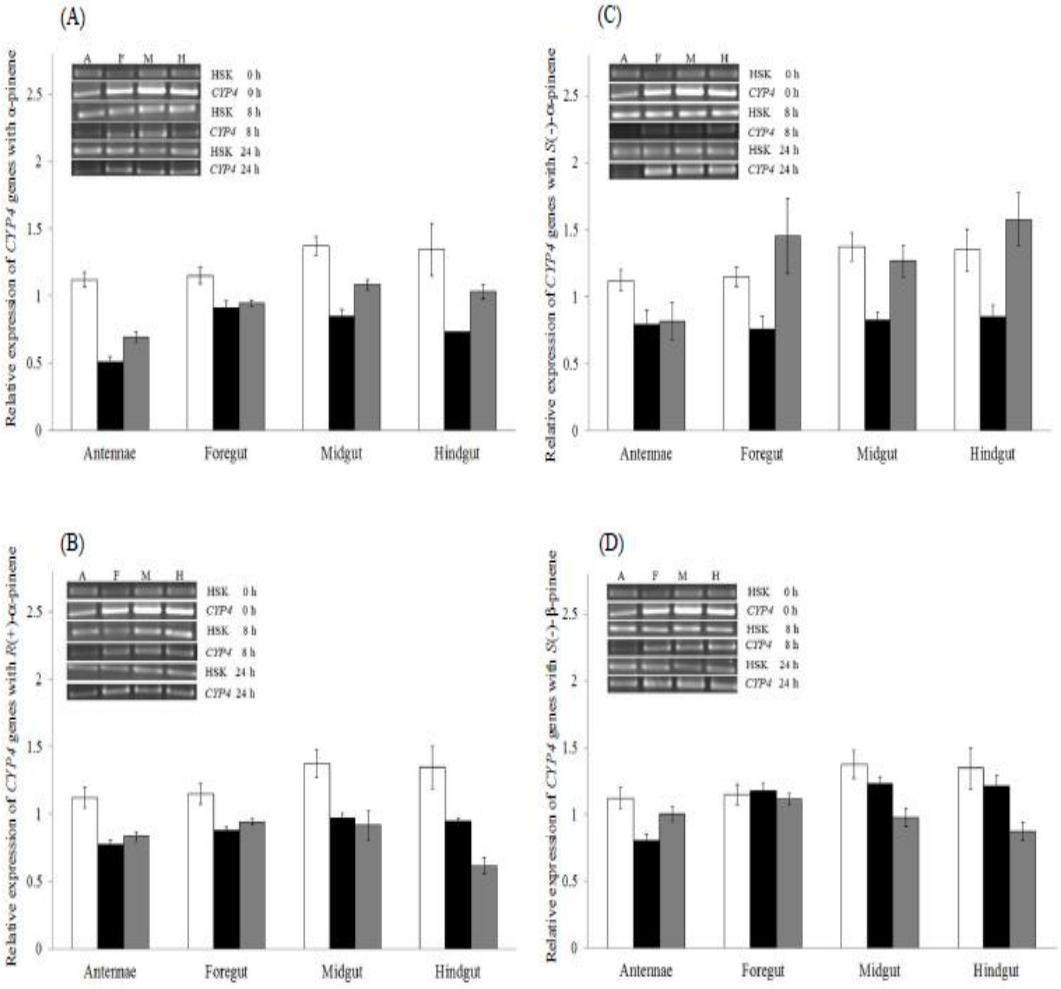
(A)
(C)
(B)
(D)
A F M H
HSK 0 h
CYP4 0 h
HSK 8 h
CYP4 8 h
HSK 24 h
CYP4 24 h
Relative expression of CYP4 genes with α-pinene
Relative expression of CYP4 genes with S(-)-α-pinene
Relative expression of CYP4 genes with R(+)-α-pinene
Relative expression of CYP4 genes with S(-)-β-pinene
0
0.5
1
1.5
2
2.5
Antennae
Foregut
Midgut
Hindgut
0 h
8 h
24 h

Figure 5.

