UNIVERSITAT DE
BARCELONA

Evaluation of biological control agents against *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) in Portugal

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Barcelona, Marzo de 2015

DOCTORAL THESIS

Departament de Biologia Animal

Universitat de Barcelona

Ph.D. Program: Biodiversidad

Thesis conducted in "Instituto Nacional de Investigação Agrária e Veterinária" (INIAV) in Unidade Estratégica de Investigação e Serviços de Sistemas Agrários e Florestais e Sanidade Vegetal

Evaluation of biological control agents against *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) in Portugal

(Evaluación de los agentes de control biológico contra *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) en Portugal)

Thesis presented by Ricardo Neves Petersen Silva to obtain the Doctorate Degree by Universitat de Barcelona

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ACKNOWLEDGMENTS

I would like to show my gratitude to all the people who contributed to the studies described here. I recognize that without their help and advices I would never have been able to finish this thesis.

Firstly I would like to thanks Juli, my thesis tutor, for his motivation, patience and huge enthusiasm which always encouraged me to go further with this research. Since the first moments, when I arrived to his office and slowly presented me the taxonomic studies and the research developed there. For all the last minute urgencies which always solved with his infinite patience, even when 1200 km apart.

Besides my tutor, I would also like to thank to my thesis directors, Edmundo Sousa and Pedro Naves. Without their advices and monitoring I would never have finished my thesis upon time. I would like to show my sincerely gratitude to Edmundo for giving me the opportunity to follow this research line. His guidance and always important commentaries were an invaluable complement to this thesis and all the work presented.

A very special thanks goes to Pedro for all the time and effort spent with my work. Despite me, he was the second person whom spent more time reviewing and helping to write this thesis. I'm really grateful for the infinite patience, friendship, knowledge and motivation that he had for the past 4 years.

I would also like to thank all my colleagues from INIAV, with who I spent most of my time during the last 4 years. They helped in almost every way possible and gave all the help they could.

I'm thankful to Lurdes Inácio and Joana Henriques for having presented me to the fungi domain which allowed great part of this thesis to be developed. I'm grateful for all the time spent in their office (even when against Joana's will), the small talks and all the advices.

I must also acknowledge, Luis Bonifácio who gave precious opinions and always supported my work. I would also like to thank him for letting me lose (almost) all the sport bets along the past 4 years. Until now I'm not sure if the reduced number that I won was due to luck or Luis mistakes. I'm also grateful to Margarida Vieira for all the conversations, comments and advices during the 3 years that I spent in that office with her.

I would also like to show my gratitude to Adérito Matos, Florinda de Medeiros, Marina Soares and Vítor Gonçalves for all the help during these years. I'm aware that without their help great part of the works would have not been finished.

I'm deeply grateful for Adérito assistance and heavy work, for all the time spent traveling across the country and cutting trees wherever I needed. Most of all, I'm thankful for the jokes that any of us had never heard. All the laboratory work was not possible without the help of Florinda and Marina. I'm grateful to Florinda for spending so much time aiding me in the lab. I also need to thank her for always having cookies or some candy to eat every time I felt hunger. I'm grateful to Marina for all the assistance during these years and above of all, for the friendship. I'm deeply aware of the patience that she needed to have me as a friend. I want also to acknowledge Vítor for the entire time spent helping, for that infinite trip to Monte Gordo and for all the lunches.

Despite not mentioning them, I'm very thankful to all the other colleagues from INIAV that were not stated. Above of all I'm really grateful to them all for having received me so well and allowed me to be part of their magnificent "team".

I would like to show my gratitude to my friends who were always there and never got tired of my complains. Firstly I would like to thank David for his assistance on finishing this thesis. For all the weekends and nights spent reviewing the successive versions of this work. I'm also very thankful to Herlander, Duarte, Edu, Fred, Sara Neves, and Gonçalo who assisted me on long and tiring field assays, even when they had no idea where they were getting into. A special thanks to Herlander for letting me stay at his house all the times that I requested making the long lasting trips a while easier. I did not forget all the other friends that year past year didn't lost their minds hearing about insects, parasitoids, fungi, and all the other "disgusting organisms". I'm thankful to Diogo, Hugo, Joana, Kika, Mónica, Pais, Raquel, Sara, Tiago, Veiga, Vidigal and all the other for being present and always paying attention to my strange conversations related to this work.

I would also like to thank my family for all the support provided during my entire life. For the encouragement to always proceed with my studies and for always giving the opportunities that I wished. Without their support it wouldn't have been possible to accomplish such goals or even start this research. I'm also very thankful to Rita for all the support and that last months of never ending "finish your thesis" moments.

I would also like to thank to all the people belonging to a vast number of Institutes, organizations, and forest associations which collaborated with the works developed for this thesis. I’m grateful to Dr. José Manuel Rodrigues, Dr. Sergey Belokobylskij, Dr Dimitri R. Kasparyan, Dr. Rita Matos Gomes and all the team from AFN Marinha Grande, Mr. Alfredo and his collegues from Monção, Eng. Teixeira and his team from Alcobaça, Eng. António Moreira and Eng. Pedro Serrasqueiro from Herdade da Comporta, Dr. Lluis Simó from UB, Dr. Paula Ramos from Instituto Superior de Agronomia, and all the people from ICNF that helped with the research for this thesis. I’m really grateful to all of them. Without their collaboration, none of this work has been possible.

I would like to express my gratitude to INIAV (Instituto Nacional de Investigação Agrária e Veterinária), UB (Universitat de Barcelona) and the team from REPHRAME project, which provided the facilities, financial assistance and administrative background needed for the developing of this research.

THESIS STRUCTURE

The structure of this thesis is based on the presentation of the research results from a compendium of publications. Following a clear line of thought, this thesis is divided in the following eight chapters:

Chapter one (I) "Abstract / Resumen" - presents a brief summary of the entire thesis and main conclusions, both in English and Spanish.

Chapter two (II) – "Introduction"- is divided in 2 different sub-chapters:

- Subchapter 1 - "The Pine Wilt Disease "

  This section offers a detailed resume about the pine wilt disease and the main aspects related to it. It also provides comprehensive information on the three relevant organisms associated with this disease, together with their biology, life cycles and interaction. It also deals with the historic facts about the pine wood nematode detections, providing information about its origins, dissemination ways and it appearances around the world.

- Subchapter 2 – "Control strategies"

  This subdivision describes the main strategies available to control the pine wood nematode and its insect vector. It presents information about cultural, chemical, biotechnical and biological control methods, which are used across the world. It also precisely focuses on the biological control approaches for *Monochamus* species and other Cerambycidae.

Chapter three (III) – "Objectives" – Includes a brief description of all the objectives planned for this thesis.

Chapter four (IV) – "Results"– is divided into 5 different sub-chapters. Each chapter corresponds to the complete PDF version of each article published or under publication/submission process. For each article a brief abstract is also present in Spanish.

- Sub-chapter IV.1. – “Rearing the Pine Sawyer *Monochamus galloprovincialis* (Olivier, 1795) (Coleoptera: Cerambycidae) on Artificial Diets”.
- Sub-chapter IV.2. – “Distribution, Hosts and Parasitoids of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) in Portugal Mainland”.
- Sub-chapter IV.3. - “Parasitoids of Monochamus galloprovincialis (Coleoptera, Cerambycidae), vector of the pine wood nematode, with identification key for the Palaearctic region”.
- Sub-chapter IV.4. – “Susceptibility of larvae and adults of Monochamus galloprovincialis to entomopathogenic fungi under controlled conditions”.
- Sub-chapter IV.5. – “Effectiveness of fiber bands impregnated with *Beauveria bassiana* for the biological control of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae)”.

Chapter five (V) – “Discussion” - Covers the careful examination and interpretation of the principal results obtained from the developed research. It also tries to evidence possible practical applications for the results and techniques obtained and probable limitations, giving a perspective into future research following this same research lines.

Chapter six (VI) – “Conclusions” – Interprets all the main points obtained during the discussion, presenting the final remarks on all the work developed.

Chapter seven (VII) – “Directors Report” - Includes the reports from the thesis directors concerning the impact factor of each published article and the work developed by each author.

Chapter eight (VIII) – “References” – Summarizes all the detailed information on the references cited and studied to elaborate the present thesis.

I - RESUMEN

La enfermedad del decaimiento súbito del pino (Pine Wilt Disease, PWD) consiste en una interacción entre tres organismos; el nematodo *Bursaphelencus xylophilus* (Steiner y Buhrer) Nickle, un insecto vector del genero *Monochamus* Dejan, y un árbol huésped del género *Pinus* Linnaeus. A pesar de la simplicidad que esta relación puede trasparecer, la realidad es bastante compleja. La biología y ecología del nematodo y del insecto vector están fuertemente sincronizadas, permitiendo a que el nematodo se propague entre huéspedes y que el insecto vector disfrute de nuevos árboles muertos para colonizar. La interacción entre estos tres organismos posibilita la dispersión natural de la PWD. Sin esta estricta asociación el nematodo no conseguiría desarrollar sus poblaciones infectando nuevos huéspedes. Por lo tanto, se puede considerar que esta asociación tiene un carácter obligatorio para el nematodo y facultativo para el insecto. No obstante, el insecto también disfruta de dicha asociación pues el aumento del número de árboles muertos aumenta sus posibilidades de crear nueva descendencia.

El nematodo de la madera del pino (Pine Wilt Nematode, PWN) presenta un ciclo de vida con dos fases distintas; la propagativa y la dispersiva (Wingfield, 1983). La fase propagativa incluye cuatro estadios larvales y un estadio adulto y ocurre cuando las condiciones ambientales son adecuadas. Esta fase ocurre dentro del huésped y eventualmente origina su muerte, pues el nematodo se continua reproduciendo y alimentando de la madera (infectada por hongos), canales resinosos, y de distintas células (Koboyashi *et al.*, 1975). Después de completar los dos primeros estadios larvales, el PWN cambia para la fase dispersiva (fase de resistencia) en la cual podrá entrar en el cuerpo del insecto vector y desplazarse hacia un nuevo huésped (Mamiya, 1975).

Los huéspedes del PWN varían según la parte del planeta en que este se encuentra. En Norte América, este nematodo no es la principal causa del decaimiento del pino en las especies nativas. Su presencia en ese continente suele ser más frecuente en las especies exóticas importadas (Akbulut y Stamps, 2012). En Europa, Portugal es el único país con una gran área de árboles afectados por esta enfermedad, siendo el único huésped el pino marítimo (*Pinus pinaster* Aiton). El hecho de existir a nivel mundial diferentes especies de árboles afectados por la enfermedad, revela las diferentes susceptibilidades de los huéspedes. Estas diferentes susceptibilidades pueden estar relacionadas con la historia evolutiva de las especies, en la cual, nematodo y huésped pueden haber evolucionado al mismo tiempo, generando resistencias. Sin embargo, especies que no presenten síntomas pueden igualmente servir de reservatorios para el PWN (Akbulut y Stamps, 2012).

La propagación del nematodo en nuevos huéspedes es totalmente dependiente del insecto vector perteneciente al género *Monochamus*. Dentro de esto género, cada una de las especies del hemisferio norte presenta huéspedes preferenciales, distribuciones geográficas específicas y ciclos de vida particulares (Morgan, 1947; Ross, 1966; Hellrigl, 1971; Naves *et al.*, 2008). Clásicamente estos coleópteros son considerados como insectos secundarios, que solamente atacan árboles debilitados o que están muriendo. (Linsley, 1959; Linit, 1987; Hanks, 1999). Algunas de las especies de esto género son los principales vectores del PWN (Linit, 1988), ya que no todas están asociadas a la enfermedad; así, por ejemplo, *Monochamus sartor* (Fabricius) y *M. sutor* (Linnaeus) se sabe que no están asociadas a la transmisión del nematodo.

Como otros miembros de la familia Cerambycidae, estas especies pasan gran parte de su ciclo de vida (larva y pupa) dentro del huésped vegetal. Antes de la puesta, las hembras abren una herida en la parte exterior de la corteza del huésped en decaimiento o recién muerto y depositan sus huevos (Evans *et al.*, 1996). Los primeros instares larvales emergen 1 o 2 semanas después de la puesta y durante varios meses se alimentan del cambium situado justo debajo de la corteza del huésped (Naves *et al.*, 2008). Después de este periodo las larvas empiezan a formar su galería por el xilema, que terminarán en una galería pupal (Campadelli y Dindo, 1994). De las pupas emergerán los adultos en el estadio de “callow” (no esclerotizado); el endurecimiento (esclerotización) lo conseguirán posteriormente después de emerger por un agujero con una forma perfectamente redonda. La emergencia de estos insectos es seguida por un periodo de 2 semanas de ‘pasto de maduración’, en la que mayoritariamente su alimentación se basa en la corteza de los ramas más delgados. Después de este periodo se inicia el periodo de puesta, originando a un nuevo comienzo y una nueva generación para el insecto (Tomiczek y Hoyer-Tomiczek, 2008).

Entre todas las especies del género *Monochamus* existe una que presenta una importancia más remarcable, *M. galloprovincialis* (Olivier), la cual está ampliamente distribuida por toda la Europa (excepcionando Chipre, Irlanda y el Reino Unido) y que puede ser frecuentemente encontrada en la zona circunmediterránea en países como España, Francia, Italia o Portugal (Francardi y Pennacchio, 1996; Koutroumpa, 2007).

En Portugal, este cerambícido fue inicialmente reportado por Oliveira (1984), que colectó especímenes de la región de Leiria y que equivocadamente lo determinó como *M. sutor*. Antes de la asociación entre el nematodo y esta especie muy pocos trabajos fueron desarrollados, existiendo apenas algunas descripciones detalladas de su morfología (Hellrigl, 1971; Francardi y Pennacchio, 1996; Francardi *et al.*, 1998). En los últimos años la distribución incierta de estos insectos se viene aclarando, con descubiertas de agujeros de emergencia por todo el país e incluso Madeira (Fonseca *et al.*, 2012) y Azores (Borges *et al.*, 2013). Desde su asociación con el nematodo, estos cerambícidos fueron más estudiados, lo que originó algunos estudios sobre su biología y ecología (e.g. Naves *et al.*, 2006a, b, 2008; Koutroumpa *et al.*, 2008; Akbulut, 2009).

En lo que respecta al ciclo de vida de esta especie, algunas variaciones ocurren conforme al país y a las condiciones climáticas que ahí existan. En países donde los inviernos son más rigorosos, con temperaturas más bajas las larvas de *M. galloprovincialis* entran en diapausa por periodos más largos, por lo que las generaciones tardan dos años en sustituirse (Tomminen, 1993). En los países Mediterráneos en que los inviernos son más cálidos con temperaturas más elevadas, las generaciones tardan apenas un año. Sin embargo, en cualquier territorio en condiciones naturales, las larvas del insecto tienden a presentar un periodo de entumecimiento invernal (Naves *et al.,* 2008). En Portugal las emergencias de adultos empiezan al final de mayo y suelen durar hasta principios de septiembre, presentando un pico de emergencias entre junio y julio (Naves *et al.*, 2008)

Hasta hace poco no existía información relativa al método de inoculación del nematodo en nuevos huéspedes por medio del cerambícido. Estudios parecidos habían sido desarrollados en otros continentes con huéspedes y vectores diferentes (e.g. Mamiya, 1984; Linit, 1990); sin embargo, trabajos desarrollados por Naves *et al.,* (2006b, c, 2007a) demuestran que el *M. galloprovincialis* suele transmitir el nematodo a nuevos huéspedes mayoritariamente durante su pasto de maturación, con un pico de transmisión en la segunda semana después de la emergencia.

La introducción del nematodo en un árbol sano activa la habilidad del nematodo para alimentarse de células tanto del parénquima como epiteliales, lo que origina el cese del flujo de agua por los tejidos del árbol. Debido a la detención del flujo de agua, la transpiración cesa, lo que origina una decoloración en las agujas, cambiando de verde a amarillo o marrón. Después de la decoloración de las agujas, usualmente el árbol empieza a desarrollar una gran diversidad y abundancia de hongos en su interior, lo que subsecuentemente originará su muerte (Mamiya, 1984).

Los síntomas característicos de la PWN no son específicos de esta enfermedad, pues varios cerambícidos y otras enfermedades suelen causar síntomas similares (Chararas y M'Sadda, 1973; Sousa *et al.*, 2011). Con este problema en mente, se desarrollaron técnicas más precisas para detectar la presencia del nematodo en la madera. El principal método utilizado para confirmar su presencia se basa en la obtención de madera del pino afectado, seguida de la extracción del nematodo del interior de la madera en un laboratorio específico para análisis de este tipo de enfermedad (OEPP/EPPO, 2013). A pesar de estos análisis, frecuentemente se utilizan técnicas como el ITS-PCR para la confirmación de la especie *B. xylophilus* pues suelen aparecer en las muestras otros nematodos bastante similares a éste (Ribeiro *et al.,* 2012).

La entrada definitiva de *B. xylophilus* en Europa ocurrió en 1999 en territorio portugués, en la región de Pegões. Se supone que este organismo, inicialmente originario de los Estados Unidos, llegó a Portugal desde Asia del Este (Mota y Vieira, 2008) por medio del comercio y transporte de madera o productos derivados de ésta (Evans, *et al.,* 1996). Después de la confirmación de su presencia en Portugal, una serie de medidas fueron implementadas para controlar la expansión de la enfermedad. Sin embargo nuevos focos aparecieron tanto en el centro del país como en la isla de Madeira, lo que originó la implementación por parte de la UE de medidas de prohibición de comercio de madera de este pino hacia el exterior.

A pesar de todas las medidas implementadas, todos los años un número bastante elevado de árboles han de ser cortados de los pinares portugueses. En el bienio de 2011/2012 aproximadamente 900.000 árboles fueron identificadas como portadores de síntomas de PWD y cortados en el terreno (Anonymous, 2012). La introducción de este nematodo en el territorio portugués ya originó un cambio drástico en la silvicultura nacional, con alteraciones en las especies dominantes y perdidas de área (alrededor del 0.3 % al año) de los pinares de *P. pinaster* (ICNF, 2013).

Las medidas de control para evitar la dispersión de la enfermedad en el territorio portugués están separadas en 3 áreas diferentes: (i) evitar la propagación de las poblaciones de nematodo por dispersión de insectos portadores del organismo, (ii) controlar las poblaciones del nematodo y (iii) la obtención de árboles resistentes a la enfermedad (Sousa *et al.,* 2011). Todas estas acciones están mayoritariamente direccionados para la identificación, corte y destrucción de los árboles sintomáticos durante los meses de otoño, invierno o primavera. Sin embargo, la enfermedad sigue propagándose y su reciente detección en España (Abelleira *et al.*, 2011) enfatiza la necesidad de descubrir nuevos métodos de control. Con esta finalidad se estudiaron los métodos de control preexistentes (culturales, químicos, bio-técnicos y biológicos) con la intención de descubrir y desarrollar un nuevo método de control para este cerambícido.
Los métodos culturales son actualmente los más utilizados (conjuntamente con los bio-técnicos) para controlar la propagación de la enfermedad, donde los árboles infectados son cortados y eliminados en las estaciones más problemáticas en relación al desarrollo del insecto vector. La legislación Portuguesa, para los materiales menores de 20cm provenientes de árboles muertos o que presenten síntomas de la enfermedad, concluye que han de ser destruidos por acción mecánica, mediante fuego o con fumigación. En el caso de pretender utilizar la madera posteriormente, ésta debe ser sometida a tratamientos a elevadas temperaturas conforme la legislación (Anonymous, 2011). También inserido en los métodos culturales, se están desarrollando algunas líneas de investigación con el propósito de seleccionar linajes de pinos resistentes a la PWD para futuros planos de reforestación (Ribeiro, 2012).

En cuanto a los métodos químicos, éstos están emarcados actualmente más como una estrategia de prevención contra nuevos focos en vez de una estrategia de control. La utilización de estas técnicas fue frecuentemente usada para controlar las poblaciones del insecto vector (Kobayashi, 1988; Jianchang *et al.*, 1998; Takai *et al.*, 2003), sin embargo, la utilización de estas técnicas suele tener impactos negativos en el ecosistema por la utilización de agentes químicos. En Portugal el uso de químicos como medida de control del PWN está bastante restringido, existiendo apenas una sustancia aprobada: el Benzoato de Emamectina. Esta sustancia sirve para prevenir ataques de diversos insectos a los pinos (Liguori *et al.*, 2010; Smitley *et al.*, 2010) y es aplicada por medio de inyecciones en el tronco (Sousa *et al.,* 2013). El uso de la fumigación, es también posible para el tratamiento de la madera infectada con el PWN (Bonifácio *et al.,* 2014).

Otro método utilizado para el control del insecto suele ser el bio-técnico, lo cual permite la captura de un elevado número de individuos, aumentando la posibilidad de reducir poblaciones. Las trampas multi-embudo con atractivos específicos son las más utilizadas en esta área (Rassati *et al.,* 2012), pues el *M. galloprovincialis* utiliza las señales químicas tanto para alimentarse, para acoplamiento e incluso para hacer las puestas (e.g. Ginzel y Hanks, 2003; Faccoli et al., 2005). Actualmente el compuesto químico más utilizado como atractivo en las trampas en la Península Iberia se llama Galloprotect 2D© y está mayoritariamente constituido por la feromona de agregación del cerambícido, capturando grandes cuantidades de adultos de ésta especie.

Los métodos biológicos de control de plagas todavía no están significativamente desarrollados para este insecto en Portugal, sin embargo por todo el globo se puede encontrar casos de éxito en el combate de diversas plagas, tanto forestales como agrícolas. Se han desarrollado nuevos métodos para combatir dichas plagas mediante el uso de hongos y nematodos entomopatógenos y el uso de poblaciones de enemigos naturales, tanto parasitoides como depredadores (Webster, 1973 Meierrose *et al.*, 1991; Snyder y Wise, 1999; Solomon *et al.*, 2000). Estos métodos son usualmente más sencillos de utilizar en áreas agrícolas en las que los cultivos están dispuestos de forma continua y no están tan dispersas como en el bosque; a presar de esto también se pueden encontrar algunos trabajos con resultados bastante interesantes en el área forestal (e.g. Reis *et al.,* 2012; Borowiec *et al.,* 2014; Matosevic *et al.,* 2014).

En el caso de *M. galloprovincialis*, que pasa gran parte de su ciclo de vida dentro del tronco de sus huéspedes, la utilización de cualquier técnica de control, especialmente las biológicas, suelen presentar algunas dificultades de implementación. Consecuentemente, el desarrollo de técnicas de control biológico deberá estar direccionado a aplicaciones de agentes mientras los insectos se encuentran más expuestos. A pesar de los diferentes métodos disponibles para desarrollar estudios de control biológico, en este trabajo solo se estudió el uso de parasitoides y la aplicación de hongos entomopatógenos.

El equilibrio de un ecosistema sano está bastante regulado y compensado entre depredadores, parasitoides y plagas, por eso, un pequeño cambio en las densidades de cada organismo puede originar abultadas desregulaciones ambientales, llevando por ejemplo a un aumento de plagas en un determinado lugar. Los humanos son uno de los agentes frecuentemente responsables de generar desequilibrios ambientales entre especies. Los humanos son los responsables de la introducción (involuntaria o intencional) de un gran número de organismos en territorios en los cuales no existían previamente (Pyšek *et al.*, 2010). A pesar de los efectos que dichas introducciones suelen tener a largo plazo, el uso de parasitoides o depredadores es utilizado actualmente como medida de control biológico. Desafortunadamente aunque el uso de agentes bióticos no nativos es más común que los existentes en determinadas regiones (e.g. Nafus, 1993; Ayalew y Hopkins, 2013), en algunos hábitats el uso de agentes nativos sigue existiendo (e.g. Mackauer y Völkl, 1993; Núñez-Campero *et al.*, 2014)

En lo que se refiere a los bióticos forestales, y más concretamente a las familias Buprestidae, Cerambycidae y la subfamilia Scolytinae, se puede encontrar una gran diversidad de trabajos direccionados al control biológico por medio de parasitoides (e.g. Azevedo y Waichert, 2006; Cooperband *et al.*, 2013). Trabajos relacionados con el género *Monochamus* se pueden encontrar fácilmente, pero para *M. galloprovincialis* hay muy pocos estudios relacionados con el control biológico de esta especie (Francardi y Pennacchio 1996; Francardi *et al.*, 1998; Martikainen y Koponen, 2001; Naves *et al.*, 2005).

En países Asiáticos, diversos trabajos relacionados con el uso de parasitoides de huevos o larvas se están desarrollando con bastante éxito. Los investigadores de estos países están trabajando con las especies nativas de *Monochamus* para encontrar parasitoides eficaces e implementarlos en programas de control biológico. De acuerdo con Xu (1998), Wang *et al.* (2009) o Hu *et al.* (2012), la especie *Monochamus alternatus* Hope ya sido ya asociada a los parasitoides *Scleroderma guani* Xiao et Wu (Hymenoptera: Bethylidae), *Bracomorpha ninghais* Wang, Chen, Wu, He (Hymenoptera: Braconidae) y *Sclerodermus harmandi* Bursson (Hymenoptera: Bethylidae). El otro *Monochamus* más frecuente en estos países, *Monochamus saltuarius* (Say), ha sido también asociado al parasitoide *Rhimphoctona* (Xylophylax) *lucida* (Clément) (Hymenoptera: Ichneumonidae). Segundo Luo & Sheng (2010) y Reagel *et al*. (2012), en Estados Unidos, *Monochamus carolinensis* (Olivier) fue parasitado por especies del género *Digonogastra* Viereck, y *Billaea monohammi* (Townsend) (Diptera: Tachinidae).

Para el *M. galloprovincialis*, Naves *et al.* (2005) analizó los registros existentes de parasitoides, donde se puede destacar las especies *Atanycolus genalis* (Thomson) (= *initiator* F.) (Hymenoptera: Braconidae), *Coeloides sordidator* Ratzeburg (Hymenoptera: Braconidae), *Cyanopterus flavator* Fabricius (Hymenoptera: Braconidae), *Dolichomitus tuberculatus* (Geoffroy) (Hymenoptera: Ichneumonidae), *Doryctes mutilator* (Thunberg) (Hymenoptera: Braconidae), *Iphiaulax impostor* (Scopoli) (Hymenoptera: Braconidae) y *Meteorus corax* Marshall (Hymenoptera: Braconidae). Estas especies fueron encontradas parasitando el vector del PWN en la región Paleártica, por lo que fueron necesarios estudios más pormenorizados para el territorio Portugués. Por esto, una parte de los estudios presentados en esta tesis se han centrado en la prospección del complejo de parasitoides asociado a esta especie de *Monochamus*.

Del mismo modo, el uso de hongos entomopatógenos como método de control biológico ha sido estudiado para diversas plagas forestales y agrícolas (e.g. Abe y Ikegami, 2005; Zhan y Morse, 2013). El uso de estos organismos en alternativa a los químicos suele ser también una buena opción, ya que no tiene las implicaciones ambientales que estos segundos pueden presentar. Como para los parasitoides, también para los hongos se puede encontrar trabajos sobre su efecto en las poblaciones de insectos que suelen representar una amenaza para los bosques (e.g. Hu *et al.*, 2010;

Castrillo *et al.*, 2010; Yao *et al.*, 2012); algunas de las especies estudiadas por los autores mencionados presentan ciclos de vida bastante similares al del *M. galloprovincialis* (Ambethgar y Mahalingam, 2002; Dubois *et al.*, 2008; Marannino *et al.*, 2010).

Gran parte de los trabajos efectuados sobre el efecto de estos hongos en el género *Monochamus* se basan en los hongos *Beauveria bassiana* (Bals.-Criv.) Vuill y *Metharizium* spp.; en China hongos como *Cephalosporium* sp., *Fusarium* sp., *Penicillium* sp., *Verticillium* sp., *Serratia* sp. o *Paecilomyces* sp., también fueron asociados a *M. alternatus* (Shimazu *et al.*, 2002; Shimazu y Sato, 2003).

En *M. galloprovincialis*, *B. bassiana* se demostró ser responsable por cerca de ¼ de la mortalidad larval dentro de la madera (Naves *et al.,* 2008), además de reducir la capacidad del insecto a alimentarse, disminuyendo así la capacidad de transmitir nematodos (Maehara *et al.*, 2007). El principal problema relacionado con la utilización de estos hongos reside en la forma como se aplican en el terreno, ya que son bastante susceptibles a condiciones ambientales como el viento, la radiación UV o la humedad. El método que se probó ser más eficaz para las aplicaciones exteriores fue la utilización de bandas de hongos impregnadas con cultivos vivos de los mismos (Shimazu, 2004; Shanley *et al.*, 2009).

Los ensayos desarrollados con hongos entomopatógenos durante el trabajo que aquí se expone fueron basados en la futura aplicación de esto tipo de bandas impregnadas con *B. bassiana*. Sin embargo, solamente fueron desarrollados ensayos de laboratorio, quedando toda la parte experimental "in situ" por realizar en futuras investigaciones.

A pesar de todas estas diferentes medidas de control, sigue siendo necesario el desarrollo de nuevas técnicas basadas en métodos de combate biológico. Descubrir y desarrollar dichos métodos ayudaría a reducir el impacto negativo que los métodos químicos puedan provocar en el ecosistema. Con este propósito se planearon y desarrollaron los trabajos presentados en esta tesis durante los últimos 4 años.

Los trabajos realizados en estos años pueden dividirse en 3 partes distintas: (i) entender la actual distribución del insecto en Portugal y desvendar los enemigos naturales parasitoides asociado al *Monochamus galloprovincialis*, (ii) descubrir las comunidades de hongos entomopatógenos que pueden servir de control biológico a dicha especie y (iii) un capitulo adicional donde se pretendió estudiar una técnica más efectiva para la reproducción en el laboratorio en gran escala de las poblaciones de este insecto.

El primer capítulo no está directamente relacionado con el control biológico del insecto, ainque fue desarrollado para intentar mejorar la capacidad de producción y manutención de las poblaciones de *M. galloprovincialis* en el laboratorio. Una de las preocupaciones más importantes, cuando se trata de poblaciones de insectos de laboratorio, es la capacidad de conseguir mantener varias generaciones sin recurrir a materiales o tejidos vivos. El objetivo principal de este capítulo fue desarrollar un "kit" que permitiría reproducir todo el ciclo de vida del cerambícido en condiciones de laboratorio sin tener que recurrir a árboles vivos. El primer punto esencial para esto, era obtener una dieta artificial que permitiera a las larvas el desarrollo durante todo el periodo de alimentación. Las dietas que presenten una tasa de obtención de adultos igual o superior a 75% sirven para producción de insectos en laboratorio. Sin duda, este resultado se logró con una dieta artificial, la cual no fue hecha a partir de tejidos vivos, y la cual se basa totalmente en el uso de productos comerciales.

A pesar de los avances, todavía es esencial la creación de un sustrato artificial que permita al insecto poner huevos fértiles. Después de dicha aportación se espera que sea posible la producción masiva y eficaz de poblaciones de *M. galloprovincialis* en condiciones de laboratorio en menos de 60 días.

Los estudios desarrollados sobre la distribución geográfica del *M. galloprovincialis* en Portugal, demostraron que sus poblaciones se encuentran ampliamente distribuidas en todo el país y están fuertemente sobrepuestas a la distribución del pino marítimo. Si las distribuciones del insecto encontradas por diversos autores del siglo XX (Oliveira, 1894) eran correctas, se presume que existió una rápida expansión de sus poblaciones en los últimos años, ayudada por una serie de factores. La preferencia clara por utilizar *P. pinaster* como huésped puede estar relacionada con la ausencia de otras especies de árboles que también sirvan como huéspedes para el insecto. Este puede ser el caso del *P. sylvestris* o del *P. nigra* que son muy poco frecuentes en el país (ICNF, 2013). Sin embargo, en países donde estas especies son más frecuentes, la transmisión de la enfermedad para estos individuos puede ser un grave peligro. Los factores climáticos y las variaciones de altitud parecen no tener una marcada influencia en la distribución del insecto, pues diversos especímenes fueron encontrados en distintas partes del territorio. Otro factor que no deberá ser ignorado, son las vastas campañas de reforestación que ocurrieron en el último siglo, y que pueden haber contribuido para la posible expansión del cerambícido. Además, y como ha sido sugerido por Moretti *et al.* (2004), los incendios forestales que ocurrieron pueden haber tenido un impacto positivo en la densidad y distribución de las poblaciones de *M. galloprovincialis*. Esta especie es conocida por ser grandemente atraída por esto tipo de eventos, pues sus poblaciones tienden a hacer las puestas de huevos en árboles quemados o en decaimiento (Bonifácio *et al.,* 2012).

La expansión de las poblaciones también fue seguramente influenciada por la biología del insecto. Diversos estudios demostraron que su capacidad de vuelo puede ser bastante elevada (David *et al.,* 2013; Mas *et al.,* 2013); sin embargo esto representaría un gran costo de energía, lo que se incompatibiliza con la teoría que los insectos recién emergidos tienden a no se apartar demasiado de su punto de eclosión (Sun and Du, 2003). Expansiones de las poblaciones de insectos para localidades como la isla de Madeira (Fonseca *et al.,* 2012) seguramente no pueden estar relacionadas con la elevada capacidad de vuelo del insecto pues están a más de 1000 km de distancia del continente donde las poblaciones existen. A pesar de todos estos factores que pueden haber llevado a la expansión de las poblaciones, no debe ser ignorado que el factor más probable puede ser que los humanos a través de transportes inadvertidos de especímenes para nuevos territorios hayan propagado involuntariamente la plaga.

En relación al trabajo de la distribución actual del cerambícido en Portugal, se prospectaron las áreas donde *P. pinaster* es la principal especie forestal. De acuerdo con la cuadrícula UTM (Universal Tranverse Mercator 10x10 Km.) se prospectó cerca de 15% de toda esta área. Se encontraron indicios de este insecto en aproximadamente 94% de toda el área analizada.

A pesar de estos resultados, lugares en los que no se detectó *M. galloprovincialis* y que no están en la red nacional oficial de trampas del estado, no deben ser considerados como ausentes del insecto. Estos individuos suelen ser poco visibles en su estado salvaje, lo que puede llevar a suponer que todavía no se han detectado. Los estudios se llevaron a cabo por medio de observaciones directas de los insectos adultos y por los registros de agujeros de emergencia en los árboles muertos. Todos los agujeros de emergencia fueron analizados cuidadosamente para aclarar a qué especie pertenecía. Además de estos análisis, otras especies forestales y ornamentales encontradas en las regiones que se prospectaba, fueron también observados para detectar la presencia del insecto vector (*Pinus sylvestris* L., *Pinus halepensis* Mill., *Pinus pinea* L., *Pinus radiata* D.Don., *Larix decidua* Mill., *Cupressus lusitanica* Mill. y *Chamaecyparis lawsoniana* (A.Murray bis) Parl.) De estos análisis, solamente las especies *P. pinaster*, *P. sylvestris* y *P. halepensis* presentaron vestigios de presencia del insecto. Las dos últimas especies mencionadas fueron nuevos registros del huésped vegetal para Portugal.