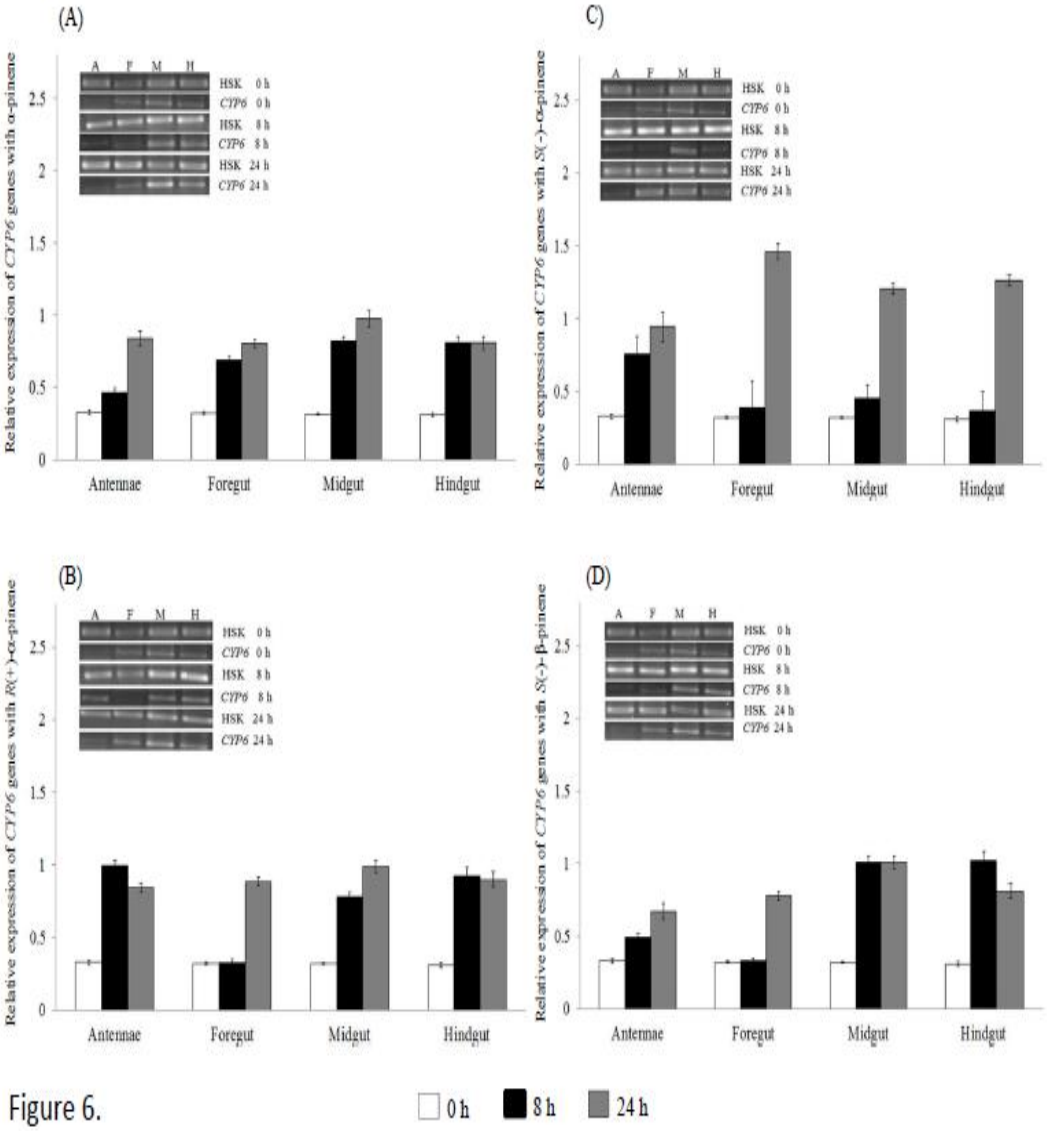
(A)
(C)
(B)
(D)
A F M H
HSK 0 h
CYP6 0 h
HSK 8 h
CYP6 8 h
HSK 24 h
CYP6 24 h
Relative expression of CYP6 genes with α-pinene
Relative expression of CYP6 genes with S(-)-α-pinene
Relative expression of CYP6 genes with R(+)-α-pinene
Relative expression of CYP6 genes with S(-)-β-pinene
0
0.5
1
1.5
2
2.5
Antennae
Foregut
Midgut
Hindgut
0 h
8 h
24 h

Figure 6.

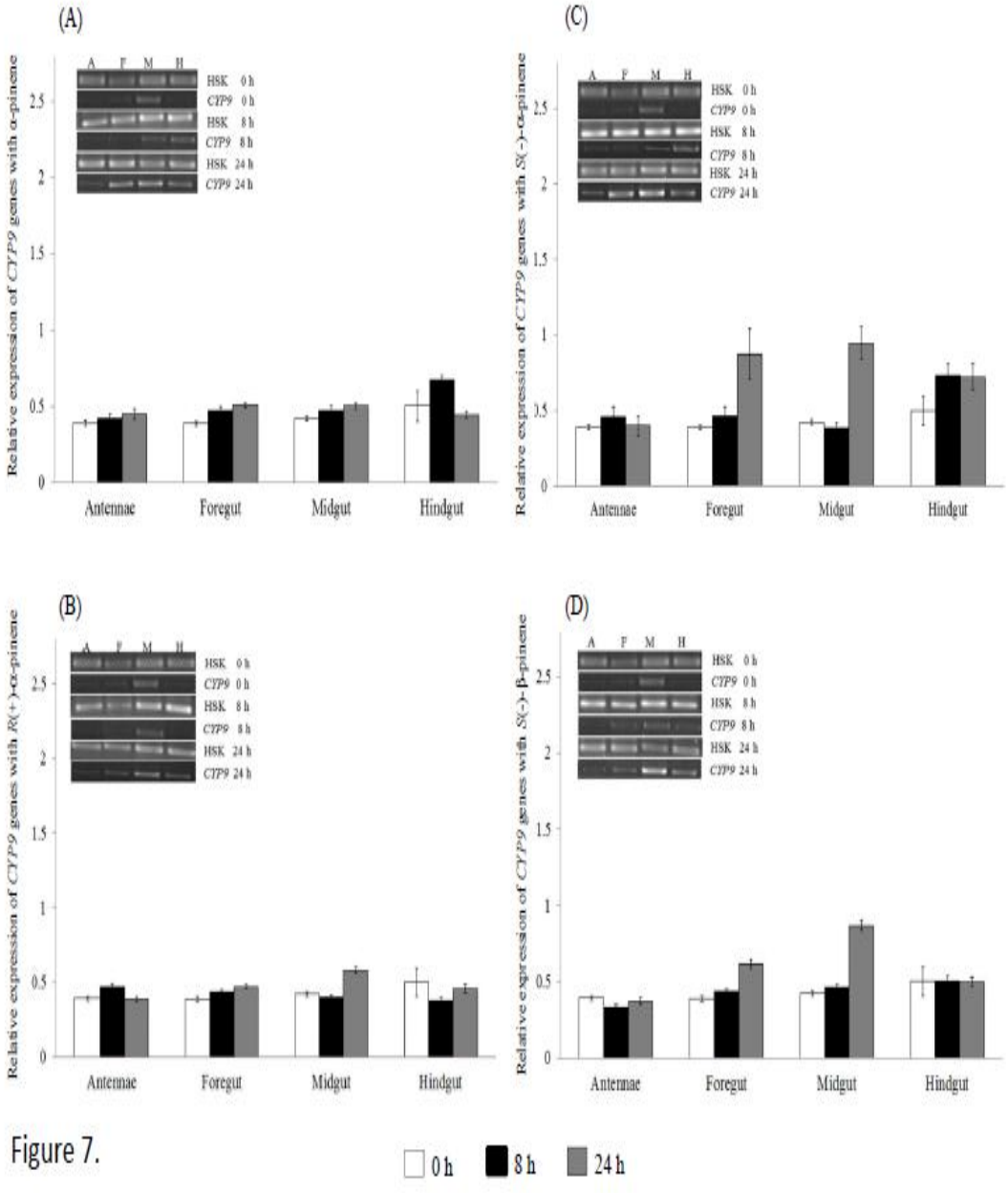
(A)
(C)
(B)
(D)
A F M H
HSK 0 h
CYP9 0 h
HSK 8 h
CYP9 8 h
HSK 24 h
CYP9 24 h
Relative expression of CYP9 genes with α-pinene
Relative expression of CYP9 genes with S(-)-α-pinene
Relative expression of CYP9 genes with R(+)-α-pinene
Relative expression of CYP9 genes with S(-)- β-pinene
0
0.5
1
1.5
2
2.5
Antennae
Foregut
Midgut
Hindgut
0 h
8 h
24 h

Figure 7.

Supplementary Material, Table 1. Amino acidic identity of cytochromes P450 cDNAs isolated of antennae and gut samples from *Dendroctonus rhizophagus*.

Partial length CYP groups name	Identity within groups	**1**	**2**	**3**	**4**	**5**
CYP4BQ1v1 (n = 36)	95.8 - 100	49 - 51	51 – 52.4	48.8 - 50	31 – 32.5	40 – 42
CYP4BD5v1 (n = 36)	87.6 – 100	43 – 44	59 – 62.3	37.6 – 39	29 – 30.4	41 – 44.5
CYP4BG2v1 (n = 14)	79.3 – 100	32 – 42	34.7 – 47	56 – 72.4	24 – 35.5	39 – 49.6
CYP4G55v1 (n = 3)	99 – 100	30.1	33-6	35	81	50.8
CYP4G56v1 (n = 3)	97.6	37 – 37.5	44 – 45.5	43.2 - 44	43 – 43.7	56.5 – 58
		6	**7**	**8**	**9**	**10**
CYP6DJ1v1 (n = 24)	98.5 - 100	48.5 - 50	47.7 - 49.2	44 – 44.6	45 – 45.4	45.4 – 46.9
CYP6DJ2v1 (n = 10)	100	46.2	52.3	43.2	49.2	48.5
CYP6BW5v1 (n = 24)	100	43.1	50.8	57.4	50.8	50.8
CYP6DG1v1 (n = 3)	100	41.5	46.9	43.8	44.6	45.4
		11	**12**	**13**	**14**	**15**
CYP9Z20v1 (n = 60)	100	57	57	54.1	59.3	57