Los trabajos sobre el complejo de parasitoides asociado al *M. galloprovincialis* demostraron que el número de especímenes encontrados en la región de Leiria, cuando eran comparadas con otras regiones, fueron bastante elevados, lo que podría explicar la reducida población del cerambícido encontrada en este territorio. Segundo Mass *et al.* (2013) este factor puede deberse al trabajo constante de manutención y gestión de este pinar, ya que esto puede favorecer el estabelecimiento de poblaciones y un equilibrio más sólido al largo del tiempo. Los bajos números de *M. galloprovincialis* pueden ser difíciles de entender pues, si su número bajara, la densidad de parasitoides también debería seguir el mismo camino. Sin embargo, todos los parasitoides encontrados presentan hábitos generalistas, pudiendo utilizar como huésped otras especies de insectos que existan en dicho momento cuando las poblaciones de *Monochamus* bajan. Como ya ha sugerido Chrysopolitou *et al.* (2013), también se debe considerar la proximidad con la mar, ya que puede influenciar positivamente el ecosistema, lo cual no quedará demasiado vulnerable a la sequía o a temperaturas extremas, reduciendo así la incidencia de patógenos y plagas forestales.

La evaluación del complejo parasitoide se fundamentó en dos métodos distintos: (i) recogida de árboles colonizados por *M. galloprovincialis* y (ii) la instalación de árboles artificiales de trampa en el campo. Esta metodología fue organizada de acuerdo con las diferentes etapas de desarrollo del insecto, repitiéndose para los huevos, larvas floémicas, larvas xilémicas, y pupas. De esta prospección solamente se obtuvieron resultados positivos de parasitismo para las larvas sub-corticales. Dichas larvas corresponden a los primeros instares de desarrollo del insecto. Entre todos los parasitoides encontrados, la especie que demostró ser la más promiscua fue *Cyanopterus flavator* (Fabricius), la cual se encontró en un número más elevado de localidades. A pesar de esta especie, también se colectaron individuos pertenecientes a las especies *Atanycolus denigrador* (Linnaeus), *Atanycolus ivanowi* (Kokujev), *Doryctes striatellus* (Nees) (Braconidae), y *Xorides depressus* (Holmgren). De estos cuatro últimos sólo *A. ivanowi* no era una nueva especie para la fauna portuguesa.

Este complejo de especies asociado al *M. galloprovincialis* en Portugal, demuestra que a pesar del elevado número de especies, ninguna puede ser considerada como un candidato relevante para un programa de control biológico ya que todos presentan hábitos generalistas. Esto puede estar relacionado con la diversidad de especies, con hábitos y ciclos de vida semejantes al *M. galloprovincialis* en Portugal, lo que permite que los parasitoides elijen entre las especie de huéspedes que presenten una abundancia más elevada, y que puede no ser constantemente *M. galloprovincialis.* Sería entonces relevante desarrollar estudios sobre el impacto de estos parasitoides sobre la reminiscente diversidad de especies de insectos que habitan en el mismo ecosistema que los *Monochamus*.

Las 14 especies de parasitoides encontradas asociadas al vector del PWN demuestra que existe una gran variedad de enemigos naturales artrópodos en nuestro territorio, y que probablemente se podría descubrir algunos más en un estudio más profundo en regiones como la de Leiria, donde la comunidad es bastante diversa. En contraste con las otras especies de parasitoides encontrados, el género *Cyanopterus* fue detectado en varias localidades. Esto puede ser interpretado como una adaptación intra-especifica de esta especie a los diferentes ecosistemas, lo que sería una ventaja en un futuro programa de control biológico.

La introducción de especies provenientes de otros territorios en que ya estén asociadas como parasitoides de otras especies de *Monochamus* debería ser considerada, pues existe una gran probabilidad de servir como parasitoide de la especie existente en Portugal. Sin embargo, la introducción de especies exóticas debe ser detalladamente estudiada, pues podria tener un gran impacto en la entomofauna local, ya que su introducción podría originar un desequilibrio en el ecosistema en que se introduce (e.g. Boughton *et al.,* 2012). Existen ya algunos casos de estudios exitosos con especies cercanas a *M. galloprovincialis*, como el caso de *Monochamus alternatus* Hope en Asia. En estos países, donde el nematodo ya está presente hace bastante, diversos ensayos con las especies *Scleroderma guani* Xiao et Wu y *Scleroderma harmandi* (Buysson) se van desarrollando con éxito (Xu *et al.*, 2008; Li *et al.*, 2009; Lai *et al.*, 2012). Estas especies podrían ser candidatos interesantes a tener en cuenta para una introducción pretendida, ya que no se conocen en Portugal o países más cercanos. Sin embargo, como las especies de parasitoides portuguesas, estos dos candidatos tendrían de ser ampliamente estudiados, ya que también presenta hábitos generalistas y podrían provocar un elevado costo en el ecosistema y la fauna de insectos portugueses.

Otra estrategia interesante seria la implementación de un programa de control biológico basado en el uso de murciélagos o aves depredadoras de insectos, como los miembros de la familia Picidae. Distintos estudios han revelado que las poblaciones de pájaros carpinteros pueden tener un impacto notable en el control de plagas (Wu y Liu, 1999; Liu y Wang, 2003; Zhang, 2003). En los bosques portugueses se pueden encontrar cuatro especies perecientes a esta familia, *Dendrocopos major* (Linnaeus), *D. minor* (Linnaeus)*, Picus viridis* Linnaeus and *Jynx torquilla* (Linnaeus), y todas presentan dietas más o menos insectívoras (Rabaça, 2008).

Sin embargo, existen algunas dificultades al respecto de la utilización de dichas especies. A pesar de presentaren dietas insectívoras, no siempre son adecuadas al control de poblaciones de Cerambycidae, las cuales pasan bastante tempo de su ciclo de vida en el interior de la madera. Como ejemplo, *P. viridis* se alimenta mayoritariamente de diversas especies de hormigas, mientras *D. minor* durante los meses de invierno (cuando las poblaciones de *M. galloprovincialis* se encuentran en estadios larvales dentro de la madera) se alimenta en gran parte de semillas y frutos

(Bruun *et al.*, 2002). Igualmente, *J. torquilla* también no podría ser un buen agente de control pues solo habita el país entre los meses de mayo y septiembre y no busca por alimento en el interior de la madera (Bruun *et al.*, 2002; Rabaça, 2008).

El pájaro carpintero que podría presentar resultados más promtedores seria *D. major.* Sin embargo esta especie solo es efectiva en el control de *M. galloprovincialis* en locales donde la PWN es endémica y no epidémica como en Portugal (Zhao *et al.* 2008). Según Kobayashi *et al.* (1984), se debe considerar que la utilización de estas especies suele ser efectiva en regiones donde las poblaciones de *Monochamus* presentan bajas densidades, lo que no suele pasar en algunas regiones Portuguesas. Además, el incremento de las poblaciones de estos pájaros, presupone que existan suficientes recursos naturales para su alimentación, lo que está fuertemente desaconsejado pues dejar los árboles muertos en el campo puede incrementar y facilitar la proliferación de la PWD, pues aumentaría los locales disponibles para las puestas del cerambícido (Naves *et al.,* 2008). Sin embargo sería interesante desarrollar estudios más profundos sobre la eficacia de dicha especie en el control de las poblaciones del insecto.

En cuanto al uso de murciélagos para el control del insecto, tendríamos el mismo problema, ya que solamente dos especies de estos mamíferos existen en los mismos bosques que el *M. galloprovincialis* en Portugal: *Pipistrellus pipistrellus* (Schreber) y *Pipistrellus pygmeus* (Leach). Los hábitos alimenticios de estas dos especies también representan una dificultad, visto que mayoritariamente se alimentan de dípteros (Rehak *et al.*, 2005).

Además de todas estas alternativas para uso en un programa de control biológico, también los hongos entomopatógenos deben ser considerados. Esta alternativa fue abordada como el desarrollar de un complemento al uso de redes impregnadas con insecticida, que son usualmente utilizadas para controlar las emergencias de insectos de la madera infecta por el nematodo. El objetivo principal seria identificar el aislado de una especie de hongo que pudiera disminuir la vida de los insectos adultos en menos de 10 días, reduciendo la probabilidad de transmitir la enfermedad en nuevos huéspedes. Se probaron seis aislados de hongos entomopatógenos. Todos los aislamientos se realizaron desde cadáveres de *Rhynchophorus ferrugineus* (Olivier) o *M.*

galloprovincialis encontrados en territorio portugués. Se testaron tres aislados de *Beauveria bassiana* (Bals.-Criv.) Vuill, uno de *Metarhizium anisopliae* (Metchnikoff) Sorokin, uno de *Metarhizium* sp., y un aislado de *Fusarium* sp. Con el objetivo de conocer las diferentes susceptibilidades de los diferentes estadios de desarrollo del insecto, dichos aislados fueron diferentemente testados en estadios larvales y adultos de *M. galloprovincialis*.

Estos ensayos tuvieron resultados bastante interesantes. Aparentemente, las larvas y adultos de *M. galloprovincialis* presentan diferentes vulnerabilidades a los hongos testados. Como otros autores (Xue-You *et al*., 2005; Hajek *et al*., 2008), también aquí los adultos probaron ser más susceptibles a las infecciones por *B. bassiana*; en cuanto que las larvas fueron más afectadas por los aislados de *Metarhizium* sp. y *Fusarium* sp. El modo de acción de dichos hongos no es completamente conocido, sin embargo las diferentes vulnerabilidades de cada estadio de desarrollo sugieren que los hongos estudiados puedan presentar diferentes preferencias mediante la composición de la cutícula de los insectos en cada estadio.

Se sabe que cuando las esporas de *B. bassiana* interaccionan con la cutícula de los insectos, desarrollan fuertes conexiones con la superficie, empezando seguidamente a penetrar el interior del insecto, matándolo en menos de 10 días (Boucias *et al.,* 1988). El mismo autor descubrió que los diferentes instares larvales cuando expuestos a las mismas concentraciones de esporas, suelen presentar diferentes capacidades de supervivencia. Esto se justificó por la diferente composición de la cutícula de las larvas de último instar, la cual es bastante menos hidrofóbica que en los adultos. La diferente vulnerabilidad de las larvas en relación a los adultos se puede también justificar por la agregación de las esporas con la cutícula, la cual empieza a liberar quitinasas y proteasas (Clarkson y Charnley, 1996). Estas sustancias suelen ser más efectivas cuando la cuantidad de quitina y proteínas es más elevada, lo que pasa en los adultos (Gullan y Cranston 1994). Como demostrado por Yanhua *et al.* (2007), un incremento en la expresión del gen responsable del desarrollo de una quitinasa especifica puede afectar positivamente la virulencia de *B. bassiana* contra los insectos. Se puede concluir que los adultos serán más afectados por los hongos que demuestren tener esporas con características menos hidrofóbicas y que presenten quitinasas y proteasas con una virulencia más elevada. Como *Metarhizium* sp. y *Fusarium* sp. probaron ser más eficaces en reducir la longevidad de las larvas, se puede concluir que estas esporas presentan una afinidad superior a la cutícula de las larvas, la cual presenta una superficie más húmeda y una relación de proteínas más baja, tal como ha sido demostrado por Li *et al.* (2010).

Con la intención de desarrollar una técnica de aplicación de dichos hongos en el ambiente, fueron testados dos métodos de aplicación para las suspensiones de esporas. Se testó un método basado en el contacto directo por parte del insecto en la suspensión de esporas, y otro fundamentado en la aplicación por medio de un spray. El primer método probó ser más eficaz que el método de la aplicación de un spray en el insecto. A pesar de los resultados positivos obtenidos con los estadios larvales, existe un punto de elevada relevancia para considerar. Considerando que los estadios larvales del insecto pasan la generalidad de su tiempo en el interior del tronco de pino, la aplicación efectiva de las suspensiones de esporos contra el insecto seria casi impracticable. Debido a que los adultos de *M. galloprovincialis* demostraron ser más susceptibles al contacto directo con las suspensiones, se desarrolló el siguiente paso del estudio.

Tal como anteriormente utilizado para otras plagas forestales, y utilizando el aislado más eficaz de *B. bassiana,* se empezó la creación de bandas de fibra de poliéster impregnadas con el hongo. Tales bandas, cuando eran impregnadas con un medio artificial, demostraron ser capaces de sostener el hongo inoculado, permitiendo así su desarrollo y esporulación. La principal preocupación con respecto a estas bandas de fibra estaba relacionada con su eficacia y durabilidad cuando expuesta a condiciones de campo. De esta manera, se ensayó en condiciones de laboratorio la eficacia de las bandas de fibra sometidas a períodos de degradación. Los períodos de degradación fueron creados artificialmente dentro de una cámara bioclimática, la cual simuló durante 15 días la temperatura y humedad existente entre noches y días en el mes de julio en Lisboa.

Similarmente a Shimazu (2004) o Hajek *et al*. (2006), los resultados mostraron que incluso cuando estuvieron degradadas por un periodo de 15 días, las bandas impregnadas siguen presentando una elevada eficacia. Los adultos del insecto que se pusieron en contacto con las bandas, murieron antes de los 10 días. Si se considera que los adultos de *M. galloprovincialis* presentan un máximo de transmisión del PWN a los nuevos pinos 10 días después de la emergencia, es de gran relevancia el resultado obtenido. Ser capaz de eliminar a los insectos antes de que lleguen a su máxima capacidad de transmisión de la enfermedad es esencial para cualquier método de control desarrollado. Además, mientras se desarrollaba el ensayo, se pude observar que los insectos infectados por los hongos, reducían significativamente la cuantidad de excrementos que producían, sugiriendo que las infecciones disminuyen su capacidad de alimentación. Esto dato juntamente con la muerte de los insectos en menos de 10 días revelan la posible importancia que este hongo podría tener en un futuro programa de control biológico.

La utilización de estas bandas tiene bastante interés no solo para el control de las poblaciones en el periodo de vuelo, sino también para la aplicación en los árboles muertos en el suelo o mismo en los residuos de corte. Actualmente y segundo la ley (Anonymous, 2011) es obligatorio la utilización de redes impregnadas con insecticida en el transporte de madera infectada, de forma a impedir las emergencias del insecto durante el transporte. Sin embargo, esta técnica tiene algunos problemas, ya que la utilización del insecticida no se restringe apenas a los *Monochamus* que emergen de la madera, sino a todos los insectos que entren en contacto con ella. Se precisa de una técnica mixta, donde se utilizarían las redes y por debajo las bandas impregnadas con hongos entomopatógenos, disminuyendo así las muertes de otros insectos y los riesgos para el ambiente. Este método aquí desarrollado podría ser usado en áreas sensibles, como áreas protegidas o parques nacionales, que pueden verse afectados por el PWD y en los que no se deben utilizar insecticidas u otras técnicas prejudiciales para el ecosistema.

Los trabajos desarrollados durante esta tesis permitieron el avance exhaustivo del conocimiento sobre algunos agentes de control biológico que pueden afectar el *M. galloprovincialis* en Portugal. El desarrollo de un programa de control biológico para esta especie deberá ser cuidadosamente analizado, ya que todos los agentes biológicos estudiados, mostraron tener efectos generalizados en el ecosistema y en la entomofauna ahí existente, no siendo específicos de este cerambícido. De entre todos los parasitoides estudiados y sus respectivas tasas de parasitismo, la especie *C. flavator* podria ser la elegida para un futuro programa de control biológico, ya que fue encontrada en diversas regiones y en más altas densidades. Sin embargo, debido a su falta de especificidad, es necesaria la realización de estudios en laboratorio más exhaustivos. Esto permitiría estudiar la posibilidad de liberación masiva de dicha especie en las zonas afectadas. Siendo una especie nativa, la cual parasita eficazmente las larvas del escarabajo, es de esperar que el ecosistema circundante no se desregularice. El uso de artrópodos debe ser integrado como un complemento y no como el principal y único agente de control biológico, debido a las bajas porcentajes de parasitismo observado.

Por otro lado, la utilización de hongos entomopatógenos parece mostrarse bastante prometedor, ya que el objetivo principal de disminuir la longevidad de los adultos de *M. galloprovincialis* (y subsecuentemente disminuir las tasas de transmisión de PWN) fue claramente demostrado. El hongo, *B. bassiana* fue el que mostró tener la mejor eficacia para la disminución de la longevidad del insecto, presentado al mismo tiempo una tasa de crecimiento y reproducción bastante elevada. Esta última particularidad hace con que sea el candidato más relevante para la aplicación en las bandas de poliéster impregnadas con hongos. A pesar del éxito observado en los ensayos con las bandas de poliéster, el uso de este hongo en el ambiente tiene también de ser cuidadosamente estudiado, ya que los efectos de *B. bassiana* en la gran variedad de insectos que se puede encontrar en determinado ecosistema es bastante incierta. Debido al gran costo asociado con la producción de estas bandas de poliéster impregnado (comparadas con las redes con insecticida), su utilización podría estar más direccionada para las áreas donde los insecticidas están completamente prohibidos de ser utilizados. Zonas como las áreas protegidas, o reservas de naturaleza se podrían beneficiar con la utilización de este método como alternativa al uso de las redes insecticidas. Sin embargo, serían necesarios bastantes testes y ensayos antes de la implementación de este método en el ecosistema.

Considerando todos los trabajos desarrollados y las regiones analizadas durante todos estos años, se puede evidenciar que la medida más segura y efectiva para el control de las poblaciones de este insecto, es el correcto cuidado y mantenimiento de las áreas forestales. En regiones donde esta gestión es minuciosamente seguida durante largos años, como la región de "Leiria", la abundancia de *M. galloprovincialis* es bastante menor. Además, el establecimiento de una diversa comunidad de parasitoides en la región, debe tener un papel fundamental en la gestión de la población de esta especie.

Ha sido pues exhaustivamente estudiada la comunidad de parasitoides asociada con el *M. galloprovincialis* y los efectos de los hongos entomopatógenos más comunes en Portugal asociados a este insecto. En el futuro, los estudios se deben basar en otros enemigos naturales como los pájaros insectívoros o los murciélagos. Trabajos sobre bacterias, virus u otros hongos entomopatógenos podrían también ser nuevas líneas de trabajo interesantes. Sería también interesante analizar los impactos que enemigos naturales de otras especies del género *Monochamus* tendrían en las poblaciones Portuguesas de *M. galloprovincialis*.

En vista de los resultados obtenidos, se puede considerar que se estudiaron algunos elementos esenciales de un futuro programa de control biológico contra *M. galloprovincialis*. Si los resultados se consideran como un todo, puede ser que sea posible aplicarlos en un futuro cercano.

II- INTRODUCTION

1- The Pine Wilt Disease

The pine wilt disease (PWD) consists of an interaction between three organisms; a nematode, *Bursaphelencus xylophilus* (Steiner and Buhrer) Nickle, an insect vector belonging to the genus *Monochamus* Dejan, and a tree host, *Pinus* spp. Despite the simplicity this interaction may disclose, the reality is a lot more complex. The biology and ecology of the pine wood nematode (PWN) is fully synchronized with the development of the pine sawyer, allowing it to use the beetle as vector and consequently infect new tree hosts. Understanding the relationship between these three organisms is essential to comprehend the mechanism of PWD. The natural spread of the PWD is fully reliant on of the interaction between the insect vector and the nematode. Without this strict association, the nematode wouldn't be able to infect new hosts and spread its population, so it can be considered as a facultative relation for the insect and mandatory for the nematode. Both, insect vector and nematode show an increased advantage with the development of this relation, benefiting the first one from the growing number of dead trees created by the nematode's propagation.

1.1- The nematode

The pine wood nematode life cycle is based in two distinct life phases; the propagative and the dispersive (Wingfield, 1983). The propagative phase consists in four larval and one adult stage (Mamiya, 1983) and it occurs when the environmental conditions are adequate. This phase occurs inside the hosts and lead to the nematodes population growth. The populations of *B. xylophilus* while in the propagative phase inside the host feed on the wood, resin canals and other living cells which will eventually result in the tree's death (Koboyashi *et al*., 1975).

After completing the first two larval stages, the PWN may change to the dispersal phase, being the subsequent life stages called third and fourth juveniles' stages (resistant phase). The JIV phase is the one used by the nematode to interact and be carried by the insect vector, gaining the necessary mobility to change tree host (Mamiya, 1975).

1.2- The hosts

Worldwide, there are various records of *B. xylophilus* living on different tree species. Species belonging to the genus *Abies* (Miller)*, Cedrus* (Trew)*, Larix* (Miller), *Picea* (Dietrich), *Pinus* (Linnaeus)*, Pseudotsuga* (Carrière)*,* were already found associated with this nematode, however, not all of them are equally affected by the disease (Wingfield *et al.,* 1982; Mamyia, 1983; Evans, *et al.,* 1996). In North America, where the nematode isn't a major cause of decaying for native trees, records of PWN infections are mainly associated to exotic trees, such as; *Pinus sylvestris* L., *Pinus nigra* J.F. Arnold, *Pinus densiflora* Siebold and Zucc., *Pinus thunbergii* Parl. (Akbulut and Stamps, 2012). In Asian countries native species like *P. densiflora, P. thunbergii, Pinus koraiensis* Siebold & Zucc., *Pinus luchuensis* Mayr, or other species belonging to different genus (e.g. *Pseudotsuga menziesii* (Mirb.) Franco, *Picea* spp.) can also be found with PWN infections (Mamyia, 1983, 1987; Ryss *et al*., 2005; Zhao *et al.,* 2008). In Europe, the only infected host is *Pinus pinaster* Aiton, however, recent studies confirmed the high susceptibility found on other tree species, like *Pinus nigra* (Inácio, *et al.,* 2014). Despite the diversity of affected tree species worldwide, each of them shows different susceptibilities according to the country where they are native from and the possibilities of having coevolved with *B. xylophilus*. Such species can act as reservoirs for the nematode even when not showing evident PWD symptoms (Akbulut and Stamps, 2012).

1.3- The vectors

Various insect species are known carriers of the pine wood nematode, such as; *Acanthocinus griseus* Fabricius; *Arhopalus rusticus* Linnaeus; *Chrysobothris* spp.; *Monochamus* spp. or *Spondylis buprestoides* Linnaeus; (Wingfield and Blanchette 1983; Mamiya 1984). Nevertheless, not all of them can be considered as effective vectors for PWN transmission, being the most reliable the species belonging to the genus *Monochamus*. Some of these species are the most important vectors of the PWN (Linit, 1988, 1990; Kishi, 1995) and are listed in Table 1. Each of the *Monochamus* species from the Northern hemisphere exhibit different host preferences, specific biological cycles and particular geographic distributions (Morgan, 1947; Ross, 1966; Hellrigl, 1971; Naves *et al*., 2008). These beetles are generally known as secondary insects that only attack weakened or dying hosts, not breeding on healthy trees (Linsley, 1959; Linit, 1987; Hanks, 1999).

Table 1 - List of confirmed *Monochamus* species vectoring the pine wood nematode worldwide

***Monochamus* species**	**Continent**	**Reference**
M. carolinensis (Olivier)	North America	Wingfield and Blanchette (1983)
M. scutellatus (Say)	North America	Wingfield and Blanchette (1983)
M. titillator (Fab.)	North America	Esser and Harkcom (1981)
M. mutator LeConte	North America	Wingfield and Blanchette (1983)
M. obtusus Casey	North America	Dwinell and Nickle (1989)
M. notatus (Drury)	North America	Wingfield and Blanchette (1983)
M. marmorator Kirby	North America	Wingfield and Blanchette (1983)
M. alternatus Hope	Asia	Mamiya and Enda (1972)
M. nitens Bates	Asia	Kobayashi *et al*. (1984)
M. saltuarius (Say)	Asia	Sato *et al.* (1987)
M. urussovi (Fischer)*	Asia	OEPP/EPPO (1986)
M. galloprovincialis (Olivier)	Europe	Sousa *et al.* (2001)

*Wallin *et al.* (2013) recently proposed *M. urussovi* to be included in the species *M. sartor* as a subspecies; *M. sartor urussovi.*

Other species such as *Monochamus impluviatus* Motschulsky, *Monochamus saltuarius* (Say)*, Monochamus sartor* (Fabricius), *Monochamus sutor* (Linnaeus), and *Monochamus urussovi* (Fischer) have also been found associated with the PWN, but transmission of the nematode has not been proved yet.

As other members of the Cerambycidae family, *Monochamus* species spend most of their life cycle inside the wood as larvae or pupae. Adult females lay eggs on the branches or main trunk of recently dead or decaying tree hosts, by opening small wounds on the outer bark, where fertilized eggs will be laid (Evans, *et al*., 1996). *Monochamus* first instar larvae emerge from the eggs 1 or 2 weeks after oviposition and throughout several months feed on phloem and cambium bellow the bark (Naves *et al.*, 2008). After this feeding period, larvae enter the xylem and start an internal gallery which ends in a pupal chamber (Campadelli and Dindo, 1994).

Late instar larvae (Fig. 1) usually overwinter inside the wood until next spring, allowing the development to pupal stage (Fig. 2), and subsequently, to callow stage (unsclerotized adults).

Figure 1 – Last instar larvae of *M. galloprovincialis*

The callow stage is the most important developmental stage concerning the interaction PWN – vector. After the callow stage, fully functional adults (Fig. 3) emerge from the host by a perfectly round-shaped emergence hole on the wood surface (Naves *et al.*, 2008). As in most of the Cerambycidae, in this genus the males emerge first, as a strategy to choose and defend the best oviposition sites (Schoeman *et al.*, 1998; Shibata, 1998). The insects eclosions' are followed by a 2 weeks feeding period, based on thinner branches out-bark. This feeding period is followed by oviposition, which will lead to a new generation (Tomiczek and Hoyer-Tomiczek, 2008).

Figure 2 – Ventral view of *M. galloprovincialis* pupal stage

1.3.1- The PWN Vector in Portugal - *Monochamus galloprovincialis*

This Cerambycidae was firstly reported in Portugal by Oliveira (1984) who collected specimens from Leiria region and erroneously identified as *M. sutor*. On the catalogues dated before 1984 no references to this species can be found (Corrêa de Barros, 1916; Neves, 1950; Serrano, 1983). Before the association between the nematode and the insect (Sousa *et al.,* 2001) only a small number of authors studied this species. Only a small number of works with detailed morphologic descriptions can be found (Hellrigl, 1971; Francardi and Pennacchio, 1996; Francardi *et al*., 1998). Nowadays, the detailed distribution of *M. galloprovincialis* in Portugal is largely unknown, although there are records from several locations in mainland Portugal, Madeira Island (Fonseca *et al*., 2012) and a few Azores islands (Borges *et al*., 2013). The increasing importance of this species has been enlarged by its role as vector of the PWN, which has recently lead to several studies on its biology and ecology (e.g. Naves *et al*., 2006a, b, 2008; Koutroumpa *et al*., 2008; Akbulut, 2009).

Figure 3 – Lateral view of adult female *M. galloprovincialis*

Regarding the species life cycle, some variations can be noticed according to the country and its edapho-climatic conditions. In locations with cold winters, *M. galloprovincialis* larvae tend to overwinter for longer periods and the development from egg to adult can take up to 2 years (Tomminen, 1993). In Mediterranean countries with milder winters, populations usually take 1 year to complete the entire cycle. However, even in warmer sites, populations always spend a period in winter dormancy (Naves *et al.,* 2008). In Portugal, adult emergences occur between the end of May and the beginning of

September, reaching their maximum in June and July (Naves *et al.*, 2008). Adults tend to live from 65 to 80 days (Hellrigl, 1971).

This species is widely distributed in Europe (except in Cyprus, Ireland and the United Kingdom), being commonly found in the Mediterranean countries, specifically in France, Italy, Portugal and Spain (Francardi and Pennacchio, 1996; Vives, 2000; Brustel *et al.*, 2002; Koutroumpa, 2007). Other areas outside Europe also have this cerambycidae, like the Caucasus region, Russia, China, Mongolia, and Northern Africa (Hellrigl, 1971). In various countries different tree species are also common hosts for *M. galloprovincialis* (Hellrigl, 1971; Evans *et al.*, 1996; Francardi and Pennacchio, 1996). This Cerambycidae has already been found associated with *P. sylvestris, Pinus halepensis* Mill. and *P. nigra* (Inácio *et al.,* 2014) and less frequently with *Pinus uncinata* Ramond ex DC., *Picea abies* (L.) H.Karst and *Picea orientalis* (L.) Peterm. (Yuksel, 1998; Behalová, 2006; Vincent, 2007).

1.4- The interaction - Nematode, Vector and Host

Until recently no information could be found on the inoculation of the PWN into new tree host by *M. galloprovincialis*. Prior studies have been made about the infection of trees by the nematode in other continents like North America or Asia, but always by other *Monochamus* beetles (e.g. Wingfield and Blanchette, 1983; Mamiya, 1984; Linit, 1990). Works by Naves *et al.* (2006b, c) clarified this interaction for Portugal and for *M. galloprovincialis*. The transmission between the insect vector and the tree host reaches its maximum during the second week after the insect emergence, mainly occurring during the beetles feeding activity (Naves *et al.*, 2007a) yet, it can also happen as a result of oviposition (Naves *et al.*, 2007b), similarly to what occurs with other *Monochamus* species (Wingfield and Blanchette, 1983; Linit, 1989; Edwards and Linit, 1992). As the nematode penetrates the new host the cycle restarts, resulting in the spread of the PWD.

1.5- Pine Wilt Symptoms and Detection

When a tree host is infected by the PWN, the nematode rapidly spreads to the entire tree, which usually leads to its death in a few months or even weeks (Malek and Appleby, 1984). The introduction of the nematode into a healthy tree triggers the nematode feeding activity on healthy parenchyma and epithelial cells, leading to tracheid cavitation with the subsequent cessation of the water flow through the tracheids. After the water stops flowing, transpiration will also cease, leading to a change in the needles coloration, from green to brownish yellow. The needles decolouration will also be followed by an increasing amount of fungi inside the wood and the consequent tree death (Mamiya, 1984).

The symptoms characteristics of the PWD are not specific to this infection, as bark beetles like *Ips* spp., *Ortothomicus* spp. or even diseases can cause similar symptoms (Chararas and M'Sadda, 1973; Capretti *et al.*, 1987; Lombardero and Ayres, 2011; Sousa *et al.*, 2011). With the great variety of diseases/pests affecting the pines, further analyses are needed to confirm PWN presence in the wood. The principal method used to identify *B. xylophilus* consists on removing wood from symptomatic trees followed by the extraction of the nematodes to water and their identification in laboratory. Besides the classic identification method based on morphological characters (Abelleira, 2011; Zhuo *et al.*, 2011; OEPP/EPPO, 2013), molecular aproaches based on ITS-PCR methods can also be used (Ribeiro *et al.*, 2012). Despite this advances, in an attempt to detect PWD infected trees in the field new methods are being developed (Joon-Bum *et al.*, 2001; Lee and Cho, 2006).

1.5.1 – First detections and dissemination of the PWN

The first records with reference to the PWN date from 1934 when Steiner and Buhrer described for the first time *Aphelenchoides xylophilus,* which had been isolated five years earlier in Louisiana, USA from a native longleaf pine (*Pinus palustris* Mill). Later, and after Tokushige and Kiyohara (1969) reported the presence of *Bursaphelenchus* sp. in the wood of dead pines in Japan, Nickle (1970) transferred the species to the genus *Bursaphelenchus*. In 1971, Kiyohara and Tokushige demonstrated the pathogenicity of *B. xylophilus* by inoculating adult Japanese red pines (*P. densiflora*), followed by the first report of the pine wilt disease (PWD) by Dropkin and Foudin (1979) in Missouri, USA. In 1982, China discovered the PWN for the first time in the Jiangsu province. Since then the damages caused by the disease have rapidly increased year after year (Zhao *et al.*, 2008).

Canada reported in 1983, *B. xylophilus* affecting the Jack pine (*Pinus banksiana* Lamb.) in Manitoba (Knowles *et al.*, 1983) and Korea first reported the nematode in 1988 (Yi *et al.*, 1989). Two years earlier in Finland, *Pinus* spp. wood chips imported from the USA and Canada alarmed the European Union by turning out positive for the presence of the nematode in the wood (Rautapaa, 1986). Following this development, the European Plant Protection Organization (EPPO) transferred the PWN to the "A-1" category pest and included *B. xylophilus* in the EU quarantine organism list Annex II, Part A – Section I, Directive 77/93/CEE. EPPO suggested furthermore that Europe should start banning the importation of softwood products from countries where the nematode or the disease were present (OEPP/EPPO, 1986; Sutherland, 2008).

Its definitive entrance in Europe only occurred in 1999 in the Setubal Peninsula, Portugal. It is believed that the entrance in Portugal originated from Eastern Asia (Mota and Vieira, 2008) and occurred by the movement of wood products like pallets, timber or other wood derived materials as it is a major dissemination method for the PWN (Evans *et al.*, 1996). Promptly a team from "Instituto Nacional de Investigação Agrária" and the University of Évora started to follow this disease situation (Mota *et al.*, 1999) and the information was immediately transmitted to the Portuguese responsible organism (Direcção-Geral das Florestas) and subsequently to the EU responsible authorities in Brussels. Considering the urge of the events, a task force and a national action program (PROLUNP) were established in the end of 1999. The PROLUNP was launched to implement measures and actions, so that the disease's spread could be controlled and the PWN eradicated. At the same time, the PROLUNP was entrusted to coordinate the scientific research on the disease in Portugal. The national action plan delineated by this task force, determined the most important pine-forested areas and high risk zones, where extensive surveys would be performed, allowing new outbreaks of the disease to be detected. Afterwards, PWN was detected in a considerable large area surrounding the Setubal Peninsula, and the region was classified as "affected zone". Surrounding this area, a 20 km wide strip was created and called the "buffer zone", classified as "free area". Simultaneously, this program implemented the orientations and procedures to handle the PWN infected wood (DGRF, 2003).

Despite these measures, in 2007 the European Union advised to the establishment of a clear cut zone surrounding the affected area, what lead to the cut of nearby five million healthy maritime pines (*P. pinaster*) (DGRF, 2007). Regardless of these actions, in the next year new wilt disease focuses were found affecting the central Portuguese region; Sarzedo and Lousã. After these findings, new legislation was created, implementing a “restriction zone” to the entire Portuguese continental territory and Madeira. Despite all the efforts, in 2008 *B. xylophilus* was also detected in the regions of Galicia and Extremadura, Spain (EPPO/OEPP, 2010; Abelleira *et al*., 2011). In the year of 2009, this nematode was also found on the Madeira Island (Fonseca *et al.,* 2012), showing that the spread of the PWN to other distant territories in the future might be a reality. Between 2000 and 2007 the number of felled pine trees with wilting symptoms (although not necessarily with the PWN) increased from 53.487 to over 200.000, causing significant economic and environmental impacts to Portugal (Sousa *et al*., 2011). Recently, these numbers have been increasing, with almost 900.000 trees felled between 2011 and 2012 (Anonymous, 2012).