I. paraconfusus **(1:** *CYP4AY1*, accession no. ABF06544; **2**: *CYP4BD1*, accession no. ABF06546; **3**: *CYP4BG1*, accession no. ABF06550; **4:** *CYP4G27*, accession no. ABF06553; **12:** *CYP9T1*, accession no. DQ471884). ***I. confusus*** **(11**: *CYP9T1*, accession no. ACK37844). ***I. pini*** (**13***: CYP9T2*, accession no. ABG74909*).* ***H. armígera*** (**5**: *CYP4G9*, accession no. AAD33078*).* ***A. minimus*** **(6***: CYP6AA3*, accession no. AY129952). ***H. sjoestedti*** **(7:** *CYP6AM1*, accession no. AB194532**).** ***L. decemlineata*** (**8:** *CYP6BJ1*, accession no. DQ117463). ***T. castaneum*** **(9:** *CYP6BK4*, accession no. NM_001130403; **10:** *CYP6BK12*, accession no. EFA12529; **14:** *CYP9F2*, accession no. NP_001127706; **15:** *CYP9Z4*, accession no.NP001164248). n = number of clones.

Supplementary Material, Table 2. Identity percentage of cytochromes P450 cDNAs isolated of antennae and gut samples from *Dendroctonus rhizophagus* with respect to known cytochromes P450

	Blastp Closest Matches in GenBank			
Full length *D.rhizophagus* CYP name	CYP450 name	Species	GenBank Accession Number	Identity (%)[a]
CYP4BQ1v1	*CYP4AY2*	*Ips paraconfusus*	ABF06545	49.9
	CYP4AY1	*I. paraconfusus*	ABF06544	48.9
CYP4BD5v1	*CYP4BD1*	*I. paraconfusus*	ABF06546	51.0
	CYP4BN1	*Tribolium castaneum*	EFA04615	38.0
CYP4BG2v1	*CYP4BG1*	*I. paraconfusus*	ABF06550	59.3
	Cytochrome P450-like	*T. castaneum*	EFA10781	50.8
CYP4G55v1	*CYP4G27*	*I. paraconfusus*	ABF06553	79.7
	CYP4G29	*Leptinotarsa decemlineata*	AAZ94273	74.1
CYP4G56v1	*CYP4G7*	*T. castaneum*	NP001107860	55.1
	CYP4G44	*Nasonia vitripennis*	NP001165993	52.4
CYP6DJ1v1	Predictiva *CYP6BK17*	*T. castaneum*	XP969813	44.9
	Cytochrome P450	*T. castaneum*	EFA12627	46.2
CYP6DJ2v1	Cytochrome P450 *6BR3*	*T. castaneum*	EFA12627	43.1
	Cytochrome P450 *6BK4*	*T. castaneum*	NP001123875	39.3
CYP6BW5v1	*CYP6BQ7*	*T. castaneum*	EFA02821	45.7
	CYP6BQ5	*T. castaneum*	EFA02819	44.7
CYP6DG1v1	Cytochrome P450 *6BR3*	*T. castaneum*	EFA12627	46
	CYP6BJ1	*L. decemlineata*	AAZ94272	40.9
CYP9Z20v1	Cytochrome P450 9Z4	*T. castaneum*	NP001164248	53.2
	CYP9V1	*L. decemlineata*	AAZ94269	59.1

[a]As predicted by MatGat (Campanella *et al*., 2003).

Supplementary Material, Table 3. Primers used in this study

Primer name	Full length (FL) and Partial (P)	Primer sequence (5'-3')
+ CYP4F	[P] CYP4 degenerate, PCR	GAG GTI CAT ACI TTC ATG TTC GAG GGI CAC CAT AC
+ CYP4R	[P] CYP4 degenerate, PCR	CTG ICC GAT ACA GTT ICG GGG ICC IGC GIA GAA GGG
+ CYP69F	[P] CYP4 degenerate, PCR	TAC GAR YIG CIR WIA AYC CYG A
+ CYP69R	[P] CYP4 degenerate, PCR	CCI AKR CAR TTI CKI GGI CC
+ CYP6EF	[P] CYP4 degenerate, PCR	GAA CTC GCC TCA ACA TCC TG
+ CYP6EF	[P] CYP4 degenerate, PCR	GCA AGC GWG AGG TCC AGC AC
* CYP4AY1FSC	[FL] *CYP4BQ1v1*	CTA TCA ATT TCG CAT ATT TTC CTG GC
* CYP4AY1RSC	[FL] *CYP4BQ1v1*	CCT GGA CTT CAG GCG GAT ATT CAC
* CYP4BD1FSC	[FL] *CYP4BD1v1*	ATG TGG ATC TTG CTG TTG TTA CCG G
* CYP4BD1RSC	[FL] *CYP4BD1v1*	TTA TTC ATT GCA TTT TAC GCT ATA AG
* CYP4BG1FSC	[FL] *CYP4BG2v1*	ATG TTA GAG GCT CAA GTG GTT CTA TG
* CYP4BG1RSC	[FL] *CYP4BG2v1*	TTA TAT TGG GAC AAA TTT AAC GTT GAT AG
* CYP4G27FSC	[FL] *CYP4BG55v1*	ATG TCT GTC GCM ATG GAA AAT CCG GAG TT
* CYP4G27RSC	[FL] *CYP4BG55v1*	TCA ACA GGG ACT AGT CTT TCT GGG CTC CA
* CYP4G7FSC	[FL] *CYP4BG56v1*	ATG ACA GTG CTG GGA GCT GAA AGC TAT
* CYP4G7RSC	[FL] *CYP4BG56v1*	CAC AAA TCA ATT GAG CAC CCG GTC CTT
* CYP6-1FSC	[FL] *CYP6DG1v1*	ATG GCG GTT GTT TCT ACC GGT TTT
* CYP6-1RSC	[FL] *CYP6DG1v1*	CTA AAG CTT CTC CGC TTT TAA ATA G
* CYP6-2FSC	[FL] *CYP6DJ2v1*	ATG ACG GTT TTA GGC ATT GTT CCC
* CYP6-2RSC	[FL] *CYP6DJ2v1*	CAG TAT AAA TTA AAT TTT GGT GGC ATT
* CYP6-3FSC	[FL] *CYP6DJ1v1*	ATG GCG GTC TTA GGT CTT TTT CCT
* CYP6-3RSC	[FL] *CYP6DJ1v1*	AAA TTA AAC TTT TGT GAC ATT CAG GTA C
* CYP6BJ1FSC	[FL] *CYP6BW5v1*	ATG GGG TTG AGT CTA GCC TAT ATA G
* CYP6BJ13FSC	[FL] *CYP6BW5v1*	TTA TAC TCT AAC AGC ATC GAC CC
* CYP9-1FSC	[FL] *CYP9Z20v1*	GATGTTTTGGTTATTATTTATATCCATT GC
* CYP9-1RSC	[FL] *CYP9Z20v1*	TTAACCGCAYACKGAAGCATTATAA TTG
* CYP4G27(HSK-F)	[P] *CYP4BG55v1*	TGG AAA GGT GCT TAT TGG A
* CYP4G27 (HSK-R)	[P] *CYP4BG55v1*	GGC AAG AAG TTG TCT GGA

+ From Snyder *et al*. (1996) and Dunkov *et al*., (1996). * Designed for this study

DISCUSIÓN GENERAL

Una sensibilidad olfativa hacia los monoterpenos de la resina (+)-α-pineno, (-)-β-pineno y (+)-3-careno de *P. arizonica* fue observada en ambos sexos de *D. rhizophagus*. El (+)-3-careno fue el terpeno más atrayente de ambos sexos en los ensayos de campo, en comparación con el (+)-α-pineno, (-)-β-pineno y los terpenos oxigenados. Otros estudios han documentado que otras especies del género *Dendroctonus* (Seybold *et al*., 2006) responde al (+)-3-careno, pero la respuesta no suele ser diferente entre los sexos, como fue documentado en el presente estudio si comparamos esta respuesta con la que tuvieron los otros terpenos. No obstante, se ha demostrado que en *D. valens*, la especie hermana de *D. rizhophagus*, responde también de manera significativa al (+)-3-careno, pero no así *D. terebrans*, una especie filogenéticamente cercana a ambas especies. Asimismo, se ha demostrado que este terpeno es capaz de atraer significativamente en condiciones naturales a los miembros de poblaciones de *D. valens* tanto en Norteamérica (Erbilgin *et al*., 2007) como en China, donde este descortezador fue accidentalmente introducido recientemente (Sun *et al*., 2004; Zhang *et al*., 2009).

Una atracción casi nula y diferencial entre sexos fue ejercida por el (+)-α-pineno y el (-)-β-pineno en *D. rhizophagus*, ya que el primero atrajo más machos que hembras y el segundo más hembras que machos, a pesar de que ambos compuestos atrajeron pocos individuos. El (*S*)-(-)-β-pineno and (*R*)-(+)-α-pineno en *D. valens* también muestran un poder de atracción bajo, sin embargo, no se reporta que los sexos tengan una respuesta diferencial a estos terpenos (Erbilgin *et al*., 2007). En el caso de *D. terebrans*, se ha demostrado que tiene sensibilidad olfativa al α-pineno, β-pineno y aguarrás (una mezcla de monoterpenos proveniente del pino) (Delorme & Payne 1990), no obstante, pruebas de campo indican que una combinación de etanol y (-)-α-pineno es una mezcla mucho

más atractiva para esta especie que algún monoterpenos en particular (Miller & Rabaglia 2009).