Due to this reality that the country faces for more than a decade now, together with the significant amount of forest fires and the insect pests, the Portuguese forest composition has clearly changed. For the last 10 years the forest area has been decreasing with a rhythm of 0.3% per year. The maritime pine (*P. pinaster*) which in the past occupied the majority of the Portuguese forest, lost approximately 263 thousand hectares of harvested area (Fig. 4), being nowadays and for the first time in history, surpassed by Eucalyptus (*Eucalyptys globulus* Labill.) (ICNF, 2013).

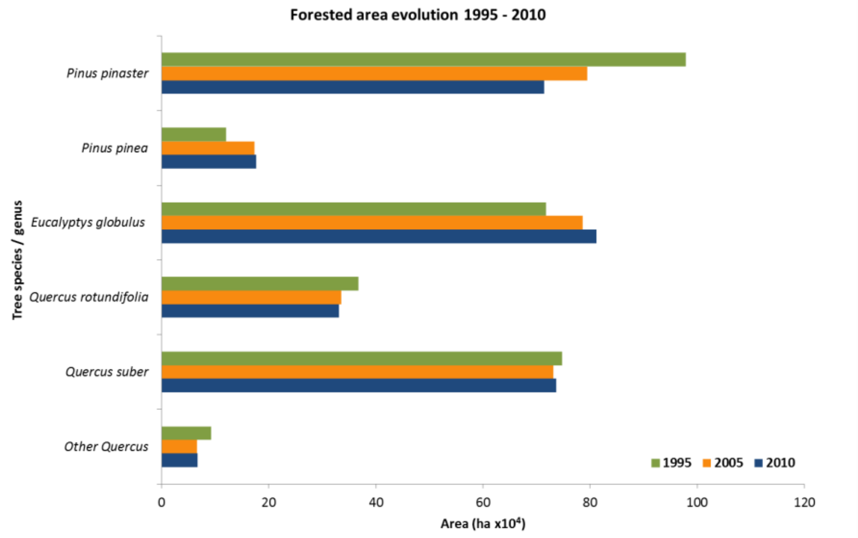

Figure 4 – Evolution of the forested area since 1995 for the principal Portuguese forest species. Adapted from ICNF (2013).

As the forest sector represents 12% of the Portuguese industrial gross domestic product, with 10% of the foreign trade, these variations have a significant impact to the Portuguese economy (Webster and Mota, 2008).

2- Control Strategies

Management and control strategies against PWN are directed in three ways; controlling the nematode’s populations; creation of trees resistant to the disease and avoiding the spread of the nematode by the dispersal of vector insects, which is presently the most effective one (Sousa *et al*., 2011). Since the detection of *B. xylophilus* in Portugal (Mota *et al*., 1999) and the association of the insect with the nematode by Sousa *et al*., (2001) strict sanitary measures are being implemented in affected forests areas. In the past years, various studies have been developed to find new ways to control this Cerambycidae and subsequently the spreading of the PWN. However, the studied methods usually have success only in localized, small-dimension areas, presenting a significant cost to implement (Naves *et al.,* 2007c). The recent appearing of PWN in pine trees in Spain (OEPP/EPPO, 2013; Abelleira *et al*., 2011) emphasizes the necessity to discover new and innovative control strategies against the PWN. The methods used to prevent the spreading of the disease can be divided among four different categories; cultural, chemical, bio-technical and biological, and are all based on the control of the pine sawyer populations.

2.1- Cultural Methods

Symptomatic trees are marked in the autumn and eliminated during the same season, winter or early spring. While eliminating the symptomatic trees, the insect specimens inside the wood as pupae or larvae are killed, reducing the number of *Monochamus* Mamiya, 1984; Kishi, 1995). According to the Portuguese legislation (Anonymous, 2011), all material (smaller than 20cm) originated from the eliminated symptomatic trees (Fig. 5) must be destroyed by mechanic action (resulting chips no bigger than 3 cm), by fire, or even fumigated (if intended for further use). It is of major importance that all left over materials, such as small branches, are destroyed, as *Monochamus* beetles tend to lay eggs on them (Naves, 2007). If intended to posterior use, the infected wood with dimensions bigger than 20cm must be submitted to a heat treatment of a minimum of 56ºC for no less than 30 minutes (measured in the center of the wood piece (Anonymous, 2011).

Figure 5 – Remainings of a Maritime Pine forest highly affected by PWD near the first detection site in Sines in July 2013.

Recently, new research lines are being developed with the purpose of growing a selection of pines resistant to the PWD (Ribeiro, 2012), using the species *P. pinaster*. In Japan, this line of studies is being developed with varieties of local pine such as *Pinus densiflora* and *Pinus thunbergii* (Kato *et al*. 1995; Toda and Kurinobu 1998, 2001, 2002). Usually the development of these research lines tends to promote the improvement of resistances or even the forest production (Mahalovich and Dickerson, 2004, Wu *et al*., 2005).

2.2- Chemical Methods

Resorting to chemical methods is today an option used more like a preventive measure than as a control strategy. In Asian countries like China, Japan or South Korea the application of chemical substances was widely used in the control of local *Monochamus* vectors and *B. xylophilus* populations (Kobayashi, 1988; Togashi, 1990; Jianchang *et al*., 1998; Lee *et al.*, 2003; Takai *et al.*, 2003), although this resulted is serious problems associated with the use of pesticides in an ecosystem (Zhao *et al.*, 2008).

In Portugal the use of chemicals to control the PWN is restricted, with the only substance approved being Emamectin Benzoate. Similarly to countries where this product is already into use to control insect pests (e.g. Liguori *et al.*, 2010; Smitley *et al.*, 2010), Emamectin Benzoate has been tested and approved to use as a preventive measure to decrease infections by the PWN. This technique is intended to use in healthy pines and it is applied by trunk injection, preventing also the attacks of bark beetles (Sousa *et al.*, 2013).

The use of chemical substances is not restricted to the prevention of new infections, being also available to use in infected wood as fumigant under controlled conditions. Recent works showed that it is possible to eliminate *B. xylophilus* populations from wood with the use of sulfuryl fluoride (Bonifácio *et al.,* 2014).

A new approach based on the use of essential oils as bioinsecticides has recently been considered (e.g. Kong *et al.*, 2006; Choi *et al.*, 2007; Faria *et al.*, 2013). These substances show a significant number of ecofriendly characteristics as they are extracted from plants, are biodegradable and do not usually present the high levels of toxicity for the environment found on the typical chemical insecticides (Figueiredo *et al.*, 2008).

2.3- Bio-Technical Methods

Besides the elimination of trees colonized by *Monochamus* beetles and the use of chemicals, bio-technical approaches are also available, allowing the capture of insects and diminishing their populations.

To simulate the shape of a maritime pine or the volatile compounds released by these trees, some different models of traps and lures have been created and developed in the past. To select the most adequate type of trap for capturing the beetle, different designs of traps have been tested (e.g. Groot and Nott 2001; Morewood *et al.*, 2002).

Fig. 6

Results have shown that multi-funnel traps (Fig. 6) are the most promising for capturing *M. galloprovincialis* (Rassati *et al*., 2012), however, other studies demonstrated that cross-vane traps (Fig. 7, 8) were the most efficient (Bonifácio *et al*., 2012).

Fig. 7

Considering that *Monochamus* beetles attractants are particular related to kairomonal activity (Allison *et al*., 2004) and that Cerambycidae species generally use the chemical clues for feeding, mating and egg-laying (e.g. Ginzel and Hanks, 2003; Faccoli *et al*., 2005) several works have been developed about the aggregation and sexual pheromones related to *M. galloprovincialis* (Ibeas *et al*., 2007, 2008; Pajares *et al*., 2010, 2013).

From these research lines, an aggregation pheromone compound has been developed. This product is called Galloprotect 2D© and it's commonly used in Portugal and Spain, being applied to pine stands susceptible to the PWD or even already affected (Pajares *et al.,* 2010; Bonifácio *et al*., 2012).

Fig.8

Figures 6, 7, 8, – Different models of traps used to capture adults of *Monochamus galloprovincialis* 6) Multi-funnel trap; 7) Colossus Cross vane trap; 8) Japanese Cross vane trap

2.4- Biological Methods

With the increasing importance of the Integrated Pest Management policies, the use of control measures not based on chemical approaches gain a considerable relevance. Biological methods to control agriculture or forest pests are used more intensively since the last century. Various authors studied the possibilities of using different biological agents to control these pests. Methods based on the use of entomopathogenic fungi, entomopathogenic nematodes and natural enemies, both predators and parasitoids were tested for a diverse number of pests (Webster, 1973, 1980; Meierrose *et al*., 1991; Bichão *et al*., 1991; Hodge *et al*., 1999; Snyder and Wise, 1999; Solomon *et al*., 2000).

Testing these techniques showed that their use may not be that easy and effective for all the ecosystems. In small localized areas where only agriculture occur and where a specific pest is attacking, these methods usually show good results (e.g. Abe and Ikegami, 2005; Oliveira *et al*., 2012; Garrido-Bailón *et al*., 2013). However, in forest areas where a pest isn't so located and not that accessible, biological control methods turn to be more difficult to implement, though, some studies can also be found (e.g. Reis *et al.,* 2012; Borowiec *et al.,* 2014; Matosevic *et al.,* 2014).

Insects belonging to the family Cerambycidae spend most of their life cycle inside the tree, bellow the outer bark or even inside the wood (e.g. Akbulut and Stamps, 2012). As the majority of their life cycle is inconspicuous, the use of control programs to attack these pests is very limited, even more if a biological method is desirable. The use of a biological program must be designed to target the moment in their life cycle where insects are more susceptible and exposed. In general, there are several biological control options; natural enemies (arthropod or non-arthropod) and entomopathogenic bacteria, nematodes or fungi.

2.4.1- Non-Arthropod natural enemies

Despite this study being only focused on the use of parasitoids or entomopathogenic fungi as biological control agents, the use of non-arthropod entomophagous species, such as bats, woodpeckers, or other insectivorous birds may have some potential to control some selected insect species, such as the pine processionary moth *Thaumetopoea pityocampa* Denis & Schiffermuller (e.g. Charbonnier *et al.,* 2014). Studies showed that increasing entomophagous bird species densities in a forest/agricultural ecosystem where an insect pest is also present may condition the insect's population dynamics and positively influence the bird population (Beckwith and Bull, 1985; Norris *et al*., 2013). Several authors proved that high populations of the Emerald Ash borer (*Agrilus planipennis* Fairmaire) or the Mountain Pine Beetle (*Dendroctonus ponderosae* Hopkins) significantly increased the populations of woodpeckers and other native birds (Edworthy *et al*., 2011; Koenig *et al*., 2013; Flower *et al*., 2014). According to Beal (1911), Hughes and Hughes (1982) and Dodds and Stephen (2000), woodpeckers have a significant effect on the reduction of Cerambycidae larvae (Fig. 9). These studies suggest that the use of woodpeckers for integrating a biological control program of *M. galloprovincialis* should not be excluded.

Figure 9 – *Pinus pinaster* branch with feeding holes made by Woodpeckers.

Similarly to entomophagous birds, bats diversity and density inside an ecosystem are closely related to the abundance of insects. Bats' flight and foraging activities can be highly modified and even changed in an opportunistic way, being able to adapt to insect population outbursts or reductions (Palmeirim, 1990; Tuttle, 1995; McCracken *et al*., 2012; Gonsalves *et al*., 2013; Thomas and Jacobs, 2013). However, comparing to birds activity, bat represent only a residual effect on insect populations, having a reduced result on diminishing it (Buckner, 1967).

The use of these mammals and birds against insect pests in a biological control program not only based on the use of non-arthropod methods may decrease pest outbursts and increase yield productions inside an agroforestry landscape (Morrison and Lindell, 2012; Karp *et al*., 2013; Mas *et al*., 2013).

2.4.2- Arthropod Natural Enemies

The equilibrium in the agroforestry ecosystems between predators, parasitoids and pest is, in general, well balanced. A subtle change in the ecosystem, as a climate change, the use of chemical products, or even the introduction of alien species may lead to the disruption of such stability. Focusing on the introduction of insect entomophagous species in an ecosystem, the main responsible can't be ignored. Humans are responsible for great part of the introduced insect alien species, either intentionally or unintentionally (Pyšek *et al.*, 2010). Insect predators or parasitoids are commonly brought from countries where they act as pest control agents into countries where they were previously absent. The introduction of these alien species may have a positive effect on local pest control programs; nevertheless, after a long period it can disclose a hidden disruption on the ecosystem, increasing their populations and even occupying the place that once belonged to native species (Evans *et al.*, 2011). Despite the long term results, the use of these species in biological control programs is nowadays common worldwide, both in agriculture and forestry. Regrettably to the native ecosystems, the number of introduced entomophagous arthropods is increasing and as a consequence, new studies concerning their interactions with the surrounding environment and their ecological impact can be found (e.g. Nafus, 1993; Duan and Messing, 1997; Avila *et al.*, 2013; Ayalew and Hopkins, 2013). Despite the tendency, not all the biological control programs are based on the introduction of alien species, but instead, on using native insect species (e.g. Mackauer and Völkl, 1993; Hernandéz-Ortiz, 1994; Pérez-Lachaud and Hardy, 1999; Gabarra *et al.*, 2014; Núñez-Campero *et al.*, 2014).

Considering the agricultural ecosystems, the use of biological control methods based on arthropods species is far more common than it is in the forest environment. Being a fragmented habitat and normally occupying extended areas, the use of any type of control programs in forests often reveals to be a challenge. Focusing on the families Buprestidae, Cerambycidae and subfamily Scolytinae, which major affect forest stands all across the world, some more specific works can be found (e.g. Senger and Roitberg, 1992; Azevedo and Waichert, 2006; Abell *et al.*, 2012; Yang *et al.*, 2012; Cooperband *et al.*, 2013; Duan *et al.*, 2013).

Inside the Cerambycidae family and taking a closer look on the genus *Monochamus*, researchers found parasitic associations of hymenopteran species with the pine sawyer. In China, some Hymenoptera have been found parasiting larvae of *M. alternatus* as it can be observed on the works by Xu (1998) and Xu *et al*. (2002), who associated *Scleroderma guani* Xiao et Wu (Hymenoptera: Bethylidae) to this pine sawyer*,* originating a diverse number of studies to its use as a biocontrol agent (e.g. Xu *et al*., 2008; Li *et al*., 2009; Lai *et al*., 2012). Besides *S. guani*, the species, *Bracomorpha ninghais* Wang, Chen, Wu, He (Hymenoptera: Braconidae), and *Sclerodermus harmandi* Bursson (Hymenoptera: Bethylidae) were too established as parasitoids of *M. alternatus* larvae in China (Wang *et al*., 2009; Li *et al*., 2010a; Li and Sun, 2011; Liu *et al*., 2011; Hu *et al*., 2012). Recently, a new *M. alternatus* egg parasitoid was also discovered in the same country, this being *Callimomoides monochaphagae* Yang (Zhong-Qi *et al*., 2014). Two Coleoptera species, *Dastarcus helophoroides* (Fairmair) (Coleoptera: Bothrideridae) and *Cryptalaus berus* (Candèze) (Coleoptera: Elateridae) were also associated parasiting and predating, respectively *M. alternatus* (Huang *et al*., 2003; Zhang *et al*., 2008). *Dastarcus helophoroides* showed to parasite both larvae, pupae and adults of the pine sawyer (Okamoto, 1999), while *C. berus* only attacked the larvae of *M. alternatus* (Yi-Nan *et al.,* 2008), what also lead to the development of other research lines (Wang *et al*., 2012a, b; Yuanling *et al*., 2013a, b).

In Japan, where *M. alternatus* is also the main culprit for the spreading of *B. xylophilus*, experimental releases of *D. helophoroides* and *Ontsira palliatus* (Cameron) (Hymenoptera: Braconidae) were also performed (Tadahisa, 2003; Urano, 2004), without effective results.

The beetle *M. saltuarius* has also been found to be a reliable host in China for *Rhimphoctona* (Xylophylax) *lucida* (Clément) (Hymenoptera: Ichneumonidae), while *M. carolinensis* is a host for a species of the genus *Digonogastra* Viereck, and *Billaea monohammi* (Townsend) (Diptera: Tachinidae) in Illinois, USA (Luo and Sheng, 2010; Reagel *et al*., 2012).

Studies concerning the Euro-Asian *Monochamus* species and their parasitoid complex have also been developed (Francardi and Pennacchio 1996; Francardi *et al*., 1998; Martikainen and Koponen, 2001; Naves *et al*., 2005), although the information is scarce and disperse. Nevertheless, the major part of the studies has been centered in the economically relevant *M. galloprovincialis*. For this species, Naves *et al.* (2005) analyzed the already existing reliable records, from which may be underlined the most important parasitoid species; *Atanycolus genalis* (Thomson) (=initiator F.), *Coeloides sordidator* Ratzeburg, *Cyanopterus flavator* Fabricius (Fig. 10), *Dolichomitus tuberculatus* (Geoffroy), *Doryctes mutilator* (Thunberg), *Iphiaulax impostor* (Scopoli) and *Meteorus corax* Marshall, nevertheless they are not specific parasitoids only of *M. galloprovincialis* and appear to be rare and infrequent. Some other parasitoid species were found by the author, however, were not considered and can be found discussed in his work.

Figure 10 – Adult male of *Cyanopterus flavator;* most abundant *Monochamus galloprovincialis* parasitoid found in Portugal.

Besides parasitoids, ants can also have an entomophagous behavior to eggs or even adults of this Cerambycidae, of which two of them are native to Portugal, namely, *Pheidole pallidula* Neylander and *Aphaenogaster gibbosa* Latreille. (Cammell *et al.*, 1996; Way *et al.*, 1997; Goméz and Espadaler, 2004).

The presented information on *M. galloprovincialis* parasitoid complex was the starting point to the development of the work concerning the arthropod natural enemies presented in this thesis, so the records needed to be confirmed and an updated parasitoid guild found. Thus far, no specific and efficient natural enemies have been detected, and therefore researching these associations could lead to an interesting and environmentally friendly option for the development of a bio-control program.

2.4.3- Entomopathogenic Fungi

The use of entomopathogenic fungi to regulate insect pests populations' is a common practice (e.g. Abe and Ikegami, 2005; Erler *et al.*, 2013; Zhan and Morse, 2013). As a complement or an alternative to the use of parasitoids, entomopathogenic fungi also offer a safer alternative to the use of chemical products like insecticides, without affecting the cultures safety and the life cycle of auxiliary insects. The use of bio-insecticides also eliminates the appearance of insect populations presenting chemical resistances

(Fernandes *et al.*, 2013; Smagghe *et al.*, 2013). However, recent studies also found that some insects are able to gain resistances to some specific entomopathogenic fungi strains (Dubovskiy *et al.*, 2013). Using entomopathogenic fungi is currently more common in the control of agriculture pests, to prevent possible harmful aliment contamination, which could affect the health of animals or humans. Several studies can be found on the associations between these fungi and insects (Stacey, 2003; Xavier and Ávila, 2005; Daniel and Wyss, 2010; Oliveira *et al.*, 2012).

Even thought, as the number of pests affecting forest stands increases, some research lines have been developed to use entomopathogenic fungi in these ecosystems. Works concerning the main insect families affecting the forests vitality are common, particularly, Cerambycidae (e.g. Hu *et al.*, 2010; Russel *et al.*, 2010; Peng *et al.*, 2011; Meyers *et al.*, 2013), Buprestidae (e.g. De la Rosa *et al.*, 2000; Liu and Bauer, 2008; Castrillo *et al.*, 2010), and the subfamily Scolytinae (e.g. Davis *et al.*, 2011; Lyons *et al.*, 2012; Yao *et al.*, 2012). The use of entomopathogenic fungi has even been studies in Cerambycidae with habits and life cycles similar to *Monochamus* (Ambethgar and Mahalingam, 2002; Ludwig and Oetting, 2002; Dubois *et al.*, 2008; Marannino *et al.*, 2010; Francardi *et al.*, 2012; Meyers *et al.*, 2013).

In China, several fungi with entomopathogenic characteristic were found associated with *M. alternatus*; *Cephalosporium* sp., *Fusarium* sp., *Penicillium* sp., *Metharizium* sp. *Trichoderma* sp., *Verticillium* sp., *Serratia* sp. and *Paecilomyces* sp., being the most abundant *Beauveria bassiana* (Bals.-Criv.) Vuill (Shimazu *et al.*, 2002; Shimazu and Sato, 2003). Most of the studies made on *Monochamus* spp. or similar species were based on the use of *B. bassiana* and *Metharizium* spp. As for M. galloprovincialis, *B. bassiana* was similarly identified to be an important mortality agent in Portugal (Fig. 11), being responsible for one quarter of larval mortality in heartwood galleries (Naves *et al.*, 2008). This fungus is also reported to inhibit the insect feeding activity and to reduce the nematode transmission (Maehara *et al.*, 2007).

An essential key issue related to the use of entomopathogenic fungi in the field is the application method or technique. Numerous methods have been tested, being the most effective the use of nonwoven fabric bands impregnated with the selected fungi and exposed to the insects (Shimazu, 2004; Shanley *et al.*, 2009).

Figure 11- Dorsal view of *Monochamus galloprovincialis* killed by infestation of *Beauveria bassiana*.

The application of entomopathogenic fungi to deal with insect pests populations isn’t as simple as the application of chemical products, as different constrains influence their effectiveness. The undesired spreading of a fungi strain from a treated area may represent the main problem associated with these techniques. Conidia dispersal and viability are highly sensitive to strong winds (Tabakovic-Tosic, 2014), temperature and humidity (Blanford *et al.*, 2012; Lopes *et al.*, 2013; Rivas *et al.*, 2014). Subtle changes in temperature can diminish the spore viability and subsequently the infection levels on the insects (Blanford and Thomas, 1999). It is then necessary to be able to predict the best biotic and abiotic conditions in which a bio-control program, based on the use of entomopathogenic fungi should be implemented.

2.4.4- Entomopathogenic bacteria and nematodes

The discovery of entomopathogenic bacteria was firstly associated with the genus *Bacillus* Cohn, however there is a wide variety of entomopathogenic bacteria associated with insects. The main finding occurred indubitably, with the specie *Bacillus thuringiensis* (Berliner) (therefore mentioned as Bt) If compared with other entomopathogenic bacteria, Bt is more effective in terms of toxicity against some insect species (e.g. *Spodoptera frugiperda* Smith and Abbot *T. pityocampa* or *Tuta absoluta* (Meyrick)) and cheaper to produce (Ruiu *et al.*, 2013). As an example, in the USA, the bio-insecticides market is fully dominated by sales of Bt, with 90% of all sold products being based on this bacteria (Sanchis and Bourguet, 2008; Sanahuja *et al.*, 2011). Most of the studies about Bt and the targeted insects are related to the larval ingestion of two toxins produced by the Bt, which will internally degrade and eventually kill the insect (Kongsuwan *et al.*, 2005; Bravo *et al.*, 2007; Zimmer *et al.*, 2013). To overpass some application difficulties, like the low action persistence after the application, scientists started to develop genetically modified vegetable crops, in order to include the genes responsible for the production of the toxins and see how does the insect pests react (Khojand *et al.*, 2013; Tian *et al.*, 2014; Du *et al.*, 2014).

Studies on other bacteria species such as *Bacillus sphaericus* Neide, which are toxic to mosquitoes, can be easily found (e.g. Singer, 1980; Charles *et al.*, 1996; Gende *et al.*, 2010; Prabhu *et al.*, 2013). Thought, works concerning the forestry ecosystem or the pests affecting it are quietly more scatter and limited. Research developed with *M. galloprovincialis* and similar species, like *Anoplophora glabripennis* (Motschulsky), or *A. planipennis* are more related with the bacterial community associated with the insect than with the control of it by the use of entomopathogenic bacteria (Vasanthakumar *et al.*, 2008*;* Reid *et al.*, 2011; Podgwaite *et al.*, 2013; Vicente *et al.*, 2013).

Likewise, studies on nematodes as bio-control agents against Cerambycidae are scarce, being most of them focused on economic relevant species like *A. glabripennis* (e.g. Solter *et al.*, 2001; Fallon *et al.*, 2006; Susurluk *et al.*, 2011; Harvey *et al.*, 2012). The use of entomopathogenic nematodes isn’t always a success, as some studies can show, this nematodes failed to control the insect pest for which they were intended (e.g. Jaques, 1967; Bélair *et al.*, 2003). The main reason for these failures is the sensitive nature of the organism used. Nematodes are extremely sensitive to temperature and humidity changes. When they are intended to control insect species living on trees surfaces or other plants exposed to the surrounding environment, the effects are not always as successful (Grewal *et al.*, 1994). However, the use of entomopathogenic nematodes in more stable environments, such as wood or soil normally results in greater success.

Some studies concerning the nematodes belonging to the families Steinernematidae and Heterorhabditidae, which carry in their guts bacteria of the genus *Zenorhabdus* and *Photorhabdusa* (Forst *et al.*, 1997)*,* have been developed (e.g. Tomalak, 2003; Yang *et al.*, 2013; Hoffmann *et al.*, 2014; Manrakhan *et al.*, 2014). These studies showed that such nematodes are lethal to various insect species as they transport these lethal bacteria from one insect host to another (Peters and Ehlers, 1994).

Studies on interaction of other nematodes, bacteria or entomophagous organisms should be performed, as interesting results on their effectiveness to control *M. galloprovincialis* populations may appear.

III- OBJECTIVES

As the consciousness about the use of pesticides increases, new techniques centered on integrated pest management strategies are called for. It is then essential to develop new methods that control the out bursting number of forest pests affecting our territory.

In Portugal, where *Monochamus galloprovincialis* is the sole vector on the spreading of the pine wood nematode (PWN), studies on the biology and ecology have been developed in the last years. However, more detailed works focused on PWN or *M. galloprovincialis* control methods need to be developed. Even with the recent studies on the biology and ecology of this pine sawyer across the continent, (Naves *et al.*, 2006a, b, 2007, Koutroumpa, 2007, Akbulut, 2009) no significant studies have been made on the biological control, parasitoid associations and entomopathogenic fungi affecting this insect.

The studies developed during the advance of this thesis are mainly directed towards building the basis for a biological control program, which will work as a complement to the use of cultural control methods.

To be able to develop assays on the biological control methods it's essential to develop a mass rearing protocol for populations of this Cerambycidae. Laboratory studies about the development, improvement and maintenance of a laboratory colony of *M. galloprovincialis* are also going to be performed, allowing faster generations and simplifying laboratory rearing procedures. These evaluations will be based on manufacturing artificial diets for the rearing of the pine sawyer. In order to diminish the generation's development time, manipulation of light cycles and temperatures under laboratory conditions will also be made tested.

Before starting the development of alternative biological control methods, it is essential to detect the actual distribution of *M. galloprovincialis* along the Portuguese mainland, its actual tree hosts, and the biological agents already affecting it. This way it will be possible to make a comprehensive list of its natural enemies in Portugal, which can be used to evaluate as possible control agents. One of the major objectives of this thesis is, in fact, to detect the potential parasitoid guild associated with the pine sawyer and develop a control program based on such species. For the survey of such guild, tree species will be prospected from north to south of Portugal, where *P. pinaster* is the dominant forest tree.

As not only the parasitoid guild associated with the vector can integrate such control programs, the entomopathogenic fungi affecting this beetle will be also under survey. Laboratory assays with the objective of revealing the susceptibility of *M. galloprovincialis* larval and adults stages to each of the fungi strains are also considered as an important objective, as they could represent an important step to control this forest species.

If all the objectives develop according to the protocol, it can be estimated that, at the end of this thesis the evaluation of potential biological control agents must be completed, allowing the development of further studies on the control of the vector of the PWN.

IV- RESULTS

Rearing the Pine Sawyer *Monochamus galloprovincialis* (Olivier, 1795) (Coleoptera: Cerambycidae) on Artificial Diets

Journal of the Entomological Research Society – 16(1) (2014): 61-70

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Resumen

El mantenimiento de una colonia en laboratorio de insectos es una de las necesidades básicas de las unidades de investigación entomológicas. Existir la disponibilidad de insectos en diferentes estadios de desarrollo en las diversas fechas del año posibilita la ejecución de los más diversos ensayos.

Una de las especies más relevantes en los corrientes años y que afectan las forestas portuguesas es el cerambícido *Monochamus galloprovincialis* (Olivier, 1795). Este insecto es el vector del nematodo del pino *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle en Portugal, lo cual es el responsable por la enfermedad del decaimiento del pino. Teniendo esto en cuenta, se ha mostrado esencial la realización de un elevado número de estudios relativos a estos organismos. Para asegurar la continuidad de los ensayos, se ha desarrollado un protocolo de creación de una población del cerambícido en laboratorio. Se han testado 8 diferentes dietas artificiales para creación del insecto en condiciones controladas. Dichas dietas están mayoritariamente basadas en el uso de productos disponibles comercialmente con bajo costo asociado, y en materiales de origen vegetal.

Entre las dietas testadas, la dieta #5 fue la que presentó una eficacia más elevada, con cerca de 80 % de eclosiones. Sin embargo, con la dieta #6 se obtuvieron los insectos en un tempo record de 53 días. Diferencialmente de todos los otros estudios encontrados, la incorporación de tejidos vegetales en las dietas no ha tenido ningún efecto positivo en la obtención de *M. galloprovincialis* adultos. Excluir este ingrediente representa una disminución en la dificultad de producción de la dieta, visto que no se tendrán de obtener los pinos sanos ni tampoco extraer los tejidos del interior de la madera. Juntamente, se presenta todos los ingredientes necesarios al desarrollo de la dieta más eficaz y su método de producción.

J. Entomol. Res. Soc., 16(1): 61-70, 2014 ISSN:1302-0250

Rearing the Pine Sawyer *Monochamus galloprovincialis* (Olivier, 1795) (Coleoptera: Cerambycidae) on Artificial Diets

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ABSTRACT

The pine sawyer *Monochamus galloprovincialis* (Olivier, 1795) is the vector of the introduced pine wood nematode *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) in Portugal, and until recently was considered a secondary insect pest. In this study eight artificial diets were tested with the purpose of rearing this insect under laboratory conditions with reduced costs and high efficiency. Tested diets included various artificial mediums commercially available for other insects and mediums based on dried or fresh pine phloem. Among the eight tested variations, diet #5 was the most efficient with 80% eclosions of adult beetles, while diet #6 allowed the fastest development, with the first adult emerging within 53 days. The best rearing method, the diet ingredients and the prices of the diets are discussed, resulting in a list of essential ingredients for the artificial rearing of the pine sawyer.

Key words: Artificial mediums, laboratory rearing, *Pinus pinaster, Bursaphelenchus xylophilus,* pine wilt disease.

INTRODUCTION

The use of artificial diets for maintaining insects in laboratory colonies is now a routine procedure worldwide (Alfazairy *et al*., 2012; Assemi *et al*., 2012). Artificial diets allow the continuous rearing of a large number of insects throughout the year, with the purpose of studying their behaviour, physiology, biochemistry, taxonomy or control, among others. Other objectives of using artificial diets include production of live material for food supply of other animals, either invertebrates or vertebrates. Artificial diets are especially useful in the case of insects whose biology, length of life cycle and habits make their study difficult in nature, such as bark and wood-boring beetles of the families Buprestidae and Cerambycidae, which usually spend a significant part of their lives under the bark and/or inside the wood. Several diets have been successfully developed for a variety of species of those families (*e. g*., Gardiner, 1970; Payne *et al*., 1975; Cannon and Robinson, 1982; Viedma *et al*., 1985).

In the case of the beetles of the genus *Monochamus* Dejean, 1821, worldwide recognised as the most important vectors of *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934), the pine wood nematode (PWN) (Linit, 1988), a few diets have been developed and tested on both the North American and Asian species (Alya and Hain, 1987; Kosaka and Ogura, 1990; Aloo and Katagiri, 1994; Necibi and Linit, 1997). The pine sawyer *Monochamus galloprovincialis* (Olivier, 1795) was found to be the vector of the PWN in Portugal (Sousa *et al.*, 2001), causing heavy losses and damages to the local forestry sector (Fig.1a). Numerous aspects of the insect's bio-ecology and interaction with the PWN have already been studied (Naves *et al.*, 2005, 2007b, 2008; Petersen-Silva *et al.*, 2012), but the lack of an artificial medium to rear the immature life stages of the beetle causes limitations to some studies. Therefore, the main objective of this study is to develop and compare artificial diets intended for mass rearing of the pine sawyer *Monochamus galloprovincialis*, testing and adapting pre-existing diets chosen by the simplicity of manufacturing and low-cost of ingredients (Carle, 1969; Alya and Hain, 1987; Kosaka and Ogura, 1990; Aloo and Katagiri, 1994; Necibi and Linit, 1997; Dubois *et al.*, 2002).

Fig. 1. Adult *Pinus pinaster* affected by the pine wilt disease a), diets #1 to #3 inside the climatic chamber b), wood boxes with *P. pinaster* prepared for *M. galloprovincialis* oviposition c), detail of *M. galloprovincialis* larvae feeding on the artificial medium d), *M. galloprovincialis* larval stages in diets #6 to #8 reared inside climatic chamber e), detail on the diets #1 to #4 (f).

MATERIAL AND METHODS

Diet preparation

Eight diets were tested, numbered #1 to #8 (Table 1). The phloem-cambium was obtained from two healthy adult pine trees (*Pinus pinaster* Aiton, 1789), which were felled and divided into small logs. In the INIAV laboratory the outer bark was removed and the phloem-cambium layer was peeled off and dried at room temperature before use. Fresh green pine needles were also removed from the pine branches and dried in an oven at 60ºC for four hours. The dried material was chopped into small pieces, and blended with a mixer followed by hand.

Diets #1 to #3 differed in the insect culture medium selected, which corresponded to around ¼ of the composition. Diets #4 and #5 excluded the culture medium, but incorporated cellulose powder as key ingredient (Fig.1b,e).

Diets #6 to #8 (Fig.1e) included an autoclaved pre mixture (pre-mix) (Table 2) with ascorbic and propionic acids added to prevent contamination with fungus or with other life forms and were adapted from the *Anoplophora glabripennis* (Motschulsky, 1854) (Coleoptera: Cerambycidae) diet developed by Dubois *et al.* (2002). In these diets the pre-mix and all the pre-autoclave ingredients were prepared in a large and sterile recipient, to which the phloem cambium was added later. All the ingredients were then autoclaved for 15 minutes at 121ºC. After sterilization, all procedures were conducted in a biological safety chamber to avoid contaminations. The methyl paraben, ascorbic and propionic acids were added to the mixture inside a fume hood using anti-acid gloves and safety mask. A total of 240 glass Petri-dishes (90mm diameter) were sterilized and filled with 50cm^3 of diet each.

Production price comparison

The price of production for each diet was calculated based on a virtual 100% survival rate for the diets, not considering the real survival observed. The values of production were calculated based on the reagent catalogue of reference chemistry international companies (Sigma-Aldrich ©, Merck © and Roche ©).