El cóctel de los tres monoterpenos (1:1:1) fue el segundo cebo más atractivo para *D. rhizophagus*, esta combinación de monoterpenos es la mezcla comercial que a diferentes concentraciones es utilizada para el manejo y control de las poblaciones de *D. valens* en China (Zhang & Sun 2006). Una característica interesante de la respuesta de *D. rhizophagus* a esta mezcla de monoterpenos fue que hembras y machos al parecer tienen una respuesta diferencial a ella. Nada ha sido reportado al respecto en *D. valens*, pero asumiendo que es la mezcla utilizada para su control, se puede inferir que ambos sexos en esta especie responden igual a la mezcla (Sun *et al*., 2004, Erbilgin *et al*., 2007, Zhang *et al*., 2009).

Por otra parte, la antena de *D. rhizophagus* presenta una respuesta electrofisiológica significativa a los terpenos oxigenados *cis*-verbenol, *trans*-verbenol, verbenona, mirtenal y mirtenol. Respuestas electroantenográficas a estos mismos compuestos y presencia de ellos en altas concentraciones en el mesenteron y proctodeo se han reportado también en *D. valens* y *D. terebrans*. Los estudios de campo realizados hasta hoy con estas dos especies no han encontrado que estos compuestos tengan alguna actividad feromonal, sin embargo, los datos de campo de este estudio sugieren un papel importante de estos compuestos aún por descubrir en la comunicación química de *D. rhizophagus*, debido a la respuesta diferente que machos y hembras tuvieron con estos terpenos oxigenados y a la mayor atracción que desplegaron los machos.

Además, la cuantificación de los monoterpenos oxigenados en insectos de *D. rhizophagus* tomados directamente en el campo, sugiere una asociación estrecha entre la producción de estos compuestos y el avance de las etapas de colonización. El incremento en la producción de terpenos oxigenados entre hembras en fase de

colonización temprana y hembras en pareja construyendo galerías, sugiere fuertemente que el aumento en la producción de estos monoterpenos podría ser una forma de atracción y retención del macho, lo que explica la atracción que estos monoterpenos tuvieron de manera individual y en conjunto en los bioensayos en campo. La disminución en la producción de estos compuestos observada después de que las hembras han ovipositado, aparentemente relaja el compromiso de los machos a permanecer en la galería. Por otra parte, una mayor producción de estos terpenos oxigenados fue registrada en machos con hembras sin ovipositar que en machos con hembras que habían ovipositado; sin embargo, las hembras de estas dos condiciones produjeron estos terpenos en cantidades mayores que los machos, a pesar de que esta diferencia fue estadísticamente no significativas.

La producción de estos terpenos en machos y hembras de *D. rhizophagus* en laboratorio fue diferente. Machos y hembras alimentados separadamente produjeron mirtenol, *trans*-verbenol y *cis*-verbenol en cantidades más altas que cualquier otro terpeno oxigenado detectado por GC-MS. Esta misma respuesta también ha sido observada en *D. valens* en la misma condición, pero la producción de estos mismos compuestos fue en cantidades tres veces más altas que lo observado en *D. rhizophagus* (Shi & Sun 2010). En *D. terebrans* la producción de estos terpenos en hembras y machos que permanecieron siete días en las galerías de los árboles en el campo, fue treinta veces mayor que la de *D. rhizophagus* (Phillips *et al*., 1989).

Se ha interpretado que la alta producción de mirtenol y *trans*-verbenol en machos y hembras de *D. valens*, es una forma diferente de atraer a sus conespecíficos para llevar a cabo la colonización masiva del árbol (Shi & Sun 2010), ya que regularmente la atracción secundaria en especies primarias (aquellas que primero arriban al árbol) se realiza a partir de feromonas de agregación y antiagregación producidas por la hembra y

el macho, respectivamente (Smith *et al*., 1993). El comportamiento atípico de *D. rhizophagus* hace difícil apegarse plenamente a esta hipótesis, ya que no realiza ataques masivos, sin embargo, hay coincidencia en la alta producción de mirtenol y *trans*-verbenol, lo que sugiere que posiblemente estos monoterpenos funcionan sinérgicamente atrayendo a los machos. Una vez que el macho se instala en la galería con la hembra, la alta producción de estos terpenos oxigenados por ambos sexos podría funcionar también sinérgicamente como una mezcla antiagregativa de otros conespecíficos. Los resultados del bioensayo de las pruebas de campo parcialmente apoyan esta hipótesis, ya que individualmente y combinados los terpenos oxigenados (excepto donde estuvo presente la verbenona) atrajeron más machos que hembras, siendo el *trans*-verbenol quien produjeron una mejor respuesta. Estudios posteriores deberán confirmar la validez de esta hipótesis.

La identificación y caracterización molecular de 10 nuevos genes citocromo P450 de las familias 4, 6 y 9, así como su expresión diferencial en antenas e intestino al exponer a los insectos de *D. rhizophagus* a los vapores de sus principales kairomonas, son un ejemplo más de la relación estrecha entre el sistema de comunicación química y los procesos de desintoxicación o biosíntesis de feromonas. Los monoterpenos presentes en la resina producen una respuesta fisiológica y comportamental en los descortezadores, pero también son compuestos fisiológicamente tóxicos para los insectos a altas concentraciones, ya que son parte del sistema de defensa de los árboles. Los procesos de desintoxicación y biosíntesis de feromonas invariablemente conducen a la biosíntesis de monoterpenos oxigenados, de diferente grado de estéreo-especificidad, los cuales al ser más polares son más fácilmente eliminados.

La baja identidad aminoacídica (≈ 40-50%) que tienen los genes citocromo P450 de *D. rhizophagus* con los de otras especies de escolítidos e insectos en general, muestra su

alto grado de exclusividad. Un aspecto importante que se deriva de los resultados de este estudio tiene que ver con conocer si los 10 genes citocromo P450 identificados en las antenas e intestino de la especie en estudio, son un muestra representativa de los genes citocromo P450 involucrados en el proceso de deintoxicación o en la biosíntesis de presuntas feromonas de esta especie. La respuesta no es simple, toda vez que existen pocos estudios de este tipo en otras especies del género. Sin embargo, dado que Huber *et al*. (2007) identificaron 13 genes, 12 de la familia cuatro y uno de la familia nueve en insectos alimentados de *Ips paraconfusus*, los datos derivados de ambos estudios hasta hoy sugieren que el número y la diversidad de genes CYP podrían ser mayores en los escolítidos. La diferencia en el número de genes identificados en ambos estudios puede ser explicado por el hecho de que Huber *et al*. (2007) analizó insectos completos y no tejido específico como fue el caso de este trabajo, y además incluyo regiones como el cuerpo graso y túbulos de Malpighi donde la expresión de genes *CYP* relacionados con la desintoxicación de los insectos es común.

Las diferencias observadas en la diversidad de los genes *CYP* en *I. paraconfusus* y *D. rhizophagus* es indicativo de la complejidad de funciones en las que pueden estar involucrados estos genes en los escolítidos. Por ejemplo, Huber *et al*. (2007) identificaron 12 genes *CYP* de la familia 4 y solo uno de la familia 9, los primeros son citocromos involucrados más en el proceso de desintoxicación *per se* de muchos compuestos ingeridos por el insecto, mientras que el gen *CYP9* se ha demostrado participa en la hidroxilación del mirceno dando origen a las feromonas de agregación ipsdienol e ipsienol producidos por los machos de esta especie vía la ruta del mevalonato. Asimismo, se ha demostrado que los ortólogos de este gen identificados en *I. pini* e *I. confusus* tienen funciones similares. En *D. rhizophagus* fueron identificados cinco genes *CYP4*, cuatro genes *CYP6* y un gen *CYP9*, lo que hace posible que en un

futuro cercano se pueda analizar la evolución de los mismos en el género y también se pueda determinar su participación relativa ya sea en el proceso de desintoxicación o biosíntesis de presuntas feromonas.

La expresión de los genes *CYP4BQ1v1, CYP4BD5v1*, *CYP4BG2v1, CYP4G55v1*, *CYP4G56v1, CYP6DJ1v1*, *CYP6DJ2v1*, *CYP6BW5v1*, *CYP6DG1v1*, and *CYP9Z20v1* in *D. rhizophagus* sugiere que éstos son inducidos directamente por los monoterpenos utilizados. En otras especies de insectos, genes de las mismas familias han sido identificadas y su expresión ha sido asociada con su exposición a xenobióticos y con la desactivación o degradación de moléculas de feromona en la antena (Wang *et al*., 1999; Wojtasek & Leal 1999; Maïbèche-Coisne *et al*., 2002, 2004a, 2005). La expresión diferencial de los diez genes *CYP* en la antena y canal alimentario sugiere que las enzimas P450 putativas podrían tener diferentes funciones dependiendo de la quiralidad del monoterpeno. Por último, el patrón de expresión de los genes *CYP* de las tres familias en antenas y regiones del intestino de *D. rhizophagus*, sugiere que cada familia es inducida en cada sitio de manera diferente en respuesta al mismo monoterpenos. La expresión de los genes de la familia 4 aparentemente es constitutiva, ya que no fueron inducidos por monoterpenos específicos. Resultados de expresión similar fueron encontrados con genes de esta familia en *I. paraconfusus* a través de PCR a tiempo real. Por el contario, el aumento en la expresión de los genes de las familias CYP6 y CYP9 ante el estímulo de diferentes monoterpenos sugiere, que estos genes podrían estar involucrados en el metabolismo de estos compuestos en las antenas y canal alimentario de *D. rhizophagus*. Hasta el momento, la función de los genes de la familia CYP6 en escolítidos es desconocida, sin embargo, en insectos fitófagos se ha asociado con el metabolismo de compuestos tóxicos de sus huéspedes (David *et al*., 2006). Por el contrario, la participación de genes *CYP9* en la hidroxilación del mirceno en ipsdienol e

ipsienol en *I. confusus* e *I. pini* (Sandstrom *et al*., 2006; 2008), conduce a pensar que estos genes pudieran tener funciones similares en *D. rhizophagus*. Estudios de expresión heteróloga e hibridación *in situ* deberán confirmar la función de estos genes.