Collection of adult *M. galloprovincialis* and obtaining neonates

Adult maritime pine trees colonised by *M. galloprovincialis* were felled in February 2012 at Comporta, Portugal, and taken to the INIAV laboratory to allow the emergence of adult beetles, which were kept in groups of 25 individuals (15 ♀♀ and 10 ♂♂) in large netted boxes (80x60x40cm) with *Pinus pinaster* branches and logs under natural photoperiod conditions (±25ºC, 70% Relative humidity, 14/10 light/dark cycle) (Fig.1c). Every two days, the adults were fed with fresh *Pinus pinaster* branches and one or two-day old eggs were collected from the branches and removed from the boxes. The eggs were immersed in 70% ethanol for 10 seconds, followed by 10 seconds immersion in distilled water and placed in a Petri dish with a moistened filter paper in a rearing chamber at 25ºC 65% relative humidity (RH) and 0:24 light/dark cycle (LD), until hatching.

Table 1. Ingredients (quantities in g or ml) used to prepare 1 kg of each diet.

IngredientS	Diet (#)							
	1	2	3	4	5	6	7	8
Agar (g)	10	10	10	10	30	-	-	-
Dried yeast (g)	49	49	49	49	40	-	-	-
Potassium sorbate (g)	1	1	1	1	-	-	-	-
Culture medium* (g)	230	230	230	-	-	-	-	-
Pinus dried needles (g)	30	30	30	30	-	-	-	-
Pinus dried phloem-cambium (g)	200	200	200	200	-	108.8**	108.8***	-
Distilled water (ml)	480	480	480	480	671	641.17	641.17	641.17
Cellulose powder (g)	-	-	-	230	160	-	-	108.8
Sucrose (g)	-	-	-	-	20	-	-	-
Potato starch (g)	-	-	-	-	20	-	-	-
Soybean powder (g)	-	-	-	-	50	-	-	-
Soybean oil (ml)	-	-	-	-	4	5.86	5.86	5.86
95% Ethanol (ml)	-	-	-	-	3	-	-	-
Propionic acid (ml)	-	-	-	-	1	1.67	1.67	1.67
Citric acid (ml)	-	-	-	-	1	-	-	-
Pre-mix (g)	-	-	-	-	-	240	240	240
Ascorbic acid (g)	-	-	-	-	-	2.50	2.50	2.50

*Culture mediums: Diet #1: Painted lady *Vanessa cardui* (Linnaeus, 1758) (Lepidoptera: Nymphalidae); Diet #2: confused flour beetle *Tribolium confusum* Jacquelin du Val, 1863 (Coleoptera: Tenebrionidae); Diet #3: silkworm *Bombyx mori* (Linnaeus, 1758) (Lepidoptera: Bombycidae) medium, all commercially-available mediums acquired from Carolina Biological Supply Company®, USA. ** Phloem-cambium extracted 1 day after the felling of an adult maritime pine tree; *** Phloem-cambium extracted 2 weeks after the felling of an adult maritime pine tree.

▨ Ingredients to mix before autoclave (exclusive for diets #6 to #8)

■ Ingredients to mix after autoclave (exclusive for diets #6 to #8)

Table 2. Ingredients (in g) used in the Pre-mix of diets #6 to #8.

Ingredients	Quantity
Potato starch	25.01 g
Sucrose	25.01 g
Defatted soybean flour	60.03 g
Casein	14.01 g
Cholesterol	1.50 g
Wesson's salt mixture	4.05 g

Ingredients	Quantity
Dried yeast	45.03 g
Cellulose	70.01 g
Agar	35.02 g
Citric Acid	2.00 g
Sorbic Acid	2.50 g
Methyl Paraben	2.50 g

Rearing and handling of *M. galloprovincialis* larvae until eclosion

A two mm round hole was created on the diet's surface to insert the recently-hatched larvae, which were placed using a paintbrush. The number of larvae tested on each diet was 30. Only one larva was used by Petri dish, as the individuals tend to be aggressive to each other (Victorsson and Wikars, 1996; Dodds *et al*., 2001). The Petri dishes were kept in rearing chambers with constant temperature and humidity (25ºC, 65% RH, 15:9 LD) until pupae were obtained (Fig.1d), being then removed from the diets and placed in a clean Petri dish with moistened filter paper. Each individual was observed once a day after reaching the pupal stage. Three days after becoming an adult, the sex and weight were recorded. Diets were evaluated and compared based on the development time, number of adults obtained (herein referred as "eclosion"), and their weight. Diets were not replaced by fresh ones, although they were humidified to prevent excessive dehydration when considered necessary. To avoid contaminations, diets #6 to #8 were never re-hydrated or opened until the pupal stage.

Comparison with pine-reared *M. galloprovincialis*

To obtain adults reared in their natural pine hosts, two adult dead *P. pinaster* trees with confirmed presence of *M. galloprovincialis* were cut in Comporta and taken to the INIAV Lab, where 30 last-instar larvae were removed from the xylem galleries with the help of a vertical chain saw. The non-feeding mature larvae were placed individually in Petri dishes with moistened filter paper and kept at 25ºC 65% RH 15:9 LD until eclosion. Adult sex and weight were recorded and compared with diet-reared *M. galloprovincialis*.

Statistical analysis

Means were compared with the nonparametric Kruskal-Wallis test followed by the Tukey HSD test, with $p \leq 0.05$. Values are presented as means ± standard error (SE) in the text and tables, unless otherwise stated. Statistical analyses were performed with the software Statistica 6.0 (StatSoft Inc. 2003).

RESULTS

Diet efficiency

Adult *M. galloprovincialis* were obtained from all tested diets. Diets #1, #5 and #7 showed the highest survival rate, with a maximum of 80% in diet #5 (Table 3). In general, mortality was higher during the first days after the larvae were placed in the diet, and lowest for subsequent larval instars.

Due to the low number of available adults (eclosion rates below 25%), diets #2, #3 and #8 were excluded from subsequent statistical analysis. When comparing the time required to obtain adult insects, a mean of 107 ± 2.5 days was registered for the other diets. Diet #6 allowed the fastest development, with the eclosion of the first insect only 53 days after egg laying, (Table 3) differing in 29 days from the diet with the longest development time (Kruskal-Wallis test: χ^2=15.76, d.f.=4, P=0.0034). Regarding the weight of the adult insects, this was also statistically conditioned by the diet (Kruskal-Wallis test: χ^2=14.11, d.f.=4, P=0.0069), with diet #6 providing the heaviest adults and diet #4 the lightest individuals. Concerning the sex of the reared *M. galloprovincialis*, the overall proportion of males and females was balanced, with a ratio of 0.91 and slightly dominated by males.

Table 3. Number of adults (percentage of eclosion), development time (days), adult weight (grams), sex ratio (male/female) and estimated production price (€) for each diet.

	Diet (#)							
	1[1]	2	3	4[1]	5[1]	6[1]	7[1]	8
Nb of adults (% eclosion)	16 (53%)	5 (16%)	3 (11%)	8 (28%)	24 (80%)	14 (46%)	17 (57%)	3 (11%)
Development time (days) (mean ± SE, range)	124±6.0a (86–162)	101±5.6 (77–110)	101±5.1 (74–96)	102±3.1ab (77–113)	107±2.7ab (84–133)	95±6.7b (53–147)	106±8.7ab (59–165)	123 (99– 147)
Adult weight (g) (mean ± SE)	0.36±0.10a	0.39±0.13	0.65±0.19	0.33±0.10a	0.39±0.12ab	0.47±0.12c	0.45±0.12bc	0.29
Sex ratio (male/female)	0.78	na	na	na	0.50	1.13	0.33	na
Estimated price per adult (€) (100% virtual survival)	1.39	0.83	1.11	1.15	1.56	1.93	1.93	1.93

[1] Mean within each line followed by the same letter do not differ, P≤0.05. na - not applicable.

Production price comparison

The prices of manufacturing the diets had significant variations, with two groups of diets that could be distinguished. The first five diets had lower prices, being the last three the most expensive (Table 3).

Comparison with field-collected *M. galloprovincialis*

The mean weight of the field collected insects was 0.26 ± 0.02g, being significantly lower than the weight of adults reared from diets #5, #6 and #7 (Kruskal-Wallis test: X^2=24.01, d.f.=5, P=0.0002). All insects, both field collected and lab reared, were able to lay viable eggs on pine logs.

DISCUSSION

Three of the diets allowed the attainment of more than 50% of adults, with a maximum of 80%. According to Singh (1983), an ideal diet for an insect mass rearing programme should produce an average yield of adults of at least 75% from the initial viable eggs, with development rates and adult size similar to those in nature. Considering our results, we observe that diet #5 fulfils all those requirements.

Furthermore, if this diet is used to rear older larvae (instead of neonate individuals), the survival can be even higher, as found by Naves *et al*. (2007b), and also supporting previous observations by other authors which found that high mortality on the diets primarily affects the newly emerged and first instars larvae (Viedma, *et al*., 1985; Kosaka and Ogura, 1990; Allo and Katagiri, 1994; Hernández, 1994).

The absence of plant tissues on the diets apparently did not influence the duration of larval development or their survival. Our results contradict previous observations, as according to Gardiner (1970) the presence of plant tissues on the diets act as phago-stimulants for the young larvae and improve their acceptance by the insects. Allo and Katagiri (1994) also found that the inclusion of host plant tissue on *Monochamus alternatus* Hope, 1843 (Coleoptera: Cerambycidae) diets had a significant benefit influence on larval growth and development.

With the diets, adult insects could be obtained with a minimum mean of just 95 days, which implies a significant reduction of the normal larval development time, thus allowing the rearing of two or three sequential generations per year instead of the single annual generation observed under field conditions (Naves *et al.*, 2008).

The disparity of prices between the two groups of diets is justified by the presence of the costly methylparaben and the propinoic and ascorbic acids in diets #6 to #8. While diets #1 to #5 were consecutively contaminated with fungi, the last 3 ones did not present any sign of degradation or contamination when stored for various months, suggesting that adding these components is important when storing the diets for a long period. Nevertheless, when considering the tentative prices presented, it has to be considered that they exclude the cost of obtaining the phloem-cambium and/or the pine needles incorporated in all diets, except #5 and #8. As the phloem-cambium almost comprehends 25% of the total weight of some diets, its extraction from the pine trunk is time and labour-consuming, increasing its price. Although diet #2 has the lowest price of production, with approximately 0.83€ per larvae, the low number of obtained adults with this diet constitute a major setback. However, the diet with the highest success did not require this ingredient, suggesting that this ingredient appears to be facultative.

Additional studies to compare the vigour and fecundity of the diet and tree-emerged insects are needed to confirm the absence of differences between them. Furthermore, the development of an artificial oviposition substrate would substitute the pine material (logs and branches) currently used to obtain *M. galloprovincialis* eggs on the laboratory colonies, thus diminishing the labour and environmental impact of the diet. The permanent availability of insects from diets, allowing the continuous laboratory rear of multiple annual generations, would be a useful contribution for research lines requiring large numbers of *M. galloprovincialis* of all life stages year round.

ACKNOWLEDGEMENTS

We are grateful to INIAV colleagues Adérito Matos for his assistance in obtaining the pine trees and the Monochamus larvae, and to Marina Soares and Maria Florinda Medeiros for all the assistance provided in laboratory handling procedures. This work was partially funded by the REPHRAME project KBBE nº 265483 “Development of improved methods for detection, control and eradication of pine wood nematode in support of European Union Plant Health policy”.

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Received: February 12, 2013 *Accepted: January 08, 2014*

Distribution, hosts and parasitoids of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) in Portugal mainland

Silva Lusitana – 22-1(2014): 67-82

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Resumen

El nematodo del pino *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle, es el responsable por la enfermedad del decaimiento súbito del pino. Su principal insecto vector en Portugal es el cerambícido *Monochamus galloprovincialis* (Olivier), lo cual viene siendo ampliamente estudiado en los últimos años, todavía su actual distribución en Portugal continental no es conocida. Para revelar su distribución geográfica ensayos fueron desarrollados en todo el territorio nacional.

Para la distribución del vector, se ha analizado cerca de 15% de toda la área forestal de *Pinus pinaster* Aiton existente en Portugal continental y para la distribución de los parasitoides una zona más restricta. Los locales elegidos han tenido como base la actual zona afectada por la enfermedad y las fronteras con España. Los resultados obtenidos han sido registrados tanto en una cuadricula UTM (10x10km.) como en las zonas de clasificación NUTS portuguesas.

Se confirmó la presencia del cerambícido en 94% de los locales estudiados, sugiriendo una ampliada y generalizada distribución del insecto. Al mismo tiempo se prospectó diferentes especies forestales para confirmar la presencia/ausencia de insectos de la especie *M. galloprovincialis* en otros huéspedes. Además del *P. pinaster* se han obtenido resultados positivos para las especies *Pinus sylvestris* L. y *Pinus halepensis* Mill., confirmando su potencial como huéspedes alternativos. Se actualizó también la distribución geográfica para un total de 7 especies de parasitoides del cerambícido *M. galloprovincialis*.

Silva Lusitana, ***22***(1): 67 - 82, 2014
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Distribution, Hosts and Parasitoids of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) in Portugal Mainland

***Ricardo Petersen-Silva, **Pedro Naves, ***Paulo Godinho, ***Edmundo Sousa and ****Juli Pujade-Villar**

Abstract. The geographic distribution of the pine sawyer (*Monochamus galloprovincialis*), vector of the pine wood nematode (*Bursaphelenchus xylophilus*), is presented for Portugal mainland, according to the UTM 10x10km square grid. Field surveys were conducted in an area of approximately 15% of the maritime pine (*Pinus pinaster*) forested area. Simultaneously, a literature review was made for the previous references on the insect in Portugal. The insect vector was found in 94% of the surveyed locations, suggesting a widespread distribution. A total of eight forest and ornamental conifers were sampled, and for the first time the pine sawyer was detected in Portugal in two of them, namely *Pinus sylvestris* and *Pinus halepensis*. The beetle was not found associated with *Pinus pinea*, *Pinus radiata*, *Cupressus lusitanica*, *Larix decidua* and *Chamaecyparis lawsoniana*. The parasitoid guild associated with the pine sawyer in Portugal was reviewed and updated, with new geographic locations for a total of seven species.

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Key words: Cerambycidae, *Pinus pinaster*; Pine Wood Nematode, *Cyanopterus flavator*, geographic distribution

Distribuição, hospedeiros e parasitóides de *Monochamus galloprovincialis* em Portugal Continental

Sumário. É apresentada e discutida a distribuição geográfica em Portugal Continental do cerambicídeo *Monochamus galloprovincialis*, vetor do nemátode da madeira do pinheiro (*Bursaphelenchus xylophilus*), baseada na grelha UTM 10x10km. Foram realizadas prospeções em aproximadamente 15% da área florestal de pinheiro-bravo (*Pinus pinaster*). Simultaneamente, foi efetuada uma extensa revisão bibliográfica sobre as referências existentes para este cerambicídeo em Portugal. Foi confirmada a sua presença em 94% dos locais estudados, sugerindo uma distribuição alargada e generalizada. Foram prospetadas um total de oito espécies florestais e ornamentais, tendo-se encontrado pela primeira vez em Portugal a associação do inseto vetor com outros hospedeiros, nomeadamente com *Pinus sylvestris* e *Pinus halepensis.* Não se detetou a presença do inseto em *Pinus pinea*, *Pinus radiata*, *Cupressus lusitanica*, *Larix decidua* e *Chamaecyparis lawsoniana*. Adicionalmente, foi também revista e atualizada a comunidade de parasitóides associada a este cerambicídeo, num total de sete espécies, para as quais são apresentadas novas localizações geográficas.

Palavras-chave: Cerambycidae, *Pinus pinaster*, Nemátode da Madeira do Pinheiro, *Cyanopterus flavator*, distribuição geográfica

Distribution des hôtes et parasitoïdes de *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) au Portugal continental

Résumé. La répartition géographique du scieur du pin (*Monochamus galloprovincialis*), vecteur du nématode du bois de pin (*Bursaphelenchus xylophilus*), est présentée au niveau du territoire continental portugais selon le quadrillage UTM 10x10km. Sur le terrain les études ont été menées en 15% environ de la surface forestière de pin maritime (*Pinus pinaster*). Simultanément a été faite une révision bibliographique des références précédentes de l'insecte au Portugal. Sa présence a été confirmée en 94% des endroits étudiés, ce qui suggère une répartition généralisée. Un total de huit conifères forestières et ornementales ont été échantillonnés, et le scieur de pin a été détecté pour la première fois au Portugal sur deux d'entre eux, a savoir *Pinus sylvestris* et *Pinus halepensis*. Le coléoptère n'a pas été trouvé associé à *Pinus pinea*, *Pinus radiata*, *Cupressus lusitanica*, *Larix decidua* et *Chamaecyparis lawsoniana*. La guilde des parasitoïdes associés au scieur de pin au Portugal a été revue et mise à jour, avec de nouvelles zones géographiques pour un total de sept espèces.

Mots-clés: Cerambycidae, *Pinus pinaster*, Nématode du Bois de Pin, *Cyanopterus flavator*, répartition géographique

Introduction

One of the most important sanitary problems affecting the maritime pine (*Pinus pinaster* Aiton) is the pine wilt disease (PWD). The causal agent is the pine wood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle (Nematoda: Tylenchida: Aphelenchoididae), which was first reported in Portugal by MOTA *et al.* in 1999. To be dispersed the PWN requires a vector insect, being the most effective the beetles of the genus *Monochamus* Dejan (Coleoptera: Cerambycidae) (KOBAYASHI *et al.*, 1984; LINIT 1988; KISHI 1995; NAVES *et al.*, 2007). In Portugal, the sole vector of the PWN is the pine sawyer *Monochamus galloprovincialis* (Olivier) (SOUSA *et al.*, 2001). This beetle was firstly reported in the end of the XIX^th^ century by OLIVEIRA (1894), who collected specimens from the Leiria region which he erroneously identified as *Monochamus sutor* Linnaeus. The species remains absent in subsequent catalogues and collection during the XX^th^ century (CORRÊA DE BARROS, 1916; NEVES, 1950), until it was collected by NEVES *et al.* (1978) in burned *P. pinaster* trees near Arganil, central Portugal.

The pine sawyer is widely distributed in Europe (except in the United Kingdom, Ireland and Cyprus), being common in the Mediterranean area, namely in Italy, France, Spain, and Portugal (FRANCARDI and PENNACCHIO, 1996; VIVES, 2000; BRUSTEL *et al.*, 2002; KOUTROUMPA, 2007). Outside of Europe, *M. galloprovincialis* can also be found in North Africa, the Caucasus, Russia, China and Mongolia (HELLRIGL, 1971).

Due to the increasing importance of this species as vector of the PWN, several recent studies have detailed various aspects of its biology and ecology (e.g. KOUTROUMPA *et al.*, 2008; NAVES *et al.*, 2008; AKBULUT, 2009), the application of chemical substances to control it (SOUSA *et al.*, 2013) and its natural enemies (NAVES *et al.*, 2005; PETERSEN-SILVA *et al.*, 2012). Despite the recently-acquired knowledge, the PWN has continued to spread and is now causing mortality on pine forests in central and northern Portugal (SOUSA *et al.*, 2011), (Figure 1). Even though the human transport of infested wood is responsible for long-distance dispersal of the disease, the beetle's flight activity and dispersal is also important when considering the spreading of PWD at a local scale. A detailed knowledge on the distribution and hosts of the vector beetle is important to understand the establishment and incidence of wilt disease in the terrain, and is lacking for Portugal. In this paper we present the first mapping of the distribution and hosts of *M. galloprovincialis* in continental Portugal, and review the parasitoid guild with new geographic records. A detailed bibliographic review on *M. galloprovincialis* distribution in Portugal was conducted.

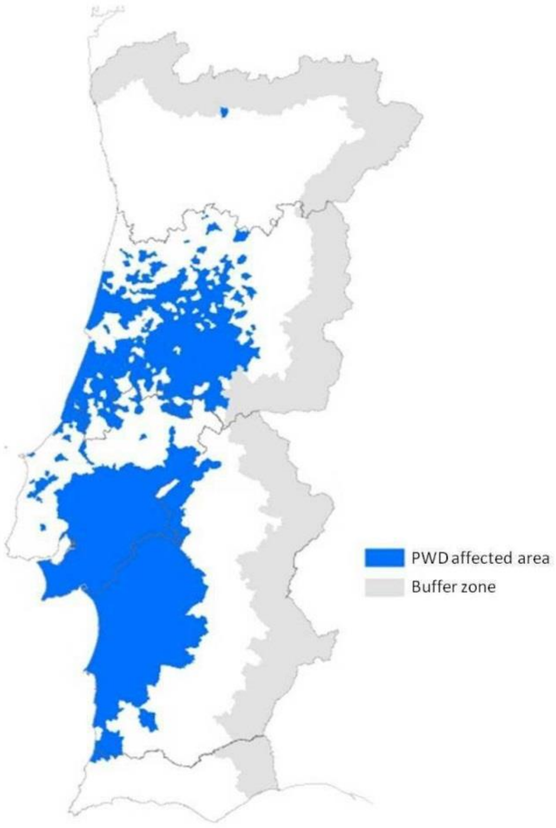

Figure 1 *– Detail of the area currently affected by the pine wilt disease in continental Portugal. Figure adapted from ICNF (2014)*

Materials and methods

Geographic distribution and hosts of Monochamus galloprovincialis

Surveys in the terrain were conducted by the INIAV (Instituto Nacional de Investigação Agrária e Veterinária, Oeiras, Portugal) entomological team. The surveys were mostly directed to areas where *P. pinaster* is the dominant tree species (ICNF, 2010), recording the locations in the UTM 10x10 km coordinates system (Universal Transverse Mercator coordinate system) and on the NUTS II system (Nomenclature of Territorial Units for Statistics). Surveys braced approximately 15% of the *P. pinaster* forested area. The surveyed areas were chosen based on the current distribution of the zones affected by the pine wilt disease (Figure 1, Annex 1), and on the proximity with Spain (buffer zone). The distribution of *M. galloprovincialis* was assessed by the presence of adult insects or larval instars inside decaying or dead trees. The characteristic round emergence holes made by the adults also allowed identifying past presence of the pine sawyer, but wood dissection was necessary to distinguish from similar holes made by carpenter bees of the genus *Xylocopa* Latreille or by wood wasps of the genus *Sirex* Linnaeus.

Although the majority of the survey was carried on *P. pinaster*, other conifers were also sporadically sampled, namely *Pinus halepensis* Mill., *Pinus pinea* L., *Pinus sylvestris* L., *Pinus radiata* Don, *Cupressus lusitanica* Mill., *Larix decidua* Mill. and *Chamaecyparis lawsoniana* (A. Murraybis) Parl.

Table 1 - *Tree species surveyed for the presence of Monochamus galloprovincialis and parasitoids associated with the species; percentage of total analyzed territory in Portugal mainland according to the NUTS II system (UTM grid of 10 x 10km)*

Tree species surveyed for the presence of *M. galloprovincialis*	**NUTS II**				
	Norte (1)	**Centro (2)**	**Lisboa (3)**	**Alentejo (4)**	**Algarve (5)**
Chamaecyparis lawsoniana	x				
Cupressus lusitanica	x				
Larix decidua	x				
Pinus halepensis			x		
Pinus pinaster	x	x	x	x	x
Pinus pinea			x		x
Pinus radiata	x				
Pinus sylvestris	x				
Percentage of UTM analyzed for parasitoid presence; (Nb)	1% (2)	4% (11)	0% (0)	2% (7)	3.5% (2)
Percentage of UTM analyzed for *M. galloprovincialis* presence (%); (Nb)	8.5% (21)	12.5% (38)	31.5% (11)	5.5% (18)	22% (13)

x- Presence of *M. galloprovincialis* confirmed; Nb- total number of analyzed UTM

Parasitoid guild associated with Monochamus galloprovincialis

The parasitoid guild distribution was studied from 2002 to 2003 and from 2011 to 2014. Locations where the surveys were conducted were randomly chosen among the locations surveyed for *M. galloprovincialis* distribution (Table 1, Annex 2). Field methods were based in the felling of trees with *M. galloprovincialis* presence and the creation of trap trees in the field. Collected wood was transported and reared at the INIAV laboratory, as described in NAVES *el al*. (2005) and PETERSEN-SILVA *et al*. (2012). The identification of parasitoid specimens was confirmed by Dr. Sergey Belokobylskij (Polish Academy of Sciences, Warszawa, Poland) and Dr. Dmitri Kasparyan (St. Petersburg, Russia).

Distribution data

Maps were created with the ESRI® ArcMap™ 10.0 program, and the presence/absence of *M. galloprovincialis* and the parasitoid guild was recorded in the UTM square grid (10 x 10 km) for continental Portugal. New records are signaled in the tables.

Results

Geographic distribution and hosts of Monochamus galloprovincialis

A total of 96 UTM squares with dominant forest cover of *Pinus pinaster* were surveyed over the years (Table 1, Figure 2, and Annex 1). The pine sawyer *M. galloprovincialis* was found in 90 of them (93.8%), being widely distributed in the Portuguese continental territory. This cerambycidae showed an obvious preference for the maritime pine, although it was also found associated with other hosts such as *P. sylvestris* in Viana do Castelo (UTM NG21), and *Pinus halepensis* in Cascais (UTM MC68). All of these are new host records for Portugal. The pine sawyer was not detected in other surveyed conifers, such as *Pinus pinea*, *Cupressus lusitanica*, *Larix decidua* and *Chamaecyparis lawsoniana*.

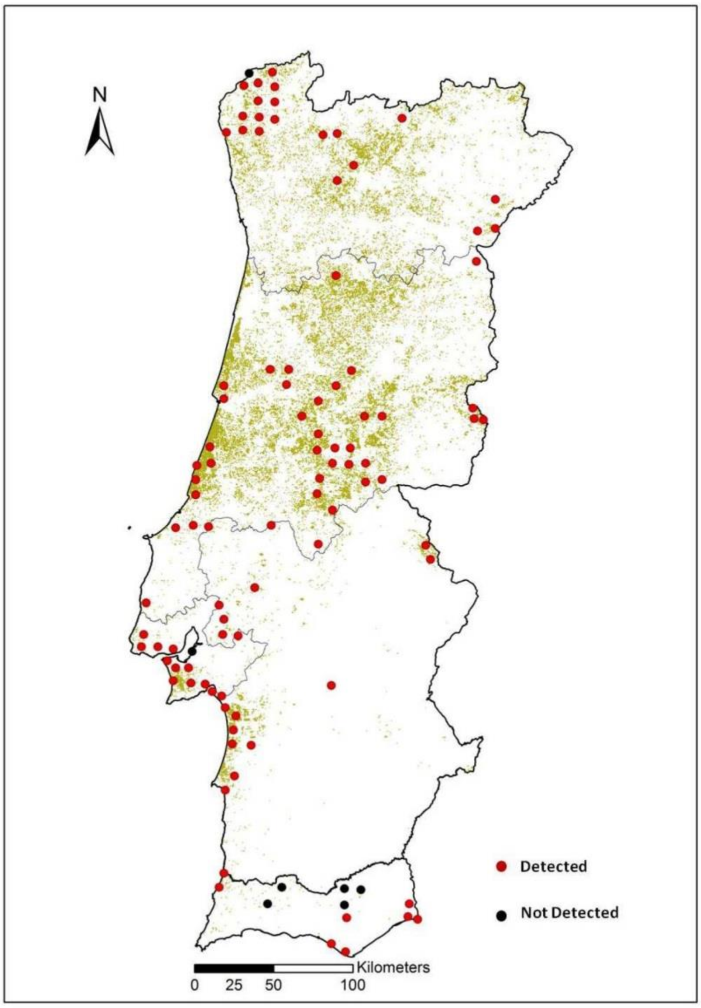

***Figure* 2** - *Distribution of Monochamus galloprovincialis in continental Portugal (NUTS II grid)*

Parasitoid guild associated with Monochamus galloprovincialis

The surveys lead to the collection of 188 parasitoid specimens found associated with *M. galloprovincialis* larvae. Specimens belong to the families Ichneumonidae and Braconidae and include seven species, namely: *Atanycolus denigrator* (L.); *Atanycolus ivanowi* (Kokujev); *Cyanopterus flavator* (Fabricius);

Coeloides sordidator Ratzeburg; *Doryctes striatellus* (Nees); *Iphiaulax impostor* (Scopoli) and *Xorides depressus* (Holmgren). Their distribution, percentage of species and the UTM grid reference can be found in Table 1, Figure 3, and Annex 2, of which most are new distribution localities for Portugal. *Cyanopterus flavator* is the more frequently detected species, being found in seven of the 15 locations analyzed.

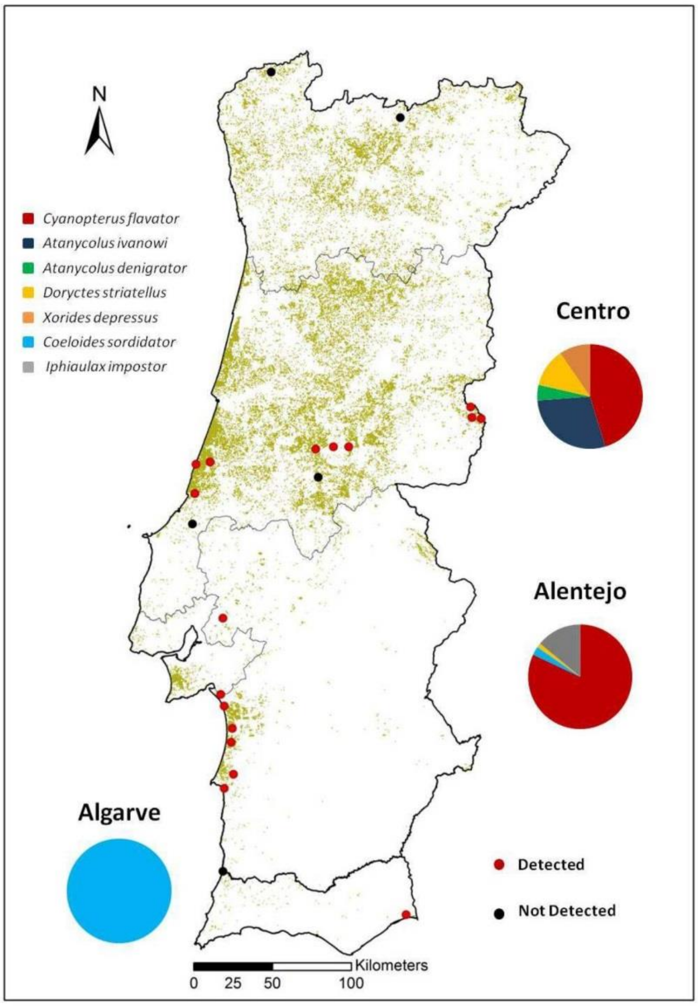

Figure 3 *- Distribution (NUTS II grid) and percentage of parasitoid species associated with Monochamus galloprovincialis in continental Portugal*

Conclusions/ Discussion

The pine sawyer was found to be widely distributed on most of the Portuguese territory where pine forests are abundant, not being conditioned by edapho-climatic variations of the different localities, which was to be expected considering its vast distribution range (HELLRIGL, 1971). The absence of this widespread and conspicuous species from the previous catalogues of OLIVEIRA (1894), CORRÊA DE BARROS (1916), NEVES (1950) and SERRANO (1983) is somewhat intriguing, although these authors were not specifically looking for this beetle, unlike the surveys conducted by the INIAV team. It is also possible that the pine sawyer may have benefited from the great campaigns of forestation with *P. pinaster* that occurred in Portugal during the XX^th^ century, experiencing a distribution expansion due to the higher availability of hosts. Insect populations may have also benefited from the sanitary problems and forest fires which occurred after this forest expansion, as the dead and scorcher pines tend to attract *Monochamus* beetles for breeding (BYERS, 1989a; b; SATO and MAETO, 2006; BONIFÁCIO *et al.*, 2012).

Although maritime pine is clearly the preferred host in Portugal, the association of *M. galloprovincialis* with other hosts such as *P. sylvestris* and *P. halepensis*is is not surprising, as these are very frequent hosts all over the Mediterranean basin (HELLRIGL, 1971; EVANS *et al.*, 1996; FRANCARDI and PENNACCHIO, 1996).Other unusual hosts include *Pinus strobus* (VINCENT, 2007), *Pinus uncinata* (BEHALOVÁ, 2006) and even *Picea abies* (L.) H. Karst. and *Picea orientalis* (L.) Peterm. (YUKSEL, 1998), although all of these species are inexistent or rare in Portugal.

Regarding the parasitoids associated with the insect vector, a fairly diverse guild was detected over the years, being dominated by *Cyanopterus flavator*, the most abundant species and with the widest distribution. Nevertheless and as already mentioned by NAVES *et al*. (2005) and PETERSEN-SILVA *et al*. (2012), these are all generalist species which attack a vast array of Cerambycidae and even other coleopteran, and therefore, their use as biological control agents seems ineffective and unlikely. Future samplings should focus on other natural enemies, such as bacteria or fungi, which have been found to affect other *Monochamus* species elsewhere, and have not yet been studied in detail in Portugal.

This is a first approach to elaborate a detailed mapping on the distribution range and hosts of *M. galloprovincialis* in Portugal, and may constitute an important tool for future assessments of pine wilt disease distribution and dispersal, and for the implementation of management and control strategies of this complex forest sanitary problem.

Acknowledgments

We would like to thank the colleagues of Instituto Nacional de Investigação Agrária e Veterinária, Mr. Adérito Matos, Dr. Luis Bonifácio and Mr. Victor Gonçalves for assistance in the field surveys and *M. galloprovincialis* laboratory handling, and Drª Lurdes Inácio for her comments and valuable text revision. We are also grateful to Dr. Sergey Belokobylskij of the Polish Academy of Sciences, Warszawa (Poland) and Dr. Dmitri Kasparyan (St. Petersburg, Russia) for confirming the identification of some parasitoid specimens. We would also like to thank Dr. José Manuel Rodrigues for the map presenting the current area affected by the pine wilt disease. This work was partially funded by the project REPHRAME Development of improved methods for detection, control and eradication of pine wood nematode in support of European Union Plant Health policy.

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Submitted for publication in April 2014
Accepted in May 2014

Annexes

Annex 1

Distribution of *Monochamus galloprovincialis* in continental Portugal (UTM grid of 10 x 10km) and hosts

(a) – New host for *M. galloprovincialis* in Portugal; 1- Ribeiro, 1992; 2- Neves, *et al*., 1978; 3- Oliveira, 1894; Unknown – *M. galloprovincialis* adult specimens collected outside the host.

Parish	**UTM reference**	**Host**	***Monochamus galloprovincialis***
Abrantes (2)	ND75	*P. pinaster*	Detected
Alcácer do Sal (4)	NC24	*P. pinaster*	Detected
Alcobaça (2)	MD99	*P. pinaster*	Detected
	ND06	*P. pinaster*	Detected
Aljezur (5)	NB13	*P. pinaster*	Detected
Arcos de Valdevez (1)	NG43	*P. pinaster*	Detected
	NG44	*P. pinaster*	Detected
Arganil[2] (2)	NE85	*P. pinaster*	Detected
Barreiro (3)	MC97	*P. pinaster*	Detected
Benavente (4)	NC19	*P. pinaster*	Detected
	ND10	*P. pinaster*	Detected
	ND11	Unknown	Detected
Caldas da Rainha (2)	MD86	*P. pinaster*	Detected
	MD96	*P.pinaster*	Detected
Cascais (3)	MC68	*P. halepensis*	Detected(a)
Castelo Branco (2)	PE13	*P. pinaster*	Detected
Castro Daire (2)	NF82	*P. pinaster*	Detected
Castro Marim (5)	PB31	*P. pinaster*	Detected
	PB32	*P. pinaster*	Detected
	PB41	*P. pinaster*	Detected
Celorico de Basto (1)	NF88	*P. pinaster*	Detected
Chaves (1)	PG22	*P. pinaster*	Detected
Évora[1] (4)	NC86	*P. pinaster*	Detected
Faro (5)	NA89	*P. pinaster*	Detected
		P. pinea	Not detected
	NA99	*P. pinaster*	Detected
Figueira da Foz (2)	NE14	*P. pinaster*	Detected
	NE15	*P. pinaster*	Detected
Figueira de Castelo Rodrigo (2)	PF73	*P.pinaster*	Detected
Figueiró dos Vinhos (2)	NE63	*P. pinaster*	Detected
Freixo de Espada a Cinta (1)	PF85	*P. pinaster*	Detected
Fundão (2)	PE03	*P. pinaster*	Detected
Góis (2)	NE74	*P. pinaster*	Detected
Golegã (4)	ND46	*P. pinaster*	Detected

Grândola (4)	NC14	*P. pinaster*	Detected
	NC15	*P. pinaster*	Detected
	NC22	*P. pinaster*	Detected
	NC23	*P. pinaster*	Detected
	NC32	*P. pinaster*	Detected
Idanha-a-Nova (2)	PE73	*P. pinaster*	Detected
	PE83	*P. pinaster*	Detected
Leiria[3] (2)	NE01	*P. pinaster*	Detected
Lisboa (3)	MC88	Unknown	Detected
Loulé (5)	NB92	*P. pinaster*	Not detected
	NB93	*P. pinaster*	Not detected
Mação (2)	ND87	*P.pinaster*	Detected
Mafra (3)	MD61	*P. pinaster*	Detected
Marinha Grande (2)	ME90	*P. pinaster*	Detected
	NE00	*P. pinaster*	Detected
Mealhada (2)	NE46	*P. pinaster*	Detected
Mogadouro (1)	PF87	*P. pinaster*	Detected
Monção (1)	NG45	*P. pinaster*	Detected
Monchique (5)	NB42	*P. pinaster*	Not detected
Montalegre (1)	NG81	*P. pinaster*	Detected
Montijo (3)	MC98	*P. pinea*	Not detected
	NC29	*P. pinaster*	Detected
Nazaré (2)	MD98	*P. pinaster*	Detected
Odemira (4)	NB14	*P. pinaster*	Detected
Oeiras (3)	MC78	*P. pinaster*	Detected
Oleiros (2)	NE91	*P. pinaster*	Detected
Oliveira do Hospital (2)	NE96	*P. pinaster*	Detected
Paredes de Coura (1)	NG33	*P. pinaster*	Detected
	NG34	*P. pinaster*	Detected
		P. sylvestris	Not detected
Pedrógão Grande (2)	NE72	*P. pinaster*	Detected
Penacova (2)	NE55	*P. pinaster*	Detected
	NE56	*P. pinaster*	Detected
Penamacor (2)	PE74	*P. pinaster*	Detected
Ponte da Barca (1)	NG42	*P. pinaster*	Detected
Ponte de Lima (1)	NG31	*P. pinaster*	Detected
		P. sylvestris	Not detected
	NG32	*P. pinaster*	Detected
Portalegre (4)	PD44	*P. pinaster*	Detected
	PD45	*P. pinaster*	Detected
Proença-a-Nova (2)	NE90	*P. pinaster*	Detected
	PE00	*P. pinaster*	Detected
Ribeira de Pena (1)	NF99	*P. pinaster*	Detected
Salvaterra de Magos (4)	ND32	*P. pinaster*	Detected
Santiago do Cacem (4)	NC20	*P. pinaster*	Detected
São Brás de Alportel (5)	NB91	*P. pinaster*	Detected
Sardoal (2)	ND78	*P. pinaster*	Detected
Seixal (3)	MC77	*P. pinaster*	Detected
	MC87	*P. pinaster*	Detected
Sertã (2)	NE71	*P. pinaster*	Detected
	NE80	*P. pinaster*	Detected
	NE81	*P. pinaster*	Detected

Sesimbra (3)	MC86	*P. pinaster*	Detected
	MC96	*P. pinaster*	Detected
	NC05	*P. pinaster*	Detected
		P. pinea	Not detected
Setúbal (3)	NC06	*P. pinaster*	Detected
Silves (5)	NB53	*P. pinaster*	Not detected
Sines (4)	NB19	*P. pinaster*	Detected
		P. pinaster	Detected
Sintra (3)	MC69	*P. pinaster*	Detected
Tavira (5)	PB03	*P. pinaster*	Not detected
Torre de Moncorvo (1)	PF75	*P. pinaster*	Detected
Valença (1)	NG25	*P. radiata*	Not detected
Viana do Castelo (1)	NG11	*P. pinaster*	Detected
	NG21	*P. sylvestris*	Detected(a)
	NG22	*P. pinaster*	Detected
Vieira do Minho (1)	NG71	*P. pinaster*	Detected
		P. sylvestris	Not detected
		C. lusitanica	Not detected
		L. decidua	Not detected
		C. lawsoniana	Not detected
Vila de Rei (2)	ND79	*P. pinaster*	Detected
Vila Nova de Cerveira (1)	NG24	*P. pinaster*	Detected
		P. radiata	Not detected
Vila Velha de Rodão (2)	PD09	*P. pinaster*	Detected
	PD19	*P. pinaster*	Detected

(a) – New *Monochamus galloprovincialis* host for the Portuguese territory; (Nb) – Number of the NUTS II region associated to each parish. 1-Norte, 2-Centro, 3-Lisboa, 4-Alentejo, 5-Algarve

Annex 2

Distribution (UTM grid of 10 x 10km) of parasitoid species associated with *Monochamus galloprovincialis* in continental Portugal

Parish (NUTS II)	**UTM reference**	**Parasitoid presence (Nb of specimens)**
Benavente (4)	ND10	*C. flavator* (6)
Caldas da Rainha (2)	MD96	Not detected
Castro Marim (5)	PB31	*C. sordidator*(a) (1)
Chaves (1)	PG22	Not detected
Grândola (4)	NC14 NC15 NC22 NC23	*C. flavator* (107) ; *C. sordidator*(5); *I. impostor* (21)
Idanha-a-Nova (2)	PE83 PE73	*A. ivanowi* (6); *C. flavator* (7)
Leiria (2)	MD98	*D. striatellus*(a) (1)
Marinha Grande (2)	NE00 ME90	*A. denigrator* (2); *C. flavator* (7); *D. striatellus* (2); *X. depressus* (3)
Monção (1)	NG45	Not detected
Odemira (4)	NB14	Not detected
Oleiros (2)	NE91	*D. striatellus*(a) (1); *C. flavator*(a) (2); *X. depressus*(a) (1)
Penamacor (2)	PE74	*A. ivanowi* (6); *C. flavator* (7)
Santiago do Cacém (4)	NC20	*D. striatellus*(a) (1)
Sertã (2)	NE71 NE81	*C. flavator*(a) (3); *D. striatellus*(a) (1)
Sines (4)	NB19	*D. striatellus*(a) (1)
Vila de Rei (2)	ND79	Not detected

(a) – New geographical location in the Portuguese territory; (Nb) – Number of the NUTS II region associated to each parish. 1-Norte, 2-Centro, 3-Lisboa, 4-Alentejo, 5-Algarve

Parasitoids of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae), vector of the pine wood nematode, with identification key for the Palaearctic region.

Zookeys – 251(2012): 29-48

Ricardo Petersen-Silva; Juli Pujade-Villar; Pedro Naves; Edmundo Sousa; Sergey Belokobylskij

Resumen

Se estudió el complejo de parasitoides asociados al cerambícido *Monochamus galloprovincialis* (Olivier), vector del nematodo del pino *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle. Se prospectó 5 diferentes regiones del territorio portugués, donde la especie forestal predominante es el pino marítimo (*Pinus pinaster* Aiton). Los ensayos han transcurrido en las localidades de Comporta, Marinha Grande, Monção, Vale Feitoso y Vimeiro, las cuales presentan diferentes condiciones edapho-climáticas. Se utilizaron dos métodos de prospección distintos; la tala de árboles muertos infectados con el insecto, donde saldrían los parasitoides, y la utilización de árboles trampa, previamente inoculadas con huevos de *M. galloprovincialis*. Fueron utilizados 96 troncos trampa divididos en 4 categorías (huevos, larvas floémicas, larvas xilémicas y pupas), los cuales fueron colocados en las regiones elegidas. Todos los ensayos fueron cuidadosamente sincronizados con el ciclo biológico del insecto en condiciones naturales.

Como resultado de esta prospección, se obtuvieron 5 especies parasitando este cerambícido en Portugal; *Atanycolus ivanowi* (Kokujev), *Atanycolus denigrator* (Linnaeus), *Cyanopterus flavator* (Fabricius), *Doryctes striatellus* (Nees) and *Xorides depressus* (Holmgren). Las especies están divididas entre las familias Braconidae y Ichneumonidae en la proporción de 4/1. Sin embargo el número total de especies parasitando *M. galloprovincialis* en la región Paleártica aumentó para 12. Se presenta juntamente con la descripción detallada de huéspedes y distribución de las especies, una clave ilustrada de identificación para dichas especies.

ZooKeys 251: 29–48 (2012)
doi: 10.3897/zookeys.251.3986
www.zookeys.org

RESEARCH ARTICLE

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Parasitoids of *Monochamus galloprovincialis* (Coleoptera, Cerambycidae), vector of the pine wood nematode, with identification key for the Palaearctic region

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Academic editor: *C. van Achterberg* | Received 11 September 2012 | Accepted 3 December 2012 | Published 17 December 2012

Citation: Petersen-Silva R, Pujade-Villar J, Naves P, Sousa E,Belokobylskij S (2012) Parasitoids of *Monochamus galloprovincialis* (Coleoptera, Cerambycidae), vector of the pine wood nematode, with identification key for the Palaearctic region. ZooKeys 251: 29–48. doi: 10.3897/zookeys.251.3986

Abstract

The parasitoid complex associated with *Monochamus galloprovincialis* (Olivier), vector of the pine wood nematode, is discussed. Four species of the family Braconidae and one Ichneumonidae were found associated with *Monochamus galloprovincialis* in Portugal, namely *Atanycolus denigrator* (Linnaeus), *Atanycolus ivanowi* (Kokujev), *Cyanopterus flavator* (Fabricius), *Doryctes striatellus* (Nees) (Braconidae), and *Xorides depressus* (Holmgren) (Ichneumonidae). *Atanycolus ivanowi, Atanycolus denigrator, Doryctes striatellus* and *Xorides depressus* are new species for Portugal fauna, and *Monochamus galloprovincialis* is recorded as a new host of *Xorides depressus*. A key for determination of the ichneumonoid parasitoids of the pine sawyer is provided for the Palaearctic fauna.

Keywords

Braconinae, Ichneumonidae, parasitoids, *Monochamus galloprovincialis*, Cerambycidae, key to species

Introduction

Worldwide, beetles of the genus *Monochamus* Dejean (1821) (Coleoptera; Cerambycidae) are the most important vectors of the pine wood nematode *Bursaphelenchus xylophilus* (Steiner and Buhrer 1934) (Nematoda, Tylenchida, Aphelenchoididae) (Linit 1988, 1990, Kishi 1995). This nematode is native to North America, where it's not considered a primary pathogen of indigenous pines, although in countries where it has been introduced it is an important agent of mortality for susceptible pines. In Portugal, where the nematode has been present for over a decade now, the pine sawyer *Monochamus galloprovincialis* (Olivier 1795) is it's sole vector (Sousa et al. 2001).

The pine sawyer *M. galloprovincialis* is widely distributed in Europe (except in the United Kingdom, Ireland and Cyprus), and is also present in Caucasus, Russia, North Africa, China and Mongolia (Hellrigl 1971, Francardi and Pennacchio 1996). The *Monochamus* beetles do not breed on healthy trees, and are attracted only to stressed, dying or recently killed trees and freshly felled timber for egg laying (Linsley 1959, Hellrigl 1971, Linit 1987, Hanks 1999). Before the introduction of *B. xylophilus* in Portugal, *M. galloprovincialis* was considered a secondary forest pest and nothing was known about the most important aspects of its biology and ecology. In the rest of Europe there is also an absence of detailed studies on its biology, with the exception of the classic paper of Hellrigl (1971).

The most efficient way to control wilt disease is to decrease the population levels of the vector *Monochamus* beetles. However, the different methods to control these insects usually have success only in localized, small-dimension areas, but are difficult to implement at low cost and have reduced efficiency over large forested areas. In Portugal, the most important management and control strategy consists in the elimination of symptomatic trees during late autumn, winter and early spring, while the insect vector is inside the host as late-instar larvae or pupae. The vector's populations can also be diminished during the beetle´s flight season with the use of traps baited with attractive lures (Naves et al. 2008).

Specific and efficient natural enemies (bio-control) would be an interesting and environmental-friendly option, but until now there are no adequate species for such control program (Naves et al. 2005). A few studies have already dealt with the parasitoids of this pine pest (Francardi and Pennacchio 1996, Francardi et al. 1998, Naves et al. 2005), although the information is scarce and disperse. In this paper we report on the diversity of parasitoids associated with *M. galloprovincialis* in Portugal, their frequency and revise all previous information on ichneumonoids parasitoids of *M. galloprovincialis* in the Palaearctic Region, resulting in a key for their identification.

Material and methods

For the parasitoid surveys, two different approaches were employed:

I – Field surveys were made in five main pine regions in Portugal, selected by their different environmental characteristics, between April and October 2011. The areas where the study was made were:

Monção: (Lat: 42.075801, Lon: -8.517426)
Marinha Grande, Leiria: (Lat: 39.751677, Lon: -8.9977)
Comporta: (Lat: 38.35808, Lon: -8.772995)
Vale Feitoso, Idanha-a-Nova: (Lat: 40.064935, Lon: -6.987579)

In each location two dead *Pinus pinaster* Aiton, trees were felled, the wood sections colonized by *M. galloprovincialis* were divided into 60 cm logs, and taken to the INIAV (Instituto Nacional de Investigação Agrária e Veterinária) laboratories in Oeiras to be stored in wood boxes prepared with a wire mesh. The boxes were completely covered with a black plastic, leaving two holes with transparent containers to collect emerged insects. Boxes were analyzed every two days for insects, which were collected and stored in alcohol for posterior identification. Additionally, the boxes were opened frequently for the evaluation of the content and to collect other emerged insects. The logs were kept inside the boxes until no emergences were registered for a period of two months, and then debarked and opened (with a vertical chain saw) to detect the hosts and life stages attacked by the parasitoids.

II – Complementary to the previous approach, artificial trap-trees were also prepared, consisting of living maritime pine trees which were cut into logs and given to adult *M. galloprovincialis* to lay eggs under laboratory controlled conditions. Each log had a medium length of 40 cm and a medium diameter of 10 cm. subsequently, the logs were taken to the terrain to be colonized by parasitoids, and in each log a hole was made to allow passing a rope to hang them in the trees, at the branches height.

A total of 96 logs were divided according to the insect´s life stages (eggs, phloem larvae, xylem larvae and pupae), with 24 replicates each. Trials were made in Monção, Marinha Grande (Leiria), Comporta and Vimeiro, Alcobaça (Lat: 39.477811, Lon: –9.022316). In each location, six trap-trees were taken to the terrain four times during the year (pupae: April; eggs: July; phloem larvae: August; xylem larvae: October), in synchrony with the natural life cycle of the insect, and hanged in a healthy adult pine tree for a period of ten days. Subsequently, the logs were taken to the INIAV laboratory, and kept in wooden boxes (similarly to the previous experiment) to allow for emergencies. All emerged insects were identified and prepared to be photographed in the stereomicroscopy and environmental scanning electron microscopy (Serveis Científico-Tècnics de la Universitat de Barcelona). The field-emission gun environmental scanning electron microscope (FEI Quanta 200 ESEM) was used for high-resolution imaging without gold-coating with the purpose of not damaging the specimens.

All the collected material was stored in INRB Forestry Entomology Collection, Oeiras, Portugal. The insects collected with the trap-tree were labeled "Artificial" and the ones from the dead trees were labeled "Natural". Terminology employed in the key for morphological features, sculpture and measurements as well as wing venation nomenclature follows Belokobylskij and Maeto (2009).

Results

Besides *M. galloprovincialis*, the following insects emerged from the wood and logs: *Arhopalus* sp. (Coleoptera: Cerambycidae), *Orthotomicus erosus* (Wollaston 1857) (Coleoptera: Scolytidae), *Thanasimus formicarius* (Linnaeus 1758) (Coleoptera: Cleridae), *Sirex noctilio* Fabricius (1793) (Hymenoptera: Siricidae), and some species of the family Anobiidae. Other bark beetles (Scolytidae) were also present in the dead tree material, although they were not analyzed.

No parasitoids emerged from the trap trees with eggs, xylem larvae and pupae of *M. galloprovincialis*. Parasitism was only found in the sub-cortical larvae, corresponding to the host´s first instars. A total of 27 specimens, belonging to five species, were recovered solely from Marinha Grande and Vale Feitoso, seven of which (all *Cyanopterus flavator*) from the trap-trees, while the remaining species were all obtained from the dead trees. *Cyanopterus flavator* (Fabricius) and *Atanycolus ivanowi* (Kokujev) were found in Vale Feitoso, and in Marinha Grande the following ichneumonids and braconids were recovered: *Atanycolus denigrator* (L.); *C. flavator*; *Doryctes striatellus* (Nees) and *Xorides depressus* (Holmgren).

By far, *Cyanopterus flavator* was the most abundant species with a total of 15 specimens from Marinha Grande and Vale Feitoso. Cocoons of this species were found in the xylem galleries of *M. galloprovincialis*, alongside with mandibles of the dead larvae.

The other cocoons found were in the inner bark associated with the larval galleries of the pine sawyer. The number of cocoons found matches exactly the number of parasitoids obtained from this surveys, and no other cocoons were found parasitizing any of the species previously mentioned. The parasitized species emerged between May and September under laboratory conditions and the precise dates are recorded in the labels of each specimen.

The following hymenoptera emerged from the wood, with both Braconidae (4) and Ichneumonidae (1):

Family Braconidae

Atanycolus denigrator (Linnaeus 1758)

http://species-id.net/wiki/Atanycolus_denigrator

Figures 7a, e

Material examined. Portugal: 1 female, "Leiria, 1/6/2011", "Ensaio Pupas Natural", "Col. Estação Florestal Nacional"; 1 male, "Leiria, 1/6/2011", "Ensaio Pupas Natural", "Col. Entomologica, est. Florestal".

Distribution. Palaearctic: Austria, Bulgaria, China, Croatia, former Czechoslovakia, Finland, France, Germany, Greece, Hungary, Israel, Italy, Kazakhstan, Korea, Mongolia, Norway, Poland, Russia, Sweden, Switzerland, Turkey, United Kingdom. **Afrotropical**: Niger (Yu et al. 2005, Wang et al. 2009). This species is here recorded for Portugal for the first time.

Hosts. *Anthaxia morio* Fabricius, *Chrysobothris chrysostigma* Linnaeus, *Ch. solieri* Laporte & Gory, *Lampra rutilans* (Fabricius), *Poecilonota variolosa* (Paykull) (Buprestidae); *Acanthocinus aedilis* Linnaeus, *A. griseus* (Fabricius), *Arhopalus syriacus* (Reitter), *Monochamus galloprovincialis* (Oliver), *M. sutor* (Linnaeus), *Rhagium indagator* Fabricius, *R. inquisitor* Linnaeus, *R. mordax* (Degeer), *Saperda populnea* (Linnaeus), *Tetropium castaneum* (Linnaeus), *T. fuscum* (Linnaeus), *T. gabrieli* Weise (Cerambycidae); *Ips sexdentatus* (Boerner) (Scolytidae) (Yu et al. 2005, Wang et al. 2009).

Biology. *Atanycolus denigrator* is an ectoparasitoid of *M. galloprovincialis* attacking *Pinus pinaster*. The species was found parasitizing the first larval instars under the bark of the tree.

Remarks. *Atanycolus denigrator* was already recorded in Italy as parasitoid of *M. galloprovincialis* (Campadelli and Scaramozzino 1994). We additionally studied this reared Italian material in Hungarian Natural History Museum in Budapest (1 female, “Italia, Ravenna, 14.IV.1992, Campadelli”, “Pinus picea”, “ex larva *Monochamus galloprovincialis* Ol., 21.IV.1992”, “*Atanycolus* ♀ *denigrator* L. det. Papp J. 2000”; 1 female, same first label, but “21.IV.1992”, second and third labels are the same ones) and confirmed present determination.

Atanycolus ivanowi (Kokujev 1898)

http://species-id.net/wiki/Atanycolus_ivanowi

Figures 4c, 5b, 6d

Material examined. Portugal: 4 females, “Vale Feitoso II, Maio 2011”, “Col. Entomologica, est. Florestal”; 1 female, same labels, but 12.VI.2011; 1 male, “Vale Feitoso II, Maio 2011”.

Distribution. Palaearctic: Armenia, Austria, Azerbaijan, Croatia, Czechia, Finland, France, Germany, Greece, Hungary, Italy, Japan, Kazakhstan, Russia, Slovakia, Switzerland, Tajikistan, Turkmenistan, Ukraine, Uzbekistan (Yu et al. 2005) and Turkey (Bolu et al. 2009). This species is here recorded for Portugal for the first time.

Hosts. *Anthaxia aurulenta* (Fabricius), *A. deaurata* (Gmelin), *Chrysobothris solieri* (Laporte & Gory), *Ovalisia mirifica* (Mulsant), *Melanophila picta decastigma* (Fabricius); *Sphenoptera tappesi* Marseul (Buprestidae); *Arhopalus syriacus* (Reitter), *Stictoleptura rubra* (Linnaeus), *Monochamus galloprovincialis* (Olivier), *Tetropium fuscum* (Fabricius), *T. gabrieli* Weise (Cerambycidae) (Yu et al. 2005, Bolu et al. 2009).

Biology. *Atanycolus ivanowi* was found to be an ectoparasitoid of first larval stages of *M. galloprovincialis* living under the bark of *P. pinaster*.

Remark. *Monochamus galloprovincialis* was already recorded by Campadelli and Scaramozzino (1994) as a host of *A. ivanowi* in Italy.

Cyanopterus flavator (Fabricius 1793)

http://species-id.net/wiki/Cyanopterus_flavator

Figures 1d, 7b, d, g

Material examined. Portugal: 1 female, “Leiria, 14/6/2011”, “Ensaio Pupas Natural”, “Col. Entomologica, est. Florestal”, “13”; 1 female, same labels, 17.VI.2011, N 12.

"Leiria, Larvas Artificial": 1 female, N 26; 1 female, N 27. "Leiria, Posturas Artificial": 1 female, 31.VIII.2011, N 25; 1 female, N 27. "Leiria, Ensaio, Posturas Artificial": 1 male, 31.VIII.2011, N 21. "Vale Feitoso": 1 female, N 9; 1 female, N 10; 1 male, N 11; 1 female, N 14; 1 female, N 15; 1 female, N 16; 1 female, N 17.

Distribution. Palaearctic: Algeria, Croatia, Cyprus, former Czechoslovakia, Finland, France, Germany, Greece, Hungary, Israel, Italy, Japan, Kazakhstan, Korea, Latvia, Morocco, Netherland, Poland, Romania, Russia, Spain, Switzerland, Syria, Tunisia, Ukraine, United Kingdom, former Yugoslavia (Yu et al. 2005) and Portugal (Naves et al. 2005).

Hosts. *Bostrichus capucinus* (Linnaeus) (Bostrichidae); *Acanthocinus griseus* (Fabricius), *Acanthoderes clavipes* (Schrank), *Hesperophanes pallidus* (Olivier), *Monochamus galloprovincialis* (Olivier), *M. sartor* (Fabricius), *Morimus asper* (Sulzer), *Phymatodes testaceus* (Linnaeus), *Pogonochaerus fasciculatus* (Degeer), *P. hispidus* (Linnaeus), *Rhagium inquisitor* (Linnaeus), *Saperda scalaris* (Linnaeus) (Cerambycidae) (Yu et al. 2005), and *Monochamus rosenmulleri* (Cederhjelm) (Watanabe 1937).

Biology. The biology of this parasitoid is poorly known, but in this study all the specimens emerged from cocoons from the xylemic galleries of *M. galloprovincialis*, which were not completely sealed with frass, as it is normal. Considering the length of the ovipositor of *C. flavator*, it is apparent that only first larval instars of *M. galloprovincialis* (found beneath the bark) are parasitized, which subsequently enter the wood carrying the parasitoid. Only the mandibles of the host larvae were found in galleries with cocoons.

Remark. *Monochamus galloprovincialis* as a host of *C. flavator* was already recorded by Campadelli and Scaramozzino (1994) for Italy and Naves et al. (2005) for Portugal.

Doryctes striatellus (Nees 1834)

http://species-id.net/wiki/Doryctes_striatellus

Figures 1b, 4b, d, 5a

Material examined. Portugal: 1 female, "Leiria, 12/8/11", "Ensaio Pupas Natural", 1 male, same labels, but 9.VIII.2011; 1 male, same label, but 29.VII.2011.

Distribution. Palaearctic: Austria, Belgium, Bulgaria, China, Czechia, Finland, France, Germany, Hungary, Italy, Japan, Lithuania, Poland, Russia, Slovakia, Sweden, Switzerland, Ukraine, United Kingdom (Yu et al. 2005). This species is here recorded for Portugal for the first time.

Hosts. *Ernobius mollis* (Linnaeus), *Dorcatoma dresdensis* Herbst (Anobiidae); *Anthaxia quadripunctata* (Linnaeus), *Phaenops cyanea* (Fabricius), *Ph. guttulata* (Gebler) (Buprestidae); *Acanthocinus aedilis* (Linnaeus), *Agapanthia* sp., *Callidium* sp., *C. violaceum* (Linnaeus), *Clytus* sp., *Exocentrus lusitanus* (Linnaeus), *Mesosa curculionoides* (Linnaeus), *Molorchus minor* (Linnaeus), *Monochamus galloprovincialis* (Olivier), *M. sutor* (Linnaeus), *Phymatodes pusillus* (Fabricius), *Ph. testaceus* (Linnaeus), *Poecilium alni* (Linnaeus), *Pogonocherus* sp., *P. hispidus* (Linnaeus), *Rhagium inquisitor* (Linnaeus), *Semanotus undatus* (Linnaeus), *Stenostola ferrea* (Schrank), *Tetropium castaneum* (Linnaeus), *T. gabrieli* Weise, *T. fuscum* (Fabricius), *T. gracilicorne* Reitter (Cerambycidae); *Pissodes harcyniae* (Herbst), *P. notatus* (Fabricius), *Rhynchaenus fagi* (Linnaeus), *R. pilosus* (Fabricius), *R. quercus* (Linnaeus), *R. testaceus* (Müller), *Magdalis violacea* (Linnaeus), *M. rufa* (Germar), *Tachyerges salicis* (Linnaeus), (Curculionidae); *Hylurgops palliatus* (Gyllenhal), *Ips typographus* (Linnaeus), *I. sexdentatus* (Boerner), *I. subelongatus* Motschulsky, *Pityogenes bidentatus* (Herbst), *Tomicus piniperda* (Linnaeus) (Scolytidae); *Xyphidria prolongata* (Geoffroy) (Xyphidriidae) (Yu et al. 2005).

Remark. This species was already recorded in Italy on the name *D. mutillator* (Thunberg) as parasitoid of *M. galloprovincialis* (Campadelli and Scaramozzino 1994).

Family Ichneumonidae

Xorides depressus (Holmgren 1860)
http://species-id.net/wiki/Xorides_depressus
Figures 1c, 3b, e

Material examined. Portugal: 1 female, "Leiria, Pupas Natural", 19.VII.2011; 1 female, "Leiria, 29/7/11, Ensaio Pupas Natural"; 1 female, N 19.

Distribution. Palaearctic: Austria, former Czechoslovakia, Finland, France, Germany, Hungary, Latvia, Poland, Romania, Russia, Spain, Sweden (Yu et al. 2005). This species is here recorded for Portugal for the first time.

Hosts. *Melanophila cyanea* (Fabricius) (Buprestidae); *Nothorhina punctata* (Fabricius) (Cerambycidae) (Yu et al. 2005). *Monochamus galloprovincialis* (Olivier) is a new host of *X. depressus* from Portugal.

Discussion

Considering the literature data and information presented in this study, the following key identifies the species of parasitoids attacking this pine sawyer in the Palaearctic Region. Only species that are reliably confirmed as parasitoids of *M. galloprovincialis* were considered for the key. Some species of parasitoids were excluded from this list and discussion about this decision is present in the final section. A key to species of Ichneumonidae and Braconidae parasitoids of *Monochamus galloprovincialis* is presented:

1 Second recurrent vein of fore wing present (Figure 1a). In hind wing, second longitudinal cubital vein always present and arising near middle of nervellus. Second and third metasomal tergites movable, not fused (Figures 1c, 2g) (Fam. Ichneumonidae). – Spiracles of first metasomal tergite placed on or before its middle (Figure 2e–f) **2**

– Second recurrent vein of fore wing absent (Figuress 1b, 4e, 5d). In hind wing, second longitudinal cubital vein absent. Second and third metasomal tergites immovable, fused (Figures 1d, 5a–c) (Fam. Braconidae) **4**

2 First metasomal sternite distinctly separated from tergite, this tergite with glymma (Figure 2e), and/or propodeum without transverse basal carina (Figure 2a). Claws of leg in female with teeth or basal lobe (Figure 2c) (Pimplinae) – Second metasomal tergite with pair of oblique furrows running from almost middle of its base to spiracles (Figure 2g). Lower valva of ovipositor apically with lateral lobes covered partly upper valva; dorsal lobe of lower valva with six–seven furrows. Ovipositor sheath 1.2–1.3 times longer than body. Body entirely black (including corner of pronotum); tegula brownish yellow; pterostigma dark; legs red, hind tibia and tarsus brownish red. Body 10.0–22.0 mm .... ***Dolichomitus tuberculatus* (Geoffroy)**

– First metasomal sternite fused with tergite, this tergite without glymma (Figure 2f); propodeum always at least with track of transverse basal carina (Figure 2b). Claws of leg in female simple, without teeth or basal lobe (Figure 2d) (Xoridinae) **3**

3 Hind femur wide, with strong median ventral tooth (Figure 3a). Temple distinctly punctuate (Figure 3d). – Middle tibia posteriorly without deep oblique groove. Second metasomal tergite transverse and finely punctuate. Ovipositor sheath about as long as body. Body blackish; flagellum of antenna rufous; legs mainly reddish, coxae blackish. Body length 5.0–9.0 mm .... ***Odontocolon quercinum* (Thomson)**

– Hind femur narrow, without ventral tooth (Figure 3b). Temple finely obliquely striate (Figure 3e). – First metasomal tergite beyond middle without dorsal longitudinal carinae, about twice longer than wide (Figure 1c). Ovipositor sheath about as long as body. Antennae brownish, without white band; hind leg brownish, but tarsus rufous; first and second metasomal tergites reddish. Body length 6.0–11.0 mm ..... ***Xorides depressus* (Holmgren)**

4 Hypoclypeal depression absent; middle of ventral margin of clypeus situated close to upper level of mandibles (Figure 4a). Brachial cell of fore wing open in distal posterior part, brachial vein absent. Second radiomedial cell of fore wing short (Figure 4e). (Euphorinae). – Second metasomal tergite striate (Figure 3c). Body mainly black. Body length 6.0–8.0 mm .... ***Meteorus corax* Marshall**

– Hypoclypeal depression deep and wide; middle of ventral margin of clypeus situated distinctly above upper level of mandibles (Figure 4b). Brachial cell of fore wing closed by brachial vein in distal posterior part. Second radiomedial cell of fore wing usually long (Figure 1b) **5**

5 Occipital and prepectal carinae present (Figure 4d). First tergite with distinct dorsope and without median area delineated by furrows (Figure 5a). Recurrent vein of hind wing present. Submedial cell of hind wing long. (Doryctinae). Body length 3.0–6.5 mm ..... ***Doryctes striatellus* (Nees) (*D. mutillator* auct.)**

– Occipital and prepectal carinae absent (Figure 4c). First tergite without dorsope and with median area delineated by furrows (Figures 1d, 5b, c, 6c, 7a-c). Recurrent vein of hind wing absent. Submedial cell of hind wing short (Figure 5d). (Braconinae)..**6**

6 Pedicel of antenna almost as long as first flagellar segment. First and second flagellar segments not longer than median segments of flagellum and concave below (Figure 6a). Second metasomal tergite without mediobasal triangle area (Figure 5c). – Ovipositor short, its sheath 1.0–1.3 times as long as metasoma, 0.60–0.65 times as long as fore wing. Second metasomal tergite about as long as third tergite, without or with fine oblique lateral furrows (Figure 5c). Body length 2.5–5.0 mm.................... ***Coeloides sordidator*** **Ratzeburg**

– Pedicel of antenna distinctly shorter than first flagellar segment. First and second flagellar segments longer than median segments of flagellum and not concave below (Figures 6b, 7d). Second metasomal tergite usually with mediobasal triangle area separated by furrows (Figures 5b, 7a, c).....................**7**

7 Second metasomal tergite without mediobasal triangle area separated by furrows (Figure 6c). Upper valva of ovipositor enlarged, distinctly larger than lower valva. Antenna setiform, longer than body. Body crimson-red with black spots. Body length 5.0–12.0 mm.......... ***Iphiaulax impostor*** **(Scopoli)**

– Second metasomal tergite usually (except *Cyanopterus flavator*) with mediobasal triangle area separated by furrows (Figures 5b, 7a, c). Upper valva of ovipositor not enlarged, not larger than lower valva. Antenna more or less filiform, not longer than body. Body never crimson-red, usually black with yellowish brown spots or areas on head and always on most part of metasoma..**8**

8 Scape of antenna with strong basal constriction and with apical collar. Pedicel distinctly projected behind scape (Figures 6d, 7e, f). Furrow between antennal socket and eye present (Figure 7f) **9**

– Scape of antenna without basal constriction and without apical collar. Pedicel weakly projected behind scape (Figures 6b, 7d). Furrow between antennal socket and eye absent (Figure 7g)..**11**

9 Second–fourth metasomal tergites of female coarsely rugose-striate at least medially (Figure 5b). Head often more or less depressed dorso-ventrally (Figure 6d). Body length 5.0–9.0 mm .... ***Atanycolus ivanowi*** **(Kokujev)**

– Second–fourth metasomal tergites of female smooth (except sculptured furrows) (Figure 7a), rarely second tergite partly with rugosity. Head never depressed dorso-ventrally...**10**

10 Head mainly brownish yellow or light reddish brown, only dorsally black and usually in large wedge-shaped black spot. Body length 7.0–10.0 mm .... ***Atanycolus genalis*** **(Thomson) (*****A. initiator*** **auct.)**

– Head mainly black, sometimes paler only near base of mandible, always with reddish stripes along inner side of eye. Body length 5.0–9.0 mm. .... ***Atanycolus denigrator*** **(Linnaeus)**

11 Ventral margin of scape (lateral view) not shorter than dorsal margin (Figure 7d). Second tergite without basomedian area delineated by furrow (Figure 7b). Metasoma brownish yellow, behind first tergite entirely smooth. Wings strongly infuscate. Body length 6.0–10.0 mm... .... ***Cyanopterus* (*Cyanopterus*) *flavator* (Fabricius)**

– Ventral margin of scape (lateral view) shorter than dorsal margin (Figure 6b). Second tergite with distinct basomedian area delineated by sculptured furrow (Figure 7c). Metasoma mainly dark brown or black, behind first tergite sculptured in furrows and suture. Wings faintly infuscate. Body length 4.0–5.0 mm... ***Cyanopterus* (*Ipobracon*) *tricolor* (Ivanov)**

With the exception of *A. ivanowi* collected from Vale Feitoso, all the other species were collected in Marinha Grande. This location is near Portugal´s oldest managed pine forest, in a pine stand with about 1700 square km and which was first planted in the XIII century. This stable and managed environment may have created favorable conditions for the establishment of a diverse entomofauna in the region. In fact, the larger number of parasitoids found in the region, and the low population levels of the vector insect suggest that *M. galloprovincialis* may be locally well controlled by its natural enemies. Further studies in the Leiria pine stand should confirm this hypothesis.