CONCLUSIONES GENERALES

- Los terpenos oxigenados mirtenal, mirtenol, *trans*-verbenol, *cis*-verbenol, verbenona, fenchyl alcohol, *trans*-pinocarveol, α-terpineol, *trans*-mirtanol, *cis*-mirtanol y 2-fenyletanol fueron los compuestos más abundantes en el mesenterón y proctodeo de ambos sexos de *D. rhizophagus*.
- La antena de *D. rhizophagus* respondió a los monoterpenos α-pineno, β-pineno and 3-careno de la resina de *Pinus arizonica* y a los terpenos oxigenados fenchyl alcohol, mirtenal, *cis*-verbenol, *trans*-verbenol, verbenona y mirtenol producidos por ambos sexos.
- En condiciones de laboratorio, la mayor producción de terpenos oxigenados se obtuvo en hembras y machos alimentados individualmente, que en insectos de ambos sexos no alimentados o en insectos formando pareja.
- Una mayor producción de terpenos oxigenados fue registrada en los insectos provenientes del campo en alguna de las diferentes etapas colonización con respecto a insectos no alimentados, obligados a alimentarse, o alimentados formando parejas en el laboratorio
- El mirtenol y *trans*-verbenol también fueron los compuestos más abundantes en el mesenteron y proctodeo de insectos de ambos sexos alimentados individualmente en condiciones de laboratorio.
- El mirtenol y *trans*-verbenol fueron los compuestos más abundantes en el mesenteron y proctodeo de insectos de ambos sexos provenientes del campo, en alguna de las diferentes etapas colonización.
- El (+)-3-careno fue el terpeno más atrayente para ambos sexos de *D. rhizophagus*

- Los terpenos oxigenados probados individualmente y combinados (excepto donde estuvo presente la verbenona) atrajeron más machos que hembras, siendo el *trans*-verbenol quien produjera una mejor respuesta.
- Diez genes nuevos citocromo P450 de las familias CYP4, CYP6 y CYP9 fueron expresados diferencialmente en las antenas y regiones del canal alimentario de *D. rhizophagus* ante el estímulo de los volátiles α-pineno y los enantiómeros (*R*)-(+)-α-pineno, (*S*)-(-)-β-pineno y (*S*)-(+)-α-pineno.
- Las proteínas putativas citocromo P450 presentan los motifs característicos de esta familia multi-enzimática y seis sitios putativos de unión al sustrato localizados en regiones con elementos de estructura variable.
- El análisis bioinformático muestra que el peso molecular de las proteínas putativas varía entre 57 y 64 kDa, y que su punto isoléctrico se encuentra entre 6.75 y 9.2.
- La localización sub-celular predictiva indica que, con excepción de la proteína que codifica el gen *CYP4G55v1*, las proteínas citocromo P450 putativas están ancladas en la membrana del retículo endoplasmico.
- La localización sub-celular predictiva del gen *CYP4G55v1* sugiere una localización citoplasmática
- El PCR semicuantitativo muestra que la expresión de los genes de la familia CYP4 es constitutiva, ya que no fueron inducidos por monoterpenos específicos.
- El PCR semicuantitativo muestra un aumento en la expresión de los genes de la familia CYP6 y CYP9 a las 8 y 24 horas.

PROSPECTIVAS

Con base en los resultados obtenidos, se propone las siguientes actividades:

- Ampliar las pruebas de campo empleando el monoterpeno (+)-3-careno en combinación con los terpenos oxigenados con el objetivo de determinar si existe un proceso de atracción mediado por feromonas en *D. rhizophagus*.
- Determinar la selectividad enantiomérica de la antena de *D. rhizophagus* y probar su funcionalidad en campo con el propósito de comprender más específicamente como esta mediada la comunicación química de esta especie.
- Probar diferentes tasas de liberación del compuesto y tipos de trampas para determinar cual es la más atrayente.
- Una vez conocida la mezcla de atracción para esta especie, comparar la respuesta del insecto en toda la aérea de distribución de *D. rhizophagus*.
- Cuantificar los niveles de expresión de los genes citocromo P450 identificados en *D. rhizophagus* empleando una técnica más precisa como es el PCR en tiempo real.
- Determinar la funcionalidad de las proteínas putativas que codifican los genes *CYP6* y *CYP9* en los procesos de desintoxicación y/o producción de feromonas a través de la expresión heteróloga de estas proteínas.
- Localizar las enzimas citocromo P450 codificadas por estos genes en la estructura de la antena y tejido del mesenteron para definir mejor su función en los procesos de desintoxicación y/o producción de feromonas a través de la hibridación *in situ* de estas proteínas.
- Evolutivamente se puede evaluar su relación filogenética con otras especies de escolítidos y tratar de determinar como se dan el proceso de duplicación para dar origen a nuevos genes.

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