There is no obvious reason for the absence of parasitoids in the other sampled locations, although factors such as the local density of *M. galloprovincialis* (and other insect hosts), and differences in the local edapho-climatic conditions may explain the absence of the natural enemies.

Despite *Atanycolus* genus being the most diverse*, Cyanopterus* is the genus were the most specimens were found. Each parasitoid was reared from one specific location, except *Cyanopterus* specimens which were found in two very distanced sites, which present completely different edapho-climatic conditions.

According to Watanabe (1937), the cocoons of *C. flavator* occurred in the trunk of *Picea jezoensis* Siebold et Zuccarini shut in by a thick corky lid at the end of the tunnel made by the larva of *M. rosenmulleri*, a conclusion which completely supports the suggested hypothesis for the parasitizing activity in Portugal.

Worldwide and including this study, there is now a total of 14 species of parasitoids associated with *M. galloprovincialis*, being six Ichneumonidae and eight Braconidae. Previous reliable records (confirmed rearing from the larvae of *M. galloprovincialis*) in the literature include references from Portugal (Naves et al. 2005), Italy (Campadelli and Scaramozzino 1994), and Siberia (Tobias and Belokobylskij 2000), among other locations (Kenis and Hilszczanski 2004, Tobias et al. 1986; Yu et al. 2005). Although not detected in this study, other groups, such as the braconids of the subfamily Helconinae (namely species of *Helcon* Nees 1812 and *Helconidea* Viereck 1914), will also likely parasitized larvae of *M. galloprovincialis* as they have been found to develop in larvae of other *Monochamus* species (Tobias et al. 1986, Yu et al. 2005).

Other records are more dubious and need further confirmation. Among these, three records of Ichneumonidae are possibly erroneous, namely *Rhyssa persuasoria* (Linnaeus) (Pimplinae)*, Perithous divinator* (Rossi) (Pimplinae) and *Stenarella domator* (Poda) (Cryptinae). The first species is a specialized parasitoid of Siricidae larvae (Yu et al. 2005), and its rearing from Cerambycidae is probably inaccurate. Likewise, the other two species are specialized parasitoids of vespoid and sphecoid wasps (Kasparyan 2010), and their associations with Cerambycidae is quite doubtful. Therefore, in the identification key only three ichneumonids were included, namely *Odontocolon quercinum* (Thomson), *Xorides depressus* (Holmgren) and *Dolichomitus tuberculatus* (Geoffroy). On the other hand, as all species of Braconidae were directly reared from *M. galloprovincialis*, they were included in our key.

Despite the relatively high diversity of parasitoids associated with *M. galloprovincialis* worldwide, all species are mainly idiobiont ectoparasitoids (except *M. corax*) and seem to be generalists attacking a vast array of other insects living in dead and dying trees. *Cyanopterus flavator*, which had already been found parasitizing young larval stages (Naves et al. 2005), appears to be the most frequent and promising candidate for studies aiming the biological control of the pine sawyer, despite its generalist habits. As mentioned, the disperse distribution of *Cyanopterus* can be considered as a major adaptation two the diverse edapho-climatic conditions characteristic for Portugal. Other options, such as the introduction of exotic natural enemies would create new parasite-host interactions, which usually offer greater changes of success for biological control than the promotion of already established associations (Hokkanen and Pimentel 1984). Nevertheless, such measures require rigorous pre-release risk assessment of the economic and environmental costs and benefits of the introduction, to evaluate its potential effectiveness, host specificity, acclimatisation and viability for mass-production (van Lenteren et al. 2006).

Detailed studies on the effect of the parasitoid guild found in Portugal on the pine sawyer´s population and the suitability of the species for biological control are being planned, with the final objective of eventually establishing an integrated bio-control program against the vector of the pine wilt disease in Europe.

Acknowledgement

We are grateful to Mr. Adérito Matos for his assistance both in the field surveys and laboratory handling, to Drª Rita Matos Gomes and to the team from the AFN Marinha Grande for all the conditions and support provided during this study, to Eng. Teixeira and his team from Alcobaça, to Mr. Alfredo and his colleagues from Monção and to Engºs António Moreira and Pedro Serrasqueiro of Herdade da Comporta for allowing the trails to take place in the private estate. We are also very thankful to Dr Dmitri R. Kasparyan (St. Petersburg, Russia) for determination of the ichneumonid parasitoid and valuable consultation.

Figure plates

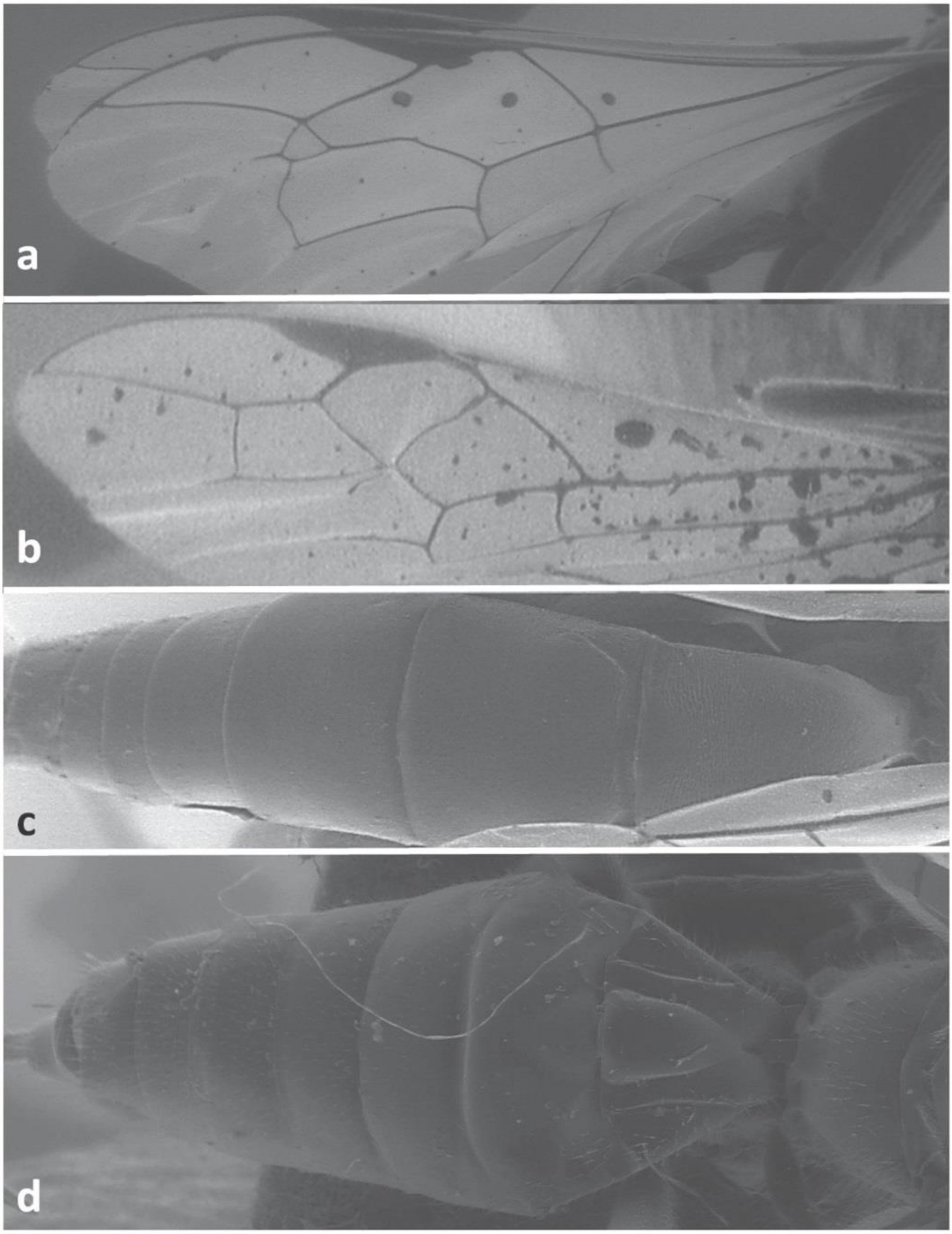

Figure 1. Forewing of *Dolichomitus tuberculatus* (**a**) and *Doryctes striatellus* (**b**); metasoma in dorsal view of *Xorides depressus* (**c**) and *Cyanopterus flavator* (**d**).

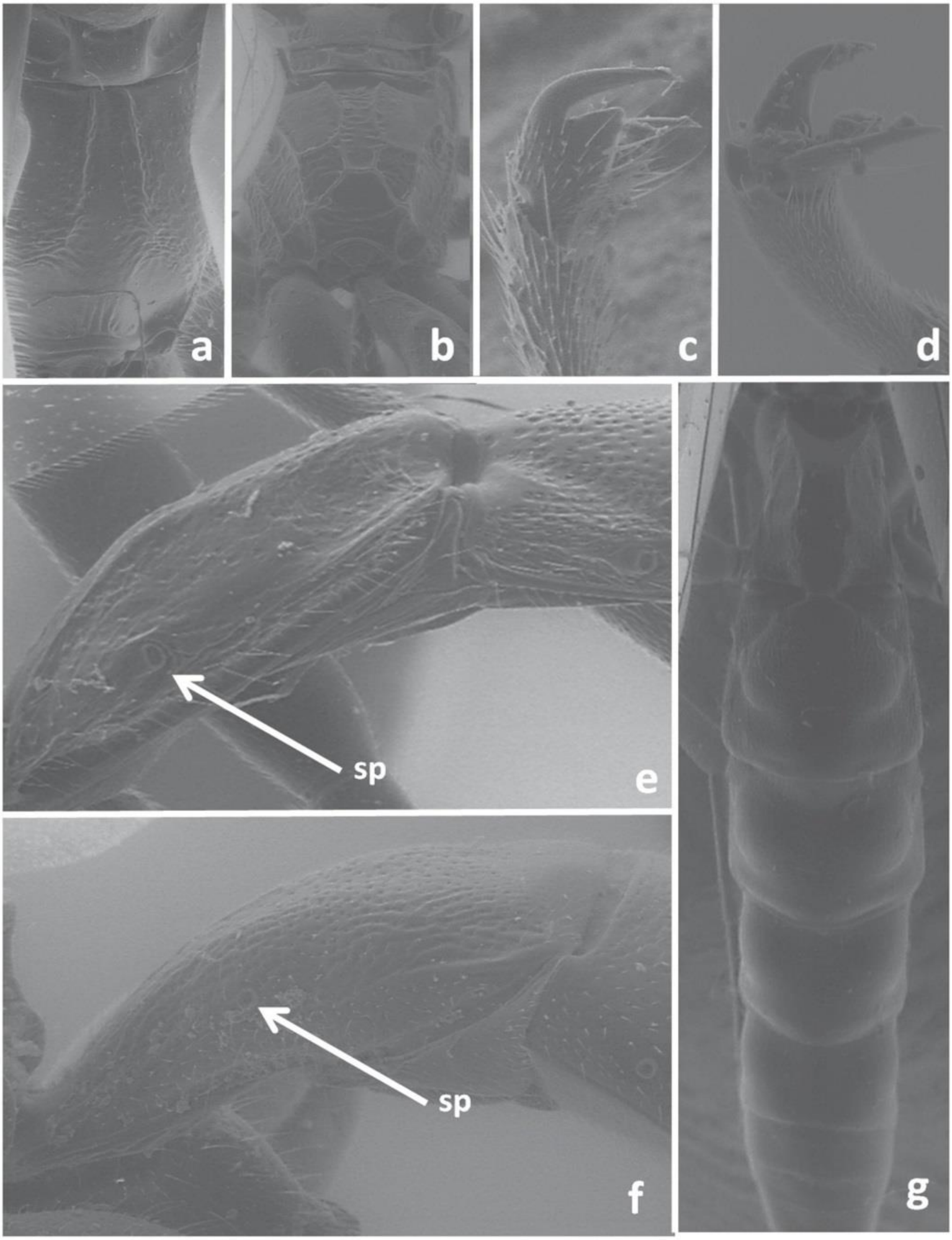

Figure 2. Propodeum in dorsal view of *Dolichomitus tuberculatus* (**a**) and *Odontocolon quercinum* (**b**); tarsal claws of *D. tuberculatus* (**c**) and *O. quercinum* (**d**); first metasomal tergite in lateral view of *D. tuberculatus* (**e**) and *O. quercinum* (f); dorsal view of metasoma of *D. tuberculatus* (**e**); sp – spiracle.

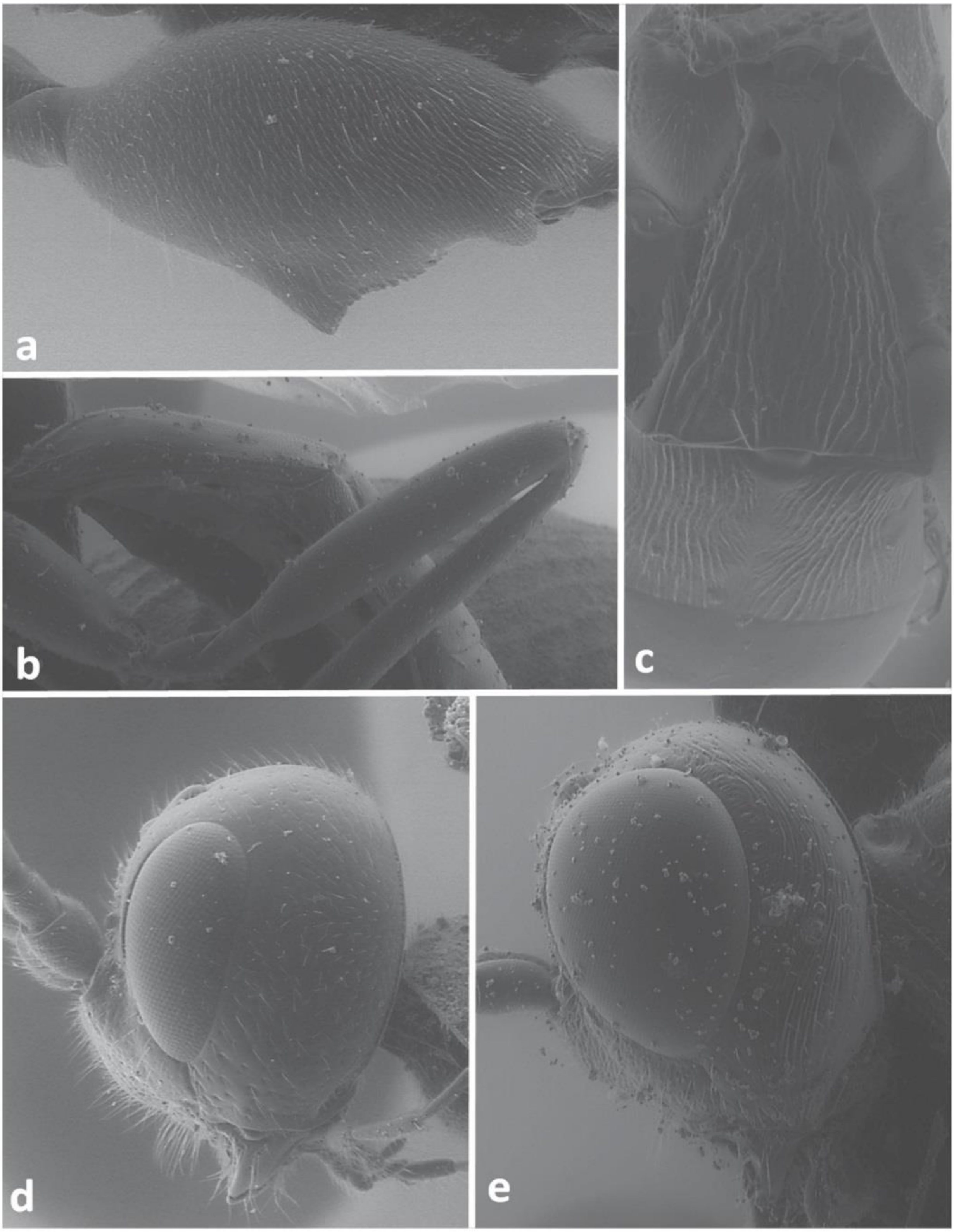

Figure 3. Lateral view of hind femur of *Odontocolon quercinum* (**a**) and *Xorides depressus* (**b**); first and second metasomal tergites in dorsal view of *Meteorus corax* (**c**); head in lateral view of *O. quercinum* (**d**) and *X. depressus* (**e**).

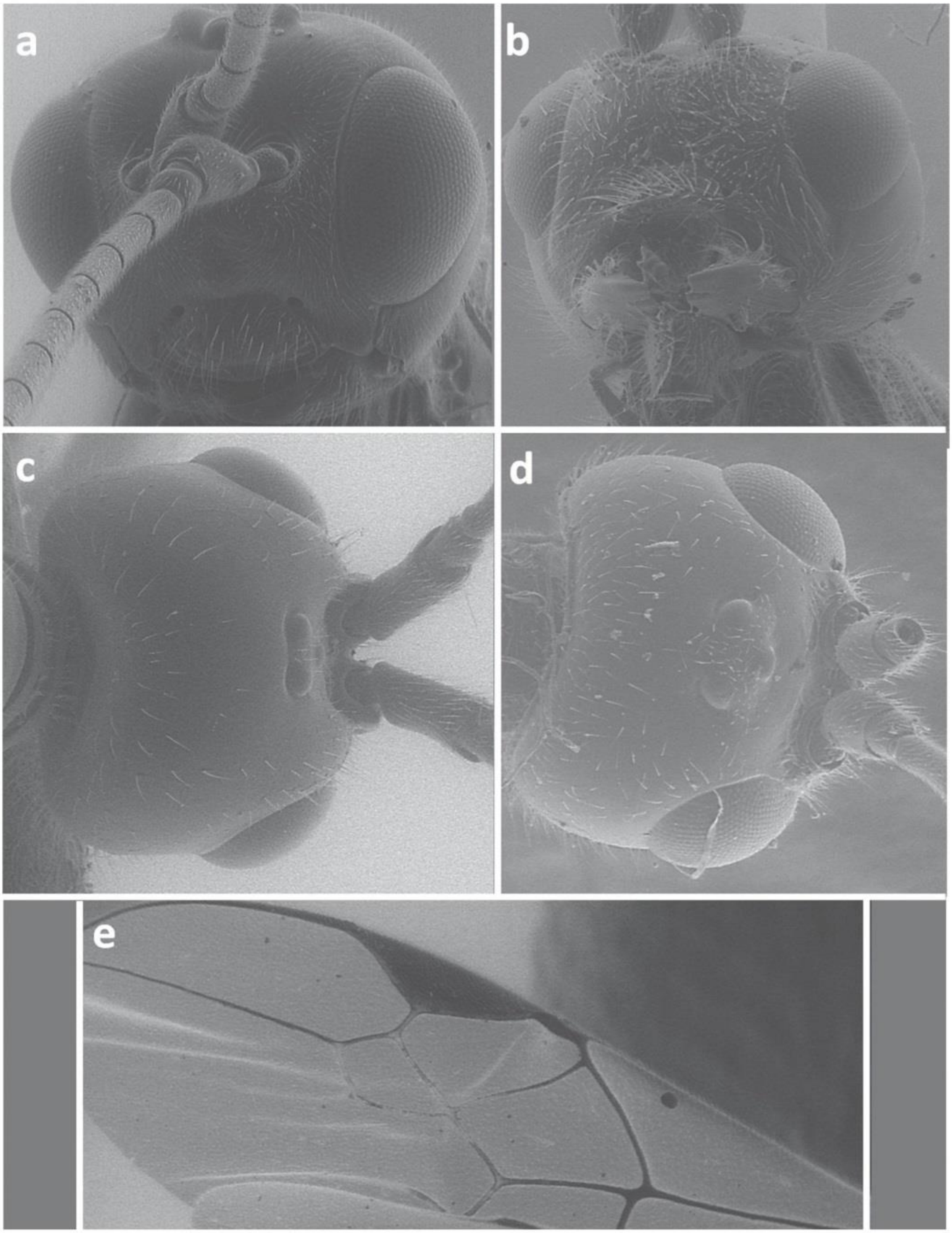

Figure 4. Face in frontal view of *Meteorus corax* (**a**) and *Doryctes striatellus* (**b**); head in dorsal view of *Atanycolus ivanowi* (**c**) and *D. striatellus* (**d**); detail of forewing of *M. corax* (**e**).

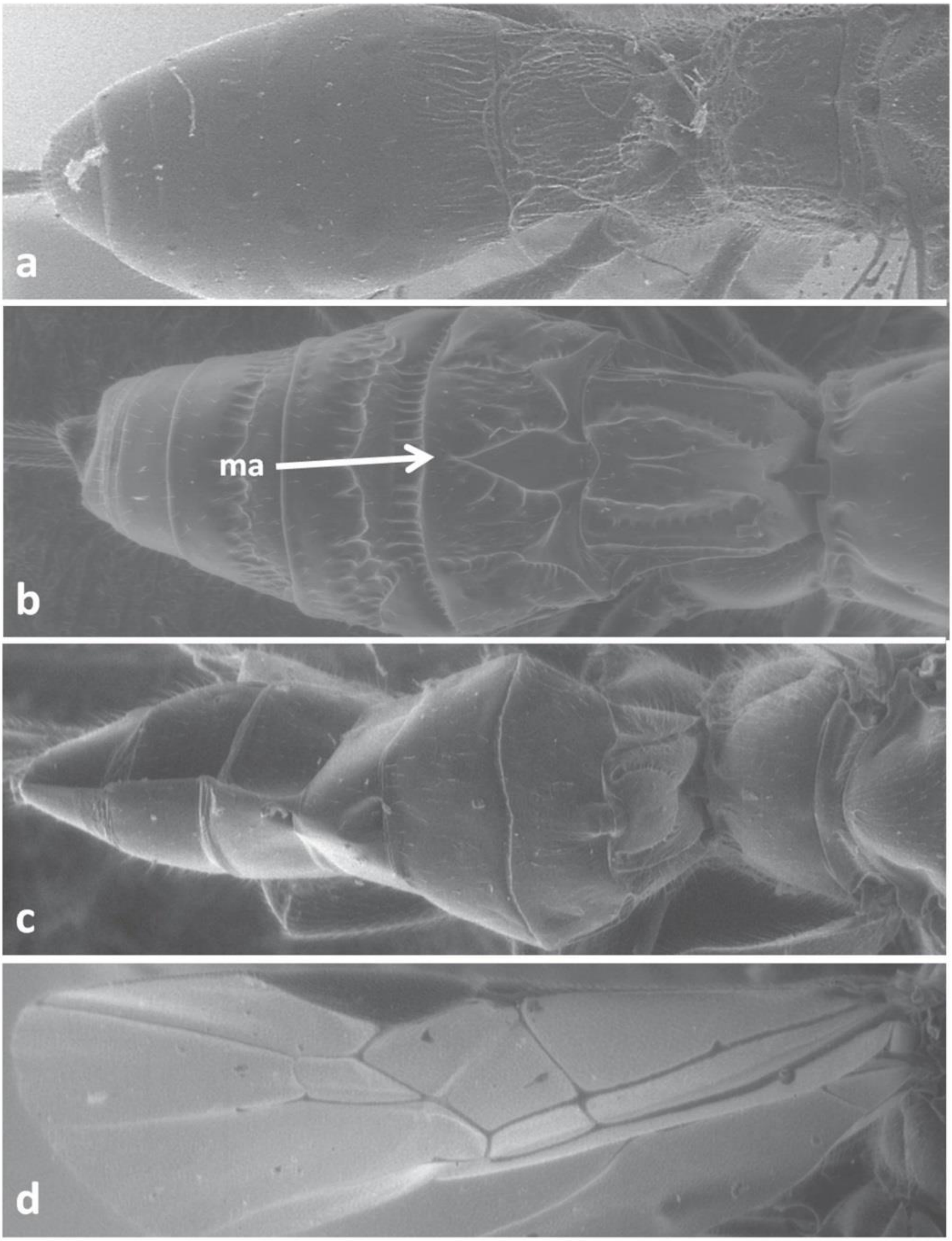

Figure 5. Metasoma in dorsal view of *Doryctes striatellus* (**a**), *Atanycolus ivanowi* (**b**) and *Coeloides sordidator* (**c**); forewing of *C. sordidator* (**d**); ma – mediobasal area.

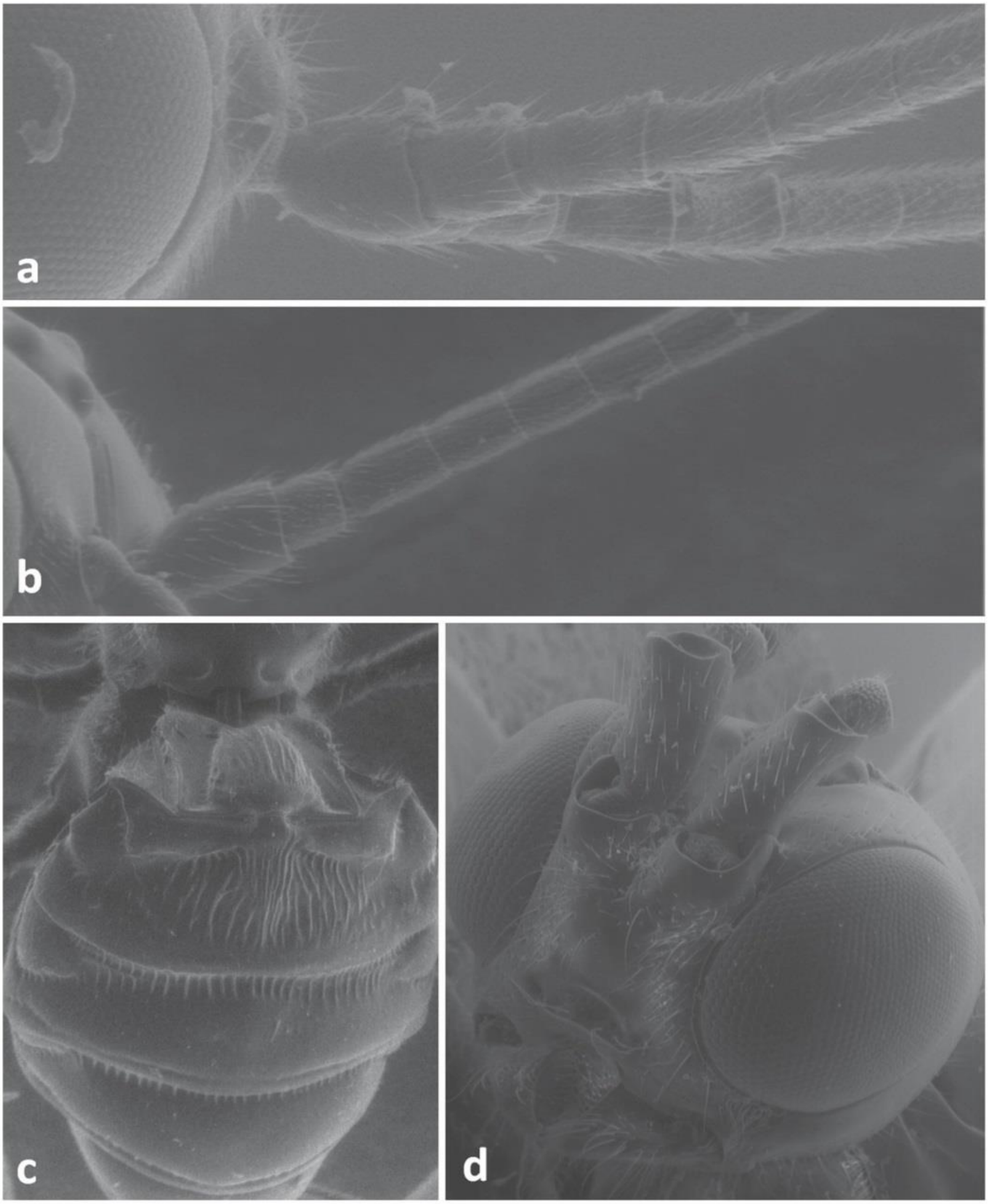

Figure 6. Basal segments of antenna in lateral view of *Coeloides sordidator* (**a**) and *Cyanopterus tricolor* (**b**); metasoma in dorsal view of *Iphiaulax impostor* (**c**); face of *Atanycolus ivanowi* (**d**).

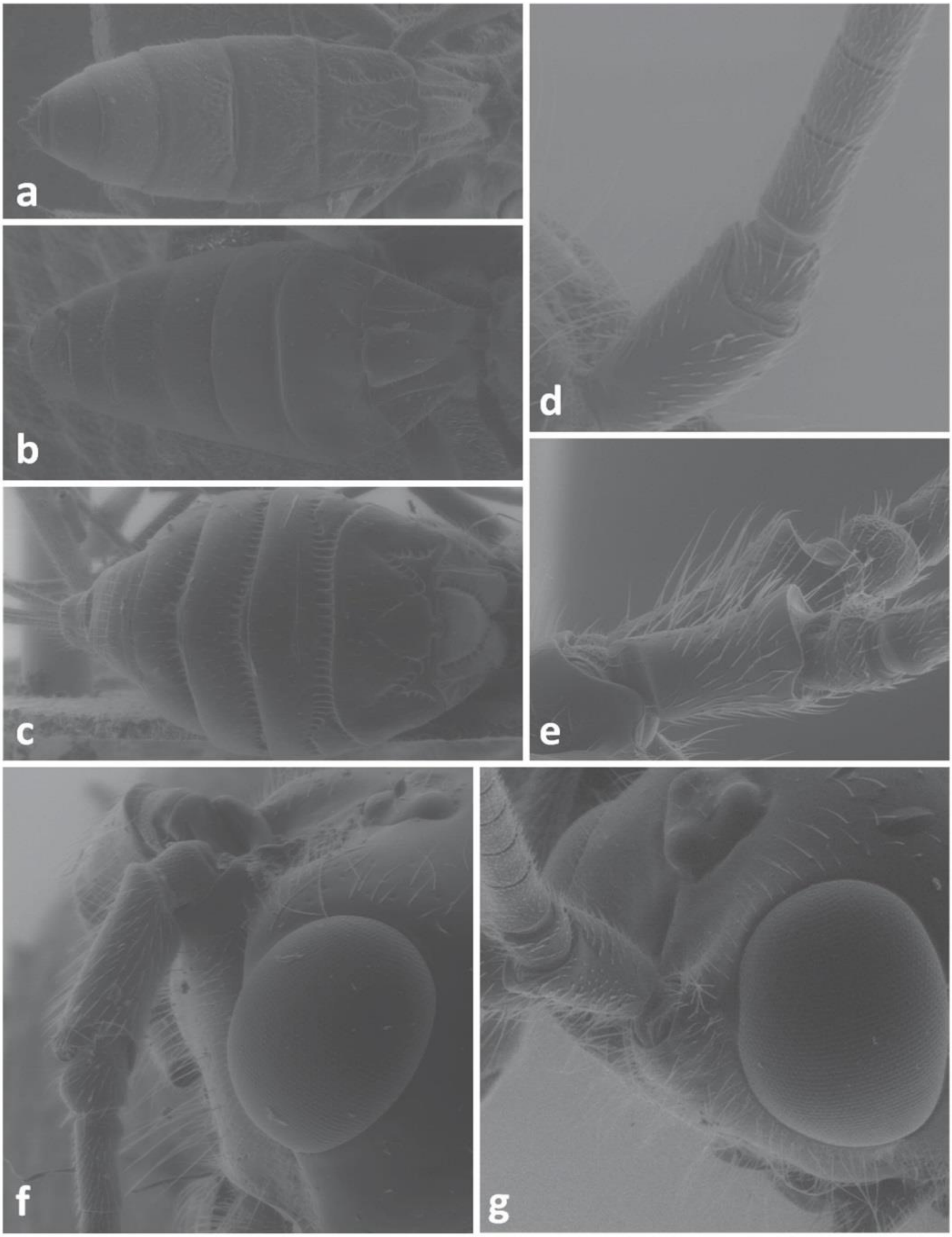

Figure 7. Metasoma in dorsal view of *Atanycolus denigrator* (**a**), *Cyanopterus flavator* (**b**) and *Cyanopterus tricolor* (**c**); scape and pedicel in lateral view of C. *flavator* (**d**) and *A. denigrator* (**e**); space of head between antennal socket and eye in laterofrontal view of *Atanycolus genalis* (**f**) and *C. flavator* (**g**).

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Susceptibility of larvae and adults of *Monochamus galloprovincialis* to entomopathogenic fungi under controlled conditions

International Journal of Pest Management –Doi: 10.1080/09670874.2015.1016472

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Resumen

Desde que fue detectado por la primera vez en Portugal el nematodo del pino *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle, y se asoció el insecto *Monochamus galloprovincialis* (Olivier) como su vector, se intenta desarrollar medidas de combate a la plaga. Sin embargo el aumento del área afectada en los últimos años indica que las mediadas existentes pueden no ser suficientes. Con este intuito se intenta desarrollar un conjunto de medidas de control biológico para el combate a la plaga. En este trabajo se demuestra cual la susceptibilidad de los insectos de la especie *M. galloprovincialis* a tres hongos entomopatógenos. Se testó tanto las larvas del insecto como adultos a diferentes aislados de las especies *Beauveria bassiana* (Bals.-Criv.) Vuill, *Metarhizium anisopliae* (Metchnikoff) Sorokin, *Metarhizium* sp., y *Fusarium* sp., obtenidos desde formulaciones comerciales o colectados desde insectos muertos y contaminados. Juntamente se comparó la eficacia de cinco productos con propiedades antimicóticas para la desinfección de las larvas del cerambícido en laboratorio.

Tanto los aislados de *Fusarium* sp. como de *Metarhizium* sp. demostraron una eficacia superior que 40% contra las larvas del insecto, mientras *Beauveria bassiana* y *M. anisopliae* mataron cerca de 50% de los adultos en menos de 5 días. Considerando que también se testó la eficacia de dos diferentes métodos de exposición al hongo, se ha concluido que el contacto directo con una suspensión de esporos puede ser más eficaz que la aplicación de espray sobre la superficie de los especímenes. Con los ensayos de desinfección de larvas se ha obtenido un resultado de 100% de sobrevivencia y desinfección con la aplicación de azoxistrobina, permitiendo así su futura utilización en ensayos en laboratorio. Se discute la utilización de hongos entomopatógenos contra la especie vector del nematodo del pino en Portugal y sus futuras utilizaciones.

Susceptibility of larvae and adults of *Monochamus galloprovincialis* to entomopathogenic fungi under controlled conditions

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This work was partially funded by the project REPHRAME Development of improved methods for detection, control and eradication of pine wood nematode in support of European Union Plant Health policy.

Abstract

Six entomopathogenic fungal isolates were tested under controlled conditions, as biological control agents against *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) (Olivier), the vector of the pine wood nematode *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae) (Steiner and Buhrer). *Beauveria bassiana* (Bals.-Criv.) Vuill, *Metarhizium anisopliae* (Metchnikoff) Sorokin, *Metarhizium* sp., and *Fusarium* sp. were tested towards larvae and adults of the insect. The fungi *Fusarium* sp. and *Metarhizium* sp. killed more than 40% of *M.*

galloprovincialis larvae, while *Beauveria bassiana* and *M. anisopliae* were found to kill 50% of the adults (Lt50) five days after direct contact with the conidia. *Beauveria bassiana* reduced the adult longevity by 19 days when compared with untreated beetles, and had a direct efficacy of 46%. Continuous contact with the fungi proved to be more effective than spraying adults. To assure that test individuals are not killed by other fungal isolates already present in the individuals, a larval disinfection protocol was developed. The implications of these results and the possible application of selected strains as bio-control agents against *M. galloprovincialis* are discussed.

Keywords

Biological control; Cerambycidae; *Beauveria bassiana*; *Metarhizium*; *Fusarium*; Application methods.

1. Introduction

Pine wilt disease is the result of a complex interaction between three distinct organisms: a nematode, a host tree and an insect vector. The pine wood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle is the causal agent, and pines (*Pinus* spp.) are the most important hosts worldwide (Linit 1988; Kishi 1995; Dwinell 1997). To be dispersed from one host to another the nematode requires a vector insect, being the most effective the cerambycid beetles of the genus *Monochamus* (Coleoptera; Cerambycidae) (Kobayashi et al. 1984; Linit 1988; Kishi 1995; Naves et al. 2007). The PWN was first detected in Portugal in 1999 (Mota el al. 1999) and in 2001 was associated with its local vector; the pine sawyer *Monochamus galloprovincialis* (Olivier) (Sousa et al. 2001).

Over the years, the PWN has been causing significant mortality in native maritime pine (*Pinus pinaster* Aiton) forests in Portugal, and between 2000 and 2007 the number of felled pine trees with wilting symptoms (although not necessarily with the PWN) increased from 53.487 to over 200.000, causing significant economic and environmental impacts (Sousa et al. 2011).

Management and control strategies against pine wilt disease focus in preventing the spread of the nematode by the dispersal of infected insects. This is done with the implementation of strict sanitary measures in affected forests, by locating, felling and removing/destroying symptomatic trees (which contain insect larvae and nematodes in the wood) during the winter months (Sousa et al. 2011). Additional strategies have recently been developed, such as trunk injection of emamectin benzoate in healthy pine trees to prevent wilt disease and bark beetle attacks (Sousa et al. 2013), although the recent spread of wilt disease to neighbouring Spain (EPPO 2009; Abelleira et al. 2011) underlines the necessity to develop and promote innovative control strategies against this sanitary problem.

The promotion and use of natural enemies would be an interesting option, and it has been employed in Japan and China to control the local *Monochamus alternatus* Hope vector (Shimazu et al. 1999; Miura et al. 2003; Urano 2004), although generally with limited success. In Portugal, surveys for parasitoids associated with *M. galloprovincialis* found a diverse number of available species, although none was suitable for biological control programs (Naves et al. 2005; Petersen-Silva et al. 2012). Nevertheless, the fungus *Beauveria bassiana* (Bals.-Criv.) Vuill was found to be an important mortality agent, being responsible for one quarter of larval mortality in heartwood galleries (Naves et al. 2008). Entomopathogenic fungi are well known as biological control agents of diverse insect pests, and have been tested with success against cerambycidae with ecology and life cycles similar to *M. galloprovincialis* (Ambethgar and Mahalingam 2002; Ludwig and Oetting 2002; Dubois et al. 2008; Marannino et al. 2010; Pramono et al. 2001; Francardi et al. 2012; Meyers et al. 2013), and therefore may constitute an interesting option to control the vector insect.

In this study we tested the effectiveness of three entomopathogenic fungal species against the larvae and adults of *M. galloprovincialis*, comparing fungi collected in Portugal from dead larvae over the years with commercially-available products, and while comparing two application methods. These two different application methods were planned to compare the effectiveness between spraying pulverizations and direct contact between the insect the conidia solution. Obtaining this data would allow the development of a specific method for field applications, based on aerial pulverizations or fibre bands impregnations.

All the tested fungi species are commonly found on *M. galloprovincialis* habitat; therefore, to assure that no other isolates from the same species may affect insect mortality, a disinfection protocol was developed. Five antimycotic products were tested for prior disinfection of *Monochamus* larvae.

2. Materials and Methods

2.1. **Collection of *M. galloprovincialis* larvae**

Last instar (L4) *M. galloprovincialis* larvae were collected from dead maritime pine trees, *P. pinaster*, from Comporta, Portugal. A total of 540 larvae were collected from the wood in January 2013 and individually placed in a Petri dish with filter paper. According to Naves and Sousa (2009), and to induce dormancy larvae were maintained at 8ºC, 65%RH and 0:24 LD until needed for the assay.

2.2. **Larvae disinfection**

To obtain aseptic larvae we tested methylparaben, commonly used as disinfectant for plants and artificial diets (Dubois et al. 2002, Petersen-Silva et al. 2014), azoxystrobin, tebuconazole, econazole nitrate and ciclopirox olamine, which are standard human pharmaceutical fungicides.

Each product was diluted to 1% to obtain 200ml, placed in autoclaved containers and stirred for five minutes. Randomly selected *M. galloprovincialis* larvae (20 individuals for each chemical) were placed inside the containers for 60 minutes, with continuous stirring, after which they were kept for 10 minutes in sterile filter paper to absorb the remaining liquid. The same procedure was repeated with 200 ml of purified water, as control.

Each larva was subsequently plated for 10 minutes in a Petri dish with 15 ml of Potato Dextrose Agar (PDA Difco®) supplemented with streptomycin ($500 mgL^{-1}$) (PDA+E). Petri dishes were kept at 25ºC 65%RH and observed daily for two weeks to detect the presence of fungi, bacteria or other contaminants.

After being removed from the culture medium, the larva were individually placed in Petri dishes with autoclaved filter paper for 96 hours and sealed with parafilm Bemis®. After 96 hours, the larvae were again plated on the PDA+E culture medium, as previously described. Larvae were kept at 25ºC, 65%RH and 0:24 LD, registering survival and adult emergence for 90 days.

2.3. **Fungi culture and conidia isolation**

Six isolates belonging to three genera of entomopathogenic fungi were tested against the larvae and adults of *M. galloprovincialis*: *Beauveria bassiana* (three isolates), *Metarhizium* sp. (one isolate), *Metarhizium anisopliae* (one isolate) and *Fusarium* sp. (one isolate). (Table I)

Table I: Details concerning fungal isolates used, their tree host, insect host, locality of collection, date and local of provenience.

Fungi Genus / species	**Isolate reference**	**Tree Host**	**Insect host**	**Locality**	**Date**	**Provenience**
Beauveria bassiana	BsI1	*Pinus pinaster*	*Monochamus galloprovincialis*	Comporta, Portugal	July 2011	INIAV[1] collection
Beauveria bassiana	BsI2	*Pinus pinaster*	*Monochamus galloprovincialis*	Comporta, Portugal	July 2011	INIAV[1] collection
Beauveria bassiana	BsI3	*Pinus pinaster*	*Monochamus galloprovincialis*	Comporta, Portugal	July 2012	INIAV[1] collection
Metarhizium sp.	MsI1	*Phoenix canariensis* Chabaud	*Rhynchophorus ferrgineus* Oliv.	Cascais, Portugal	August 2011	ISA-LPVVA[2] collection
Metarhizium anisopliae.	MsI2	NA	NA	NA	NA	®*BioMagic Comercial company*
Fusarium sp.	FsI1	*Phoenix canariensis* Chabaud	*Rhynchophorus ferrgineus* Oliv.	Funchal, Portugal	April 2011	ISA-LPVVA[2] collection

([1]INIAV: Instituto Nacional de Investigação Agrária e Veterinária; [2]ISA–LPVVA: Instituto Superior de Agronomia Laboratório de Patologia Vegetal Veríssimo de Almeida; NA- Not applicable)

Pure cultures of different isolates were obtained and kept in dark chamber at 25ºC, in PDA culture medium with half concentration of nutritive components (therefore named PDA/2) to induce faster sporulation (Li and Holdom 1995). The method used for the development of each fungus under laboratory conditions was based on Shimazu (2004a).

To separate the spores from the mycelium, 10ml of an aqueous solution of Tween 20 at 0.4% was applied on the fungus surface when spores became evident. The spores obtained were quantified by direct counting using a hemocytometer. The suspensions obtained from each isolate were used according to the highest available conidia per millilitre and are presented in table II. These concentrations are based on spore availability and previous references (Shimazu et al. 2002, Oliveira et al. 2004, Dubois et al. 2008, Marannino et al. 2010). The suspensions were then stored until use, for less than a week at approximately 5ºC.

Table 2: Conidia concentrations (conidia per ml) used in the larval and adults assays

	M. galloprovincialis life stage	
Fungal Isolate	**Larvae**	**Adult**
BsI1	$1x10^7$	$1x10^7$
BsI2	$1x10^7$	$1x10^7$
BsI3	$1x10^7$	$1x10^7$
FsI1	$1.5x10^7$	$1.5x10^7$
MsI1	$2x10^7$	$5.6x10^6$
MsI2	$2x10^7$	$2x10^6$

2.4. **Application of insect larvae and adults**

To access conidia viability, the technique suggested by Goettel and Inglis (1997) was applied. Dilutions of 1/10 ml were performed for each isolate solution, followed by 0.125 ml pulverizations into three PDA Petri-dishes (90cm diameter), which were kept at 25ºC, 65%RH and 0:24 LD for 1 week. To obtain the percentage of conidia viability, the number of developed conidia were then counted with a stereo magnifier and compared to the density of conidia obtained from the hemocytometer counting.

The larvae were exposed to the conidial suspension by submersion, following the methodology of Shimazu et al. (2002). A total of 20 larvae per isolate were submerged for 60 seconds in the conidia suspension, dried on sterilized filter paper and stored in Petri-dishes at 25ºC until adult emergence or death.

As for the exposure of adult beetles, insects were kept individually in closed containers with *P. pinaster* twigs for feeding, previously autoclaved twice, and changed every two weeks or if showing signs of degradation. Twigs were autoclaved twice to prevent the development of fungi isolates already present in the wood, which may affect insect longevity. Half of the adults were sprayed with 0.125ml pulverizations directly on each individual, with the remaining placed in recipients containing 5 ml of the conidia suspension at the bottom, with 20 individuals per method/isolate.

Dead insects showing visible fungal growth were plated for some seconds in PDA Petri-dishes, which were kept in a climatic chamber at 25ºC. Fungi were isolated and the axenic cultures used to identify the species.

For both life stages, 20 individuals were treated with sterilized distilled water, as control.

2.5. **Statistical analysis**

Analysis of variance (ANOVA) was used to compare the number of days to obtain adults, longevity and interactions between the fungal isolates and the inoculation method. The Fisher least significant difference (LSD) test was used to compare means within each significant factor in the ANOVA. A Kruskal–Wallis nonparametric analysis of variance test using $p \leq 0.05$ was used to compare larval survival between fungal isolates. Values in charts and tables are presented as means ± standard deviation. Lethal time (LT_{50}) represents the number of days required to kill 50% of the individuals. Isolate effectiveness against the adults was calculated using Abbott formula (Abbott 1925) modified. This formula was modified to incorporate longevity as days instead of the number of individuals remaining after the treatment, as in this case it was always constant. Statistical procedures were performed using STATISTICA 6 software (StatSoft Inc. 2003)

3. Results

3.1. **Larvae disinfection**

Econazole nitrate caused 100% larval mortality so it was excluded from further trials. Methylparaben caused low mortality, although it did not eliminate the fungi. Both azoxystrobin and ciclopirox olamine showed to be effective against the fungi of the larvae, being azoxystrobin chosen due to its lower mortality (0%) when compared to the other product (10%), (Fig. 1 and Table 3).

Table 3: Effectiveness of antimycotic products for disinfection of *Monochamus galloprovincialis* larvae

Antimycotic	**N**	**Observation (hours)**	**Fungi presence** (%)	**Other contaminants** (%)
Azoxytrobin	20	0	0	70
		96	0	90
Ciclopirox Olamine	20	0	0	10
		96	0	0
Econazole Nitrate	20	0	0	0
		96	NA	NA
Methylparaben	20	0	100	100
		96	100	100
Tebuconazole	20	0	60	10
		96	0	0
Control	20	0	100	100
		96	100	100

(N- nb of individuals used, NA – Not Applicable)

3.2. **Exposure Bioassays**

Petri-dishes sprayed with the fungal isolated and observed under the stereo magnifier, exhibited conidia development without contaminations. The number of available conidia when compared to the conidia density found with the hemocytometer reached 100% for all the tested isolates, confirming the inoculums viability.

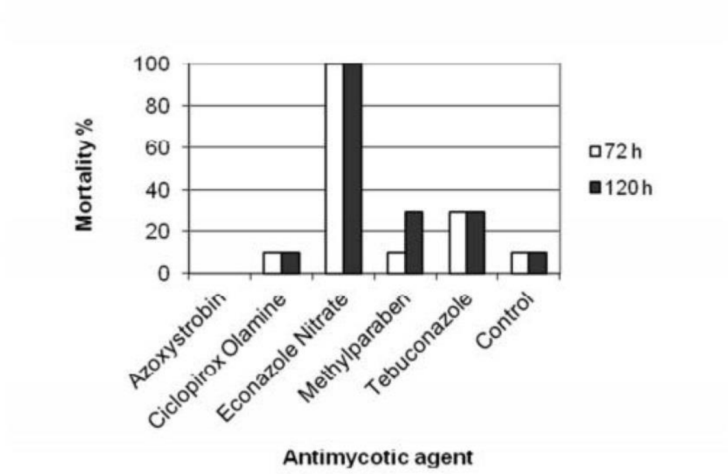

Fig. 1 Mortality (%) of *Monochamus galloprovincialis* larvae after disinfection with different antimycotic agents

3.3. **Larvae**

The fungal isolates tested caused different mortality to the larvae, with MsI1 and FsI1 being the most effective (Kruskal-Wallis test: χ^2=20.42437, d.f.=6, P=0.0023). Inversely, BsI3, BsI1, BsI2 and MsI2 showed to be inefficient (Table 4).

Comparing the time needed to obtain adults after the inoculations, no differences were observed (F=1.171 df=6, P=0.328), with a mean number of 57.6 ± 6.9 days for all treatments combined.

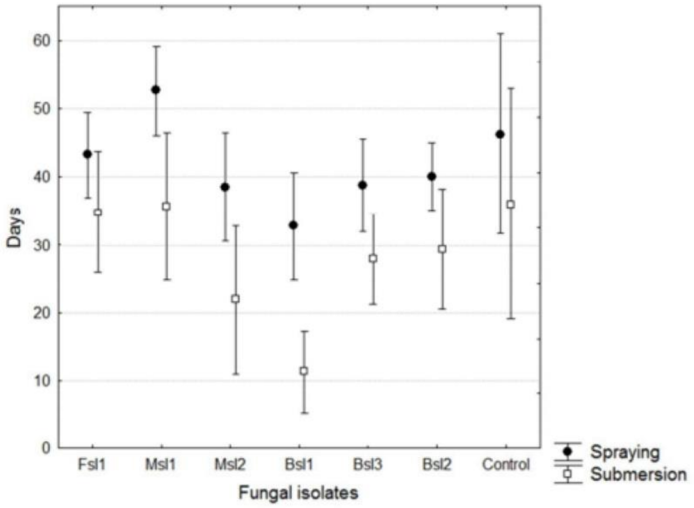

Fig. 2 Longevity (mean ± SD) of *Monochamus galloprovincialis* adults exposed to fungal isolates by spraying and direct contact

Table 4: Mortality (%) and days required to obtain adults of *Monochamus galloprovincialis* larvae exposed to fungal isolates.

Fungal isolate	**N**	**Mortality** (%)	**Days to adult** (Mean ± SD; Range)
BsI1 [1]	20	6a	60 ± 6.1 (47–74)
BsI2[1]	20	22ab	56 ± 8.3 (41–74)
BsI3 [1]	20	5a	57 ± 6.4 (39–67)
FsI1[1]	20	50c	60 ± 4.0 (53–67)
MsI1[1]	20	40bc	57 ± 6.3 (42–62)
MsI2[1]	20	15a	55 ± 4.1 (48–62)
Control[1]	20	10a	56 ± 9.6 (42-77)

([1]Means within each line followed by the same letter do not differ, P≤0.05. N- nb of individuals)

3.4. **Adults**

The longevity of adults treated with the different isolates significantly differed (F=5.9865 df=6, P<0.001), being *Beauveria* BsI1 the most effective, while, inversely, MsI1 and FsI1 showed the lowest effectiveness (Table 5).

Table 5: Longevity (mean ± SD; range), lethal time (LT50, days), corrected effectiveness (E, %) and fungi re-isolations (%) of *Monochamus galloprovincialis* adults exposed to fungal isolates.

Fungal isolate	**N**	**Longevity** (Mean ± SD; Range)	**Lethal time (LT_{50})** (days)		**E** (%)	**Fungi re-isolations** (%)
			Spraying	Direct contact		
BsI1 [1]	40	22 ± 18.3a (1-60)	21	5	46.3	63.3
BsI2[1]	40	35 ± 16.0bc (1-60)	40	40	14.6	36.3
BsI3 [1]	40	33 ± 15.2bc (1-60)	40	30	19.5	34.7
FsI1[1]	40	39 ± 16.7cd (2-60)	44	44	4.9	90.4
MsI1[1]	40	44 ± 20.7d (7-60)	60	44	0	61.9
MsI2[1]	40	30 ± 21.9ab (1-60)	37	5	26.8	90.9
Control[1]	40	41 ± 22.4cd (2-60)	52	42	NA	10.0

([1]Mean within each line followed by the same letter do not differ, $P \leq 0.05$. N- nb of individuals used. E – Corrected effectiveness (percentage) according to the modified Abbott formula. NA- Not applicable)

Fungi were differentially re-isolated from dead adult insects, with over 90% in FsI1 and MsI2, contrasting with only 35% from BsI3. In the control individuals, none of the tested fungi were found (Table 5).

Concerning the two inoculation methods tested, the submersion proved to be significantly faster in killing the insects (27.6 ± 20.8 days) when compared to the spraying (41.4 ± 15.8) ($F=36.6864$ $df=1$, $P<0.001$). Nevertheless, when analysing the LT50 (number of days required for half the population to die), relevant differences between the two methods were noticeable only for some fungi, namely BsI1 and both *Metarhizium* isolates (Table 5). When analysing the interaction of the isolate with the inoculation method, significant differences can be found ($F=0.7295$ $df=6$, $P=0.6262$), which can be visualized in Figure 1. Overall, the combination of the BsI1 and MsI2 fungal isolates applied by submersion were the fastest and most efficient in killing the adult beetles (Table 4, 5 and Fig. 2).

4. Discussion

Effective larval disinfection was achieved with the application of azoxystrobin, which had no effect on larvae survival. This antimycotic is commonly used in crops destined to the human and animal alimentary industry (Clough et al. 1996, Heaney 2000, Toffolatti et al. 2007), and our results suggest that it is a valid option for decontaminating insects for laboratory studies or for mass rearing protocols requiring sterile larvae. Despite the successful development of this protocol, we were aware that using such procedure strongly reduces the amount of fungi present on the larvae surface. As the main objective of this study was to access the susceptibility of *M. galloprovincialis* larvae to the tested isolates, reducing the interactions between the native fungi and the tested isolates was understood as an advantage to the assay. Further studies on these interactions should be considered.

The entomopathogenic fungi tested affected differently the survival of *M. galloprovincialis* larvae and adults. Only the isolates FsI1 and MsI1 had some effectiveness against the larvae, reducing the populations by 40-50%. Inversely, the same two fungal isolates showed low effectiveness against the adults, being BsI1 the most effective. The reasons why the two life stages have differential sensitivity to the fungi are unclear, however, similar results have been found for *M. alternatus* in China (Xue-You et al. 2005), where larvae were more affected by *Metarhizium* and adults by *Beauveria*. Inversely, other authors found *B. bassiana* to be more effective against *Monochamus* larvae when compared to adults (Shimazu and Kushida 1980; Hajek et al. 2008).

Our results suggest that control of *M. galloprovincialis* larvae with entomopathogenic fungi is difficult to achieve in natural conditions, as even under controlled conditions and with prolonged direct exposure the larval mortality was generally low. Furthermore, mature larvae spend a large portion of their development (several months) in galleries inside the wood, which are isolated and sealed with frass debris and fecal material (Naves et al. 2008), so the application of the fungal suspension against this life stage is difficult to be conducted in the field.

Much higher effectiveness was found for adults, with half of the insects dead five days after exposure for some treatments. This rapid mortality of adult beetles is important because *M. galloprovincialis* insects begin transmitting the pine wood nematode with high frequency two weeks after emergence (Naves et al. 2007), so any control strategy should be determined to eliminate the adult beetles shortly after they emerge, as this is the only way to efficiently limit the spread of pine wilt disease.

Besides the fungal species and isolates, the application method also appears to affect the adult´s mortality, as “direct contact” was more effective than spraying. With this method, there is a continuous contact between the insect and the conidia, increasing its effectiveness, as already reported by Shimazu (2004b). Nevertheless, prolonged periods of contact are difficult to achieve in the field, while the effectiveness of the spraying method could be improved by increasing the conidia concentrations or the amounts of solution applied. Other methods of exposing the beetles, such as the use of fabric strips impregnated with conidia suspensions (Shimazu 2004b; Hajek et al. 2006; Shanley et al. 2009), can also be used to control the populations of *M. galloprovincialis*, and will be tested in future assays.

There is an obvious need for additional and alternative control methods against the pine sawyer and the pine wood nematode, because existing control options are limited, costly and, in general, have low efficiency (Sousa et al. 2011). The application of entomopathogenic fungi may have some interest to control adult beetles shortly after emergence, according to our study. This can be achieved with the spraying of fungi on cut waste material and wood debris just before the insect´s emergence, or with the attraction of beetles to traps or fabric strips where they can contact directly with the fungi. These and other strategies are currently being developed and tested in order to offer alternative methods to manage and control this serious sanitary problem which affects Europe's pine forests.

Acknowledgement

We are grateful to Lluis Simó from Universitat de Barcelona, for important suggestions and advices for the development of the larval disinfection protocol. We would also like to thank Instituto Nacional de Investigação Agrária e Veterinária colleagues Adérito Matos for assistance in the field, Marina Soares and Maria Florinda Medeiros for laboratory handling and assays preparation. The Herdade da Comporta kindly allowed for the collection of pine wood infested with *M. galloprovincialis*. We are also grateful to Drª Paula Ramos from Instituto Superior de Agronomia for the entomopathogenic

fungus isolates of *Metarhizium* sp. (MsI1) and *Fusarium* sp. (FsI1) provided to this study.

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Effectiveness of fiber bands impregnated with *Beauveria bassiana* for the biological control of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae)

Biological Control – Submitted

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Resumen

El nematodo del pino *Bursaphelenchus xylophilus* es el agente responsable por la enfermedad del decaimiento súbito del pino. En un elevado número de países, los pinos (*Pinus spp.*) son la especie vegetal más afectada por dicha enfermedad. La capacidad de propagación a nuevos árboles está totalmente dependiente de su insecto vector; los insectos del género *Monochamus*. En Portugal, este nematodo es transportado entre arboles por el Cerambícido *Monochamus galloprovincialis*. Los métodos existentes para controlar este insecto son bastante reducidos y en gran parte basados en técnicas culturales o biotécnicas. Con el objetivo de desarrollar una estrategia de controlo basada en el uso de hongos entomopatógenos, testamos la efectividad de impregnación de bandas de fibra de polyester con aislados del hongo *Beauveria bassiana.* Las bandas impregnadas fueron degradadas por periodos de 24 horas durante 15 días. En el laboratorio, los cerambícidos adultos fueron expuestos a las tiras impregnadas y posteriormente alimentadas hasta su muerte. Degradar las tiras por periodos consecutivos de 15 días no tuve influencia tanto en la viabilidad como en la densidad de los esporos presentes. Todos los insectos expuestos a las tiras impregnadas murrieron en menos de 10 días (7.6 ± 2.5), mientras las insectos no expuestos vivieron hasta 27 días (27.1 ± 10). Reducir las poblaciones de *Monochamus* antes que lleguen a su pico de transmisión de nematodos es un importante avance en el control de la propagación de la enfermedad.

Effectiveness of fiber bands impregnated with *Beauveria bassiana* for the biological control of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae)

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Abstract

The pine wood nematode (PWN) *Bursaphelenchus xylophilus* is the causal agent of the pine wilt disease, which affects mainly pines (*Pinus* spp.). The ability to infect new tree hosts is entirely dependent on its insect vector, which are the insects from the genus *Monochamus*. In Portugal *B. xylophilus* is carried by a sole vector, *Monochamus galloprovincialis.* Until now the methods available to control the insect populations are limited and based on cultural or bio-technical techniques. With the purpose of developing a control technique using entomopathogenic fungi, we tested the effectiveness of impregnating polyester fiber bands with *Beauveria bassiana* fungal isolates. Impregnated fiber bands were artificially degraded for periods of 24 hours up to 15 days. Pine sawyer adults were exposed to the bands under laboratory conditions and their longevity evaluated. Degrading the fiber bands for 15 consecutive days had no influence on conidia viability or density. All the insects exposed to the impregnated bands died in less than 10 days (7.6 ± 2.5), while beetles not exposed to the impregnated bands lived a mean of 27 days (27.1 ± 10). This technique shows promising prospects for diminishing *Monochamus* populations shortly after the insects emerge and before they transmit the PWN to pine hosts, which may represent an important contribution for control of pine wilt disease.

Key Words

Biological control; Cerambycidae; Entomopathogenic fungi; Pine wilt disease; Pine wood nematode.

Introduction

Pine wilt disease (PWD) is responsible for the decline and death of pine trees around the world (Evans *et al.*, 1996; Webster and Mota, 2008). This is also a major sanitary problem in Portugal, where the causal agent, the pine wood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle was detected in 1999 (Mota *et al.*, 1999).

The implementation of management or control strategies against PWD has to focus on preventing the spread and dissemination of the nematode. Within short-distances (at a local scale) this spreading is done by its insect vector, which carry the nematodes on their body during the flight activity. The most noticeable insect vectors are species of the genus *Monochamus* (Linit, 1988). In Portugal, the main culprit for this spreading is the species *Monochamus galloprovincialis* Olivier. The populations of this Cerambycidae are controlled by the felling and elimination of symptomatic trees during late autumn, winter and early spring, when larvae or pupae are found in galleries inside the wood. Nevertheless, for these procedures to be effective there is the need to collect and destroy very small branches and the apical section of the pine trunk, where *M. galloprovincialis* can be found in highest abundance (Sousa *et al.*, 2001, 2011; Naves *et al*., 2008). Although this material can be shredded or transported for processing or burning, these procedures lead to significant delays and substantially increase the control costs in the areas affected by the PWN. Therefore new alternatives to manage the problem of the wood material infested with nematode and insect vectors are urgently needed.

In the absence of homologated chemical control options to treat the branches and upper trunk (Sousa *et al*., 2011), the use of specific and efficient natural enemies to be employed as bio-control agents would be an interesting and environmental-friendly alternative, although no option is currently available (Petersen *et al*., 2012). Entomopathogenic fungi have recently been investigated against adults and larvae of *M. galloprovincialis* (Petersen-Silva *et al.*, 2015), with six isolates from three different species studied, and the results showed that nearly 50% of the vector population was killed by *Beauveria bassiana* (Bals.-Criv.) Vuill under laboratory conditions. Field studies also found *B. bassiana* to be responsible for one quarter of *M. galloprovincialis* larval mortality (Naves *et al.*, 2008), suggesting this well-known fungi species has the most promising agent to be used as bio-control agent.

There is nevertheless an important constrain on its use against the target insect, which is the suitability of the application method. For pests which spend part of their cycle underground, the inoculation of fungal strains into the soil can be very simple and effective (Keller *et al*., 1997; Eilenber, *et al.,* 2006; Lozano-Tovara, *et al.,* 2013). Yet, for insects with quite different life cycles, such as bark and wood boring beetles inhabiting galleries under the bark or inside the wood, there are fewer alternatives. For a various number of forest pest whose ecology and life cycles are similar to *M. galloprovincialis*, the use of fiber bands to sustain live fungal culture has been successfully employed (Shibata, *et al.,* 1991; Dubois *et al.,* 2004; Hajek *et al.,* 2006; Shanley and Hajek, 2008; Shanley *et al.,* 2009; Ugine *et al.,* 2013). If applied in the surface of cut-waste material piles, recently-emerged *Monochamus* beetles could contact with fungi-infested fiber bands long enough to become contaminated; therefore this method could eventually help diminish population levels of the vector insect shortly after emergence. In this paper we proposed to evaluate under laboratory conditions the effectiveness of polyester fiber bands impregnated with *B. bassiana* isolates against adult *M. galloprovincialis*, and assess their efficacy over time when exposed to simulated environmental conditions.

Materials and Methods

Fungi culture

Fungal isolate of *B. bassiana* were obtained from the INIAV live collection (Instituto Nacional de Investigação Agrária e Veterinária). To induce faster sporulation they were kept in dark chamber at 25°C in PDA culture medium with half concentration (PDA/2) of nutritive components (Li and Holdom, 1995) until hyphae was observed near the petri dish boarder. This isolate was selected due to its high virulence under laboratory conditions against adults of *M. galloprovincialis* (Petersen-Silva *et al.,* 2015).

The method used for the culture of the fungal isolate under laboratory conditions was based on Shimazu (2004). Eighteen sterilized Erlenmeyer flasks were filled with 200 ml of Sabouraud Dextrose Yeast liquid medium (SDY, Table 1) and inoculated with four mycelial plugs (10mm diameter) from the margins of actively one week old cultures of *B. bassiana*. All Erlenmeyer flasks were then placed in a climatic chamber at 27°C, without light and with continuous steering (175 RPM) for 4 days for fungal development. Additionally, 12.6 liters of Sabouraud Dextrose Agar + Yeast (SDAY) were prepared accordingly to the quantities found on table 1. After 4 days of steering, the liquid SDY solutions were removed and mixed with the SDAY (pre-heated at 45°C) in a biological safety chamber (Sterilgard Class II Type A/B3, The Baker Company, Sanford, Maine, USA) to prevent contaminations.

Fiber band impregnation

One hundred and sixty squares of polyester fiber (10 x 10cm; 200g/m^2) were cut and placed inside the liquid mixture, decreasing its temperature by 10ºC to solidify the medium. Impregnated fiber bands were then placed horizontally on a rack and pilled inside sterilized plastic boxes (50x20x40 cm) with 2 liters of purified water in the bottom to create high humidity levels. Boxes were then maintained inside a climatic chamber at 27°C, no light for 1 week or until the fungi completely covered the entire band. All the bands were then individually sealed in a biological safety plastic bag and stored until usage at 4°C. Ten polyester fiber squares (10 x 10cm; 200g/m^2) were also cut and isolated to use as negative control.

Table 1

Ingredient	Culture medium	
	SDY	SDAY
Agar (g)	Na	18.6
Dextrose (g)	40	36.5
Peptone (g)	10	9.3
Water (l)	1	1
Yeast (g)	10	10

Table 1 - List of ingredients and quantities needed to prepare one liter of Sabouraud Dextrose Yeast medium (SDY) and Sabouraud Dextrose Agar + Yeast (SDAY); na- not applicable

Collection and rearing of *M. galloprovincialis* larvae

Fourth instar (L4) *M. galloprovincialis* larvae were collected from dead maritime pine trees, *Pinus pinaster* Aiton, from Comporta, Portugal. A total of 170 larvae were removed from the wood in January 2014. Larvae were disinfected in sterile containers with 200 ml of azoxystrobin (1% v/v azoxystrobin) and softly steered for 60 minutes, and subsequently placed on sterile filter paper for some seconds to absorb the remaining liquid (Petersen-Silva, *et al* 2015). Until needed for the assay, the larvae were individually kept in a sterilized plastic Petri dish with filter paper sealed with parafilm Bemis® and maintained at 8ºC, 65%RH (Relative Humidity) and 0:24 LD (Light / Dark). To obtain adults, larvae were transferred to 25ºC 16:8 LD.

Exposure of adult *M. galloprovincialis* to the fiber bands

The fungi-impregnated bands were removed from the biological safety plastic bags and artificially degraded in a climatic chamber for 15 days, simulating the normal climatic conditions of temperature (maximum and minimum), photoperiod and humidity for Lisbon, Portugal, during the first 15 days of July: 27.5ºC/17.5ºC, 16/8 L/D, 60/80% RH (Anonymous, 2014). This period of the year was chosen to coincide with the peak of *M. galloprovincialis* emergence in Portugal (Naves *et al.*, 2008).

Every day, during the 15 days, ten units of impregnated fiber squares were randomly selected and placed in direct contact with ten recently emerged *M. galloprovincialis* adults (five of each sex), which were allowed to walk on it for 30 seconds. Subsequently, each adult was placed in a sterile sealed plastic cup (0.5 l capacity) with fresh *P. pinaster* twigs supplied for feeding. Adults were observed daily until death and twigs were changed every 7 days or earlier if presenting severe signs of degradation. An additional 10 *M. galloprovincialis* were also allowed to walk individually on ten pre-cut squares of polyester fiber without fungus impregnation, being similarly kept in sealed plastic cups and serving as control treatments.

Each insect was registered for its elytra length, weight and sex. Dead individuals were placed in a humid chamber to confirm *B. bassiana* contamination and for fungal re-isolation. To evaluate the amount of conidia on each fiber square, a small sample (1x1cm) was removed from each fiber band square and placed in a glass container. To separate the conidia from the mycelium 10 ml of Tween 20 aqueous solution (0.4% v/v Tween 20) was poured inside the container and manually steered for 60 seconds. The spores obtained were quantified by direct counting using a hemacytometer and a stereomicroscope. Conidia counting were also accessed for control treatments.

Evaluation of conidia viability over time

To determine the conidia viability (percentage) for each degradation period, 8 dilutions of 1/10ml were consequently performed and one µ was carefully laid on three PDA Petri dishes (90cm diameter) inside a biological safety chamber. All the Petri dishes were then stored at 25°C until mycelium growth could be observed under stereo-magnifier. Number of developed conidia were counted with a stereo-magnifier and compared to the initial number of conidia in the counting chamber for each degradation period to determine the percentage of viable conidia.

Statistical analysis

For statistical analysis individuals were grouped each four consecutive days (table 2). Insect weight was grouped in three different classes for statistical analysis (a, b and c). Analysis of variance (ANOVA) was used to compare the longevity between groups for conidia densities, insect weight, elytra length and sex. The Fisher least significant difference (LSD) test was used to compare means within each significant factor after ANOVA. Values in charts and tables are presented as means ± standard deviation. Statistical procedures were performed using STATISTICA 6 software (StatSoft Inc. 2003)

Results

Insect longevity was significantly affected by the contact with *B. bassiana* impregnated bands. Beetles subjected to the control bands lived for 27.1 ± 10.4 days, (mean ± SD) while insects that contacted with the impregnated bands survived only for 7.6 ± 2.5 days. Among the degradation periods, no significant difference was found on insects longevity ($F(_{1,3})$=0.220; p=0.8824). However, differences were found between fungi-exposed groups and the control ($F_{(1,4)}$=75.553; p=0.0001).

Regarding conidia density on the impregnated fiber bands, a small decrease was observed after an initial burst (Table 2; Figure 1). When conidia density raised, insect longevity tended to diminish and vice-versa (Figure 1). Regarding conidia viability, it was always 100% except for days #13 and #14 (Table 2), and therefore had no effect on insect longevity.

From the polyester bands used as control, no conidia were found or isolated (Table 2). No visible signs of mycelial growth were observed on the control insects.

No significant differences were found on longevity for both sexes ($F_{(1,1)}$ =0.045; p=0.8327). Insect longevity was not significantly affected by body size ($F_{(1,2)}$=1.027; p=0.3603), however, larger insects submitted to the impregnated bands tended to live 0,67 days longer than smaller ones. Adult's mean body size was 1.79 cm ± 0.27 cm with a mean weight of 0.25 g ± 0.09 g.

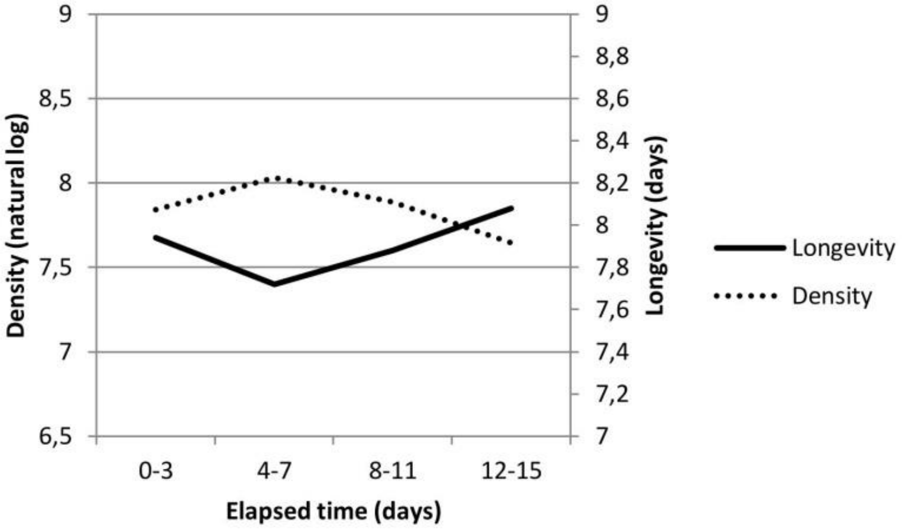

Figure 1: Evolution of *Monochamus galloprovincialis* longevity after exposed to impregnated fiber bands degraded for different periods and *Beauveria bassiana* conidia density (natural logarithm). Elapsed time: periods of four consecutive days.

Discussion

Our laboratory trials confirm that exposure to *B. bassiana* for short periods of time of just 30 seconds can affect the longevity of adult *M. galloprovincialis*, likewise previous reports for other insects (Onofre, *et al.,* 2002; Shimazu *et al.*, 2002; Marannino *et al.,* 2010; Francardi *et al.,* 2012; Meyers *et al.,* 2013). The slightly faster mortality of insects submitted to higher conidia density could result from a faster penetration of the fungi in the hemocoel of the insect, resulting in their precocious death (Augustyniuk-Kram and Kram, 2012). As the density of conidia on the fiber bands remained relatively stable throughout the experiment, the lower threshold affecting insect longevity could not be determined, but even if they didn't affect the insects longevity, entomopathogenic fungi may have other sublethal effects on insects, like the reduction of their fecundity (Dubois *et al.*, 2004), which was not evaluated in this study.

Conidia viability remained stable and with high rates when exposed to (simulated) daily ambient conditions for July, suggesting the adequacy of the *B. bassiana* isolate to the temperature and humidity tested. Nevertheless, further experiments are required to test the viability under more extreme temperature conditions, and also for the effects of wind and UV radiation, which have been found to affect its virulence (Cagán and Švercel, 2001; Thompson *et al.,* 2006). The fiber bands were also able to successfully sustain the fungal populations for two weeks, similarly to results described by other authors (Shimazu, 2004; Dubois *et al.*, 2004; Hajek *et al.,* 2006), but their longevity needs to be evaluated for prolonged periods of time.

Using fiber bands impregnated with entomopathogenic fungi was firstly developed to apply around the tree trunk of standing dead pine trees, to control emerging Japanese pine sawyers, *Monochamus alternatus* (Hope) (Shimazu, 2004). This procedure would be difficult for *M. galloprovincialis*, as this insect tends to lay eggs on the upper part of the tree canopy (Koutroumpa, 2007; Naves *et al.,* 2008), in inaccessible locations in standing trees. Nevertheless, once the tree is felled the fiber bands could be applied to branches and wood debris concealing *M. galloprovincialis* larvae, which might be new focus of wilt disease if left unprotected in the terrain (Sousa *et al*., 2011). Our results confirm that exposures of just a few seconds (similar to what recently-emerged adults would experience) to the impregnated fiber bands are sufficient to eliminate insects in less than 10 days, well before the adults start to reproduce (Naves *et al*., 2006) and to transmit the PWN to new hosts, which mostly occurs after 10 days of emergence (Naves *et al*., 2007).

This is a preliminary approach to evaluate the use of entomopathogenic fungi to control field populations of the pine sawyer. Further studies are needed on the effect of the use of impregnated fiber bands on the auxiliary fauna and their durability/viability under field conditions, although results suggest that this technique may play an important role on the management and control of wilt disease in Portugal.

Acknowledgements

We are grateful to INIAV colleague Adérito Matos for his assistance in obtaining the pine trees and the *Monochamus* larvae, to Maria Florinda Medeiros for all the assistance provided during the laboratory procedure and to Eugénio Diogo for his comments and advices on conidia isolation and viability procedures. This work was partially funded by the REPHRAME project KBBE nº 265483 “Development of improved methods for detection, control and eradication of pine wood nematode in support of European Union Plant Health policy”.

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Table 2 – Longevity (days) of the *Monochamus galloprovincialis* adults; *Beauveria bassiana* conidia density ($\times10^{8}$ conidia ml^{-1}) and viability (%) according to each period of elapsed time.

Groups	Conidia viability (%)	Conidia density ($\times10^{8}$ conidia ml^{-1})	Longevity (Mean ± Std.Dev)	Elapsed time (days)
Negative Control*	NA**	0	27.1 ± 10.4^{a}	0
A	100	1.03	7.9 ± 0.9^{b}	0
	100	1.90	7.0 ± 0.5^{b}	1
	100	1.00	8.6 ± 1.3^{b}	2
	100	1.00	7.2 ± 0.8^{b}	3
B	100	1.50	6.7 ± 0.9^{b}	4
	100	1.70	8.4 ± 2.5^{b}	5
	100	1.43	7.7 ± 1.1^{b}	6
	100	2.20	6.8 ± 1.1^{b}	7
C	100	1.10	6.3 ± 1.4^{b}	8
	100	2.05	7.2 ± 2.2^{b}	9
	100	1.89	8.7 ± 2.5^{b}	10
	100	0.65	8.2 ± 3.6^{b}	11
D	100	0.42	6.5 ± 1.7^{b}	12
	98	1.00	8.8 ± 4.1^{b}	13
	96	1.40	9.0 ± 5.7^{b}	14
	100	0.79	7.1 ± 2.2^{b}	15

* Insects exposed to polyester fiber bands without fungi impregnation. ** NA – Not applicable. Mean within each line followed by the same letter do not differ, P≤0.05. For each elapsed time (0-15) 10 individuals were used. Groups (A-D) were formed by 40

V- DISCUSSION

The works developed during this thesis may be considered the foundations for the knowledge on selected natural enemies of *M. galloprovincialis* and a preliminary step to a future development of a biological control strategy against the vector of the PWN in Portugal.

In order to study the parasitoids and entomopathogenic fungi against associated with *M. galloprovincialis* was essential to improve the rearing procedures of *Monochamus* and its parasitoids under laboratory conditions. In the case of Cerambycidae beetles and particularly *M. galloprovincialis*, it was necessary to develop an artificial diet simulating the characteristics of dead trees and healthy branches, for being able to not depend on fresh pine materials extracted from living trees.

Such diet was created based on the use of cellulose powder instead of *Pinus* derived material, successfully allowing the attainment of more than 75% of the individuals, which according to Singh (1983) is reliable enough for mass rearing insect populations. Including host plant tissues in the artificial mediums was showed by some authors to improve the insect's acceptance (Allo and Katagiri, 1994). Nevertheless the diets tested which included plant tissues did not show to be more successful, suggesting that it is possible to not incorporate this component, economizing the amount of work necessary to cut live trees and to extract the phloem. It becomes also clear that the use of antifungal agents, propionic and ascorbic acids it's not mandatory, which can significantly decrease the cost of production. However, its inclusion in the diet allows the storage for several months without major contaminations.

An artificial egg laying substrate was also tested to allow the development of the complete life cycle of the pine sawyer in artificially conditions. The several pre-assays done, suggested that the most adequate substrate should mimic the *P. pinaster* thin bark, selected by the female pine sawyer for oviposition. Nevertheless the insects seems to not only be attracted by the bark extracted from the maritime pines, suggesting that the attraction should probably be reinforced by the use of chemical substances from pines or even visual clues like the shape of the branch, as it has been suggested for other Cerambycidae species (Suckling *et al.*, 2001; Allison *et al.,* 2004; Shui-Qing *et al.*, 2007). Another possibility for the lack of acceptance must be related to some odors released by the materials used in the manufacturing.

An artificial medium to develop *M. galloprovincialis* in artificial conditions will be probably difficult to obtain although it would solve various problems, like the death of neonates or the portability of the entire device. As found by Naves *et al.* (2007b), diminishing the necessary handling of eggs between the various steps of the life cycle may decrease the death rates. Also, having a practical rearing substrate is essential, so it can be used inside bio-climatic chambers to improve generation development time. Likewise, studies from Addesso *et al.* (2009) or Lu *et al.* (2011) also suggested that it's possible to reduce larval mortality if only older larvae are handeled, corroborating the need to develop an all-in-one rearing model.

When comparing the species life cycle under laboratory conditions with the natural life cycle that occur in distinct countries, it becomes evident that the length and number of annual generations is fully dependent on the environmental parameters (Naves *et al.,* 2008; Tomminen, 1993). This data suggests that it is possible to manipulate under laboratory conditions the temperature, humidity and light cycles to obtain more generation in a shorter period. During the assays performed in the artificial rearing, it was possible to rear an insect from egg to adult in less than 55 days. The assays showed that not only the artificial medium can affect the development time of the insect, but also the temperature and the light/dark cycles can have some influence too. Like other insects (e.g. Rogers *et al.,* 2002; Shintani, 2011; Garcia-Ruiz *et al.,* 2012), *M. galloprovincialis* Portuguese populations present a diapause during the cooler season, which isn't yet fully understood (Naves *et al.,* 2008). The assays performed with the artificial diets suggested that when facing constant warmer temperatures and constant light, the pine sawyer can skip diapause and reach the adult stage in fewer days, ate least for part of the population. It is essential to perform further assays on the manipulation of such factors to fully understand this particular component of the insect's biology, to be able to decrease the time needed to rear a single generation.

On the studies performed about the species geographic range in Portugal, it become evident that its populations are well spread across the country, consistently following the geographic distribution of *P. pinaster* forests. If the absence of data regarding the distribution of this pine sawyer in Portugal before the findings by Oliveira (1894) and other authors from the XXth century is correct, several factors may have influenced its current distribution pattern. The clear preference for *P. pinaster* as host may be related to the lack in abundance of other tree species also favored by the pine sawyer. This is the case of *P. sylvestris* or *P. nigra*, which are extremely rare in our country (ICNF, 2013). In other European countries where they are more frequent, the risks of spreading *M. galloprovincialis* populations' carrying the PWN may be higher. Factors like edapho-climatic variations or altitude showed to have no influence on the pine sawyer distribution in our country. Other event that shouldn't also be neglected is the occurrence of vast *P. pinaster* forestation campaigns in the last century which may have led to the spreading of the pine sawyer populations. The results obtained during the surveys for *M. galloprovincialis* populations and its parasitoids in Leiria, suggests that the establishment of this pine stand around the year of 1300 may have created a stable and well balanced ecosystem, resulting in a stable population for the pine sawyer.

As suggested by Moretti *et al.* (2004), the forest fires may have a positive impact on insects' biodiversity in the post-fire ecosystem. The forest fires that repeatedly occurred in Portugal in the past may have positively influenced the *M. galloprovincialis* population density and distribution. It's known that *M. galloprovincialis* and other Cerambycidae are highly attracted to these events, increasing its populations by laying eggs on burned and decaying wood (Bonifácio *et al*., 2012).

Also, the basic biology of the insect may have influenced its current distribution pattern. The widespread pattern should be viewed in face of the flight capacity of the pine sawyer, studied in recent works (David *et al.,* 2013 Mas *et al*., 2013). However, insects tend to stay as close as possible to the emergence point, as long dispersals would represent a great cost of energy (Sun and Du, 2003). It must also be considered the artificial and involuntary dispersal of the insect, which may occur during wood transportation by road, railroad or sea routes, could have led to new populations' outbursts. A good example may be the appearing of *M.galloprovincialis* beetles infected with PWN in Madeira island (Fonseca *et al.,* 2012), which is close to 1000 km apart from the continent, showing that the most obvious dispersal way must have been by Human activity.

Regarding the work developed during the prospection for *M. galloprovincialis* parasitoid guild, the results showed that, the number of these species found in Leiria comparing to other *P. pinaster* forested areas were really high, which can explain the lower population of *Monochamus* found.. Similarly to Mass *et al.* (2013), finding such a complex parasitoid guild in a specific site, together with the low number of *M. galloprovincialis* beetles, may evidence that the continuously and carefully managed environment can favor the establishment of more diverse and balanced communities of insects and subsequently parasitoids. The low number of *M. galloprovincialis* found it's harder to explain from this point of view, however it must be considered that the parasitoids found aren't specific of the pine sawyer, therefore can be hosting different insects when *Monochamus* is not present. Similar findings relating species diversity and managed forest areas can also be found on various works (FAO, 2001), evidencing the necessity to establish such zones to diminish pest expansions. Together with this data, and as suggested by Chrysopolitou *et al.* (2013), also the proximity to the coast must be considered as it may positively influence the ecosystem by not subjecting it to severe climate changes, such as drought and extreme temperatures. This may have an impact on the forest's health, diminishing the incidence of pathogens and forest pests.

Concerning the unveil of the parasitoid guild associated with *M. galloprovincialis,* the results showed that despite the numerous species related to this Cerambycidae, none of them is at the moment a reliable candidate to establish a biological control program. Several species of parasitoids were found during the surveys; however it was not possible to find specific parasitoids which weren't associated to other insect host. It may be hypothesized that this factor may be directly related to the availability of different and similar possible hosts in our ecosystems, allowing these parasitoids to choose between host species and select the most common, which may sometimes not be *M. galloprovincialis.* Then it would be interesting to develop further studies on the biology and ecology of the parasitoids found parasiting the pine sawyer, to understand if the alternative hosts are also insect pests and understand the parasitoids impact on the auxiliary entomofauna. The total of 14 parasitoid species associated with *M. galloprovincialis* after this study shows that a great variety of natural enemies associated with the pine sawyer; however, several more may be found in a deeper and more exhaustive study on the regions which presents the higher diversity and abundance of parasitoids, like Leiria.

Unlike the other parasitoid species, which were found on specific locations, the genus *Cyanopterus* was found on various sites. This could be interpreted by a higher intraspecific adaptability to different ecosystems. For the implementation of a future control strategy against PWN vector integrating the use of parasitoids, the species *C. flavator* should be one of the species analyzed.

Due to the already mentioned unspecific parasitoid-host associations found among the studied parasitoids, a possible alternative would be the introduction of an exotic parasitoid already associated with the *Monochamus* genus in a foreign country. However, the use of exotic insect species in the control of pests does present some intrinsic risks, as no information is known about the possible interactions with the local entomofauna. Importing these species into new habitats may lead to a large disruption of the ecosystem equilibrium, favoring the outburst of some species and leading to the diminishing of others (e.g. Boughton *et al.,* 2012). Studies on the impact of such species on the ecosystem must be performed prior to their use in “in-situ” situations. The introduction of alien species to be used in biological control programs takes long to implement but usually work (e.g. Zong-Qi *et al.*, 2013). However, in some cases, the result is not what was expected (Ayalewa and Hopkins, 2013). Regarding the well succeeded case studies and closely related to *M. galloprovincialis*, several works with parasitoid species for the control of *M. alternatus* can be found. In Asian countries where the nematode is already present for a long time, assays with the species *S. guani* for the control of *M. alternatus* are now being successfully developed (Xu *et al.*, 2008; Li *et al.*, 2009; Lai *et al.*, 2012). *Scleroderma guani*, *S. harmandi* or the recently discovery egg parasitoid, *C. monochaphagae* could be an interesting alternative, as no similar individuals are known in Portugal or any adjacent country. However, like the parasitoid species found in Portugal, *S. guani* or *S. harmandi* aren’t also species with specific parasitoid habits, as their normal activity targets a vast number of possible hosts.

Another interesting approach would be the implementation of biological control strategies based on the use of bats or selected bird species such as woodpeckers. Researchers have found that natural populations of woodpeckers can effectively diminish the impact of notorious pests in their ecosystems (Wu and Liu, 1999; Liu and Wang, 2003; Zhang, 2003). In the Portuguese forest four species of woodpeckers can be found; *Dendrocopos major* (Linnaeus), *Dendrocopos minor* (Linnaeus)*, Picus viridis* Linnaeus and *Jynx torquilla* (Linnaeus). All of them have insectivore diets, feeding on ants, moths and larval stages of bark-living or wood boring beetles from several species (Rabaça, 2008).

Nevertheless, there are some concerns about the effectiveness of using such species for biological control techniques in Portugal. The woodpecker *P. viridis* is not a reliable candidate for control of wood boring beetles, as they specifically feed on soil living ants and typically live in deciduous forests, which not always occur in Portuguese forests (Bruun *et al.*, 2002). According to the same authors, *D. minor* isn’t also a promising candidate as its winter feeding habits are mainly focus on coniferous seeds, instead of insect larvae, the most abundant life stage at this period of the year. Likewise, *J. torquilla* also does not appear to be a good candidate for a biological control program as it only occurs in Portugal between May and September and doesn’t penetrate the wood to search for food (Bruun *et al.*, 2002; Rabaça, 2008). The most promising woodpecker species would be to *D. major,* however according to Zhao *et al.* (2008) this species is only effective to control the vector of the PWN in regions where the disease is endemic and not epidemic, as in Portugal. Likewise, it should be considered that the use of woodpeckers only has a significant effect on low density insect populations (Kobayashi *et al.,* 1984) which may not be the case of some Portuguese regions affected by *M. galloprovincialis.*

Besides, to increment woodpecker populations, it would be necessary to leave accessible feeding options in the ecosystem, what is strongly discouraged against the control of PWN infected areas, as the main strategy to control this nematode directs to the removal and destruction of infested and potentially infested trees where *M. galloprovincialis* lay eggs (Naves *et al.,* 2008). Nevertheless and despite all this negative setbacks, it would be interesting to develop biological and ecological studies on the hypothesis of using *D. major* as a biological control agent against the pine sawyer in Portugal.

Concerning the use of bats for the control of the pine sawyer, a similar problem arises. In Portugal, only two bat species have their main habitat in coniferous forests; *Pipistrellus pipistrellus* (Schreber) and *Pipistrellus pygmeus* (Leach), being the other typically more associated with human-built structures, caves, or similar habitats. Nevertheless, even these two species are not mandatory forest habitants, even preferring urban environments. The feeding habits of these bats are also a problem if using them as biological control agents is intended in our case, as they mainly prey on Diptera species (Barlow, 1997; Vaughan, 1997; Rehak *et al.*, 2005). Also, these mammals prefer to forage near fresh water habitats, what sometimes cannot be found close to the pine forests where the pine sawyer exists (Vaughan, 1997).

An alternative or a complement to the use of parasitoids or non-arthropod predators could be the development of a strategy based on entomopathogenic fungi for the control of *M. galloprovincialis* populations. This option was firstly approached, as an opportunity to provide a complement for the use of nets impregnated with insecticides (to prevent eclosions from infected felled wood). It was also developed with the intention of diminishing *M. galloprovincialis* populations in less than 10 days, reducing the chances of spreading the PWN. The assays on the prospection about the effect of different entomopathogenic fungal species revealed some interesting results. Apparently, *M. galloprovincialis* larvae and adults show different vulnerability to specific entomopathogenic fungi. Similarly to what was found by other authors (Xue-You *et al.*, 2005; Hajek *et al.*, 2008), adult beetles proved to be more susceptible to infections by *B. bassiana*, while larvae were highly affected by isolates from *Metarhizium* sp. or *Fusarium* sp. It is not completely clear the mode of action in which these fungi work. However the different vulnerability of each insect development stage, suggests that the studied fungi may present different cravings according to the composition of the outer cuticle of the insect.

It is known that when *B. bassiana* conidia contact with the insect cuticle, it starts to develop strong bindings and growing hyphae that will penetrate the insect's body, eventually killing it in less than 10 days (Boucias *et al.,* 1988). The same author found that first instar and fourth instar larvae of *Anticarsia gemmatalis* Hubner (fourth instar was used in the assays presented in this thesis) when exposed to the same amount of conidia showed completely different conidia-cuticle aggregations. This fact was justified by the less hydrophobic cuticle found on the fourth instar larvae. This result could justify the differences found between fourth instar larvae and adults concerning the vulnerability to *B. bassiana* infections. It may also be possible to consider a different approach. According to Clarkson and Charnley (1996) *B. bassiana* conidia attaches itself to the insect's body and releasing specific chitinases and proteases that slowly degrades the insects cuticle (chitin matrix fused with proteins), resulting in a strong virulence against the insect. As recently showed by Yanhua *et al.* (2007), increasing the expression of the gene responsible for the development of a specific chitinase significantly increase the effect of

B. bassiana virulence against insect species. It is then possible to hypothesise that the differences between a larvae cuticle and an adult cuticle regarding the amount of chitin and proteins involved (being considerably lower in larvae) (Gullan and Cranston 1994), should be manifested in different virulence when submitted to the same amount of conidia, as the amount of virulent proteases and chitinases wouldn't be so abundant in the larvae. As *Metarhizium* sp. and *Fusarium* sp. showed to be more appetent to develop on larval stages, it would be possible that the affinity of such conidia to the cuticle surface may be influenced by the hydrophobic relations in a different way. In 2010 Li *et al.* demonstrated that, when resorting to genetic changes and integrating some of the genes responsible for the expression of the highly hydrophobic proteins found on the surface of conidia from *Metarhizium* sp. into *B. bassiana*'s genomic sequence, a strong influence was noticed on the hydrophobicity of *B. bassiana* conidia.

Concerning the application methods tested; spraying, constant contact and impregnated fibre bands, it remained clear that it's more efficient to apply the conidia in a constant available solution or through an artificial medium where the conidia are always exposed. When comparing between the two preferable solutions, the one consisting in the use of fibre bands impregnated with artificial medium where the fungi can grow is clearly the most promising. The differences obtained between the 3 methods can perhaps be justified by the amount of water in which the conidia are immersed. The first two techniques involved a constant wet medium for the conidia, which could probably decrease its adherence to the insect cuticle, as conidia tend to easily adhere to more hydrophobic surfaces (Boucias *et al*., 1988). However, the use of a constant wet medium would increase the durability of such conidia in the environment. Recent works about new techniques for appliance of *B. bassiana* conidia against insect pests have evolved to the use of wet solutions based on oils (e.g. Ming-Guang *et al*., 2004; Robert *et al*., 2009; Rondelli *et al.,* 2011). These techniques increase the adherence of the conidia, and do not allow for the quick deterioration found when exposed to direct UV radiation and temperatures. Therefore, the method of impregnating fibre band with artificial medium that allow a longer hydration for the conidia may be a reliable solution.

Despite the influence of humidity levels and the fungi effectiveness, the technique used with impregnated fibre bands showed to be highly reliable for a considerable amount of time. The use of the fibre band technique seems to be of particular interest if intended not for controlling the spread of the insect during it flight between pines, but instead for the control of already felled trees or even to apply on cut waste. Applying impregnate fibre bands with artificial medium and inoculated with the fungi, provide the necessary nutrient required for the fungi to survive for a longer period without resorting to artificial substrates. Similar techniques are already being used for other insect pests around the world (Shimazu 2004; Hajek *et al.*, 2006; Shanley *et al.*, 2009) with promising results.

Nowadays one of the measures imposed by law (Anonymous, 2011) consists in surrounding all the material with a net impregnated with insecticide, not allowing any successful *Monochamus* emergence into the surrounding environment. Nevertheless, this method shows some inherent problems. Appling a net impregnated with insecticide may not only affect the insects emerging from it interior, but also some other species that eventually touch it from the exterior. If a mixed technique is used with the fibre bands, it would be possible to apply the bands on the top of the waste materials and therefore apply a net without insecticide. This way, any insect that touch the net from the outside won't be affected. It would be also possible to leave the wood and derived remains in the field, lowering the cost of transport associated with their external elimination. The use of this mixed technique may be of particular interest in areas where the use of insecticides is not allowed, and a control strategy is essential. Areas like natural parks, natural reserves or protected areas can greatly benefit from this technique.

The goal of eliminating recently-emerged adult *M. galloprovincialis* in less than 10 days was achieved. *Beauveria bassiana* showed 100% effectiveness in killing all the tested individuals in less than 8 days for almost all the tested variations. In addition to these results, and as corroborated by Maehara *et al.,* (2007) the amount of feeding waste found on each container belonging to fungi infected individuals was significantly low when compared to the control subjects. Besides such data not being present in the results section they may suggests that after entering the insect's body, the fungi interfere with the ability to feed on the provided branches. This way, infecting the insects with *B. bassiana* not only kills them in lees then 10 days, but may also prevents them to pass the disease to the next host trough maturation feeding, what slowly occurs on the first days after emergence but exponentially increases with the time, reaching its peak in the 15th day (Naves, 2007a). Nevertheless, future field studies are required to evaluate the efficacy of this technique under all the natural conditions and with exposure to adverse

UV radiation and strong winds. Similarly, studies on the effect of this method on the auxiliary entomofauna should be made, in order to diminish its potential environmental risks.

Analysing all the results obtained, it remained clear that these approaches may constitute the foundations for future biological control program. However, it is essential to analyse that the effects on the ecosystem and effectiveness of such techniques under field conditions, as all the assays were performed under laboratory conditions. Studies on the effect of both methods on the surrounding environment, auxiliary entomofauna or even the effect on other live organisms must be carried.

VI- CONCLUSIONS

The introduction of pine wood nematode in Portugal has led to the development of various research lines. Each of these approaches was focused on a detailed particularity of the pine wilt disease complex. However, they were all committed to avoiding and even eradicating this serious disease.

As previously detailed in the first chapter, different methodologies are currently used to combat PWD, both targeting the nematode or the insect vector. Though, methodologies based on the use of biological control techniques are not yet available for these organisms. With the purpose of altering the common practices in the control of the PWN and its vector, the assays performed and presented in this thesis were conducted.

The maintenance of a self-sustaining laboratory mass reared population of *M. galloprovincialis* specimens requires the accomplishment of several steps. Before being able to maintain such population from egg to adult it's essential to develop an artificial rearing diet that doesn't need to resort to fresh *Pinus* materials. Completing an entire generation without using these components would allow the diminishing of the time needed to obtain *M. galloprovincialis* adults, at the same time as it would reduce the ecological cost of cutting live trees to obtain fresh components. The results obtained during the assay (Sub-chapter IV-1) demonstrated that it is now possible to rear an entire generation from egg to adult in a minimum of 50 days and only with the use of commercial available products. Nevertheless it wasn't yet possible to find a reliable alternative substrate for egg laying. It is clear that *M. galloprovincialis* adults only lay eggs if presented with real *Pinus* branches. The reason why egg laying is so dependent on this component is still unclear, as several tests were performed with materials extracted from branches without success. Despite the advances in developing the artificial diet it's still essential to investigate and experiment with an alternative egg laying subtract, in order to enable the creation of an artificial device for mass rearing the pine sawyer under laboratory conditions.

To start the survey of the natural enemies (parasitoids and entomopathogenic fungi) affecting the pine sawyer, it was essential to reveal the current situation on the distribution of *M. galloprovincialis* in Portugal. The presence of the pine sawyer was detected in most of the surveyed area (Sub-chapter IV-2), with only some regions showing no signs of the beetle. It may be concluded that *M. galloprovincialis* is well adapted to the different edapho-climatic variations occurring along the territory. The high percentage of records showed that it's highly probable that this Cerambycidae exists in almost the entire country where *Pinus pinaster* is present, however as it is a inconspicuous species, it is sometimes extremely difficult to confirm its presence or absence.

Concurrently with the survey on the parasitoid guild associated with the pine sawyer there was an attainment of new records for Portugal on two host tree species; *P. sylvestris* and *P. halepensis*. Despite the novel findings for these tree species in our country, records of *M. galloprovincialis* hosting these trees are not in Europe. This *Monochamus*-host relation was already found in countries where *P. sylvestris* or *P. halepensis* are more frequent, and where the maritime pine may not be the *Pinus* dominant species. These alternative hosts, should be subjected to extensive surveys, as the risk of getting infected by PWD is extremely high.

Regarding the parasitoid complex associated with the pine sawyer, 14 species of the families Braconidae and Ichneumonidae were found. Resorting to the identification key and to electronic microscope images, (Sub-chapter IV-1 and IV-2) it is now possible to identify any parasitoid already associated with the pine sawyer in the Palearctic region. Among these species, the more common and diffuse was *Cyanopterus flavator.* For a biological control program, this species would represent the most interesting candidate, as it seems to be quite adaptable (being found in locations with diverse climatic conditions) likewise *M. galloprovincialis*. Yet, its generalist habits and the lack of information about its biology and ecology make this Hymenoptera a lesser interesting option to launch a biological control program, although further studies concerning the interactions between *C. flavator* and the surrounding ecosystem are required.

In relation to the use of entomopathogenic fungi in the control of *M. galloprovincialis* populations, the assays performed lead to some interesting results. Firstly, it was successfully developed a disinfection protocol, which allows the attainment of *M. galloprovincialis* larvae decontaminated from fungi and bacteria (Sub-chapter IV-4). Following this procedure permits the use of live specimens in laboratory trials which need uncontaminated individuals. Such method seems to be adequate to apply to other Cerambycidae species that show similar biological cycles, nevertheless this was not tested.

Concerning the entomopathogenic fungi tested against larval and adult stages of *M. galloprovincialis*, different results were obtained. Isolates from *Fusarium* sp. and *Metarhizium* sp. highly affected the larval stages, while *M. anisopliae* and *B. bassiana* diminished the insect populations by 50% in less than 5 days (Sub-chapter IV-4). Despite the promising results, the use of entomopathogenic fungi for the control of larval stages inside the wood or below the bark do not seem as realistic as using them against adults, being unpractical in a field situation.

The subsequent assay intended to develop a field application method for the fungus to control insect populations showed that it is possible to impregnate polyester fiber bands with culture medium to sustain fungi cultures. The ability to eliminate *M. galloprovincialis* population in less than 10 days, underlines the importance of this technique, however, as the assays were all conducted under laboratory conditions, it is essential to perform field confirmations before implementing *B. bassiana* into a biological control program.

The work developed during this thesis enabled the development of an exhaustive study on the effectiveness of various biological control agents against the pine sawyer *M. galloprovincialis*. The development of an integrated pest management program that uses the biological agents examined here should be carefully considered. All the analyzed agents showed to have general effects on the ecosystem and the entomofauna, not being specific to the pine sawyer. The use of arthropods species must be integrated as complement and not only as a single option, as the percentages of attack seem not to be highly significant. The entomopathogenic approach on the other hand, looks like a promising result, as the main objective of eliminating the population before it reaches the peak of nematode transmission was successfully accomplished. Besides the success showed by the application based on polyester fiber bands, the use of this type of fungi on the environment must be carefully considered, as several records can be found about the effects of *B. bassiana* on other insects. Due to the higher cost associated with the production of these fiber bands (when comparing to the use of nets to cover infected wood and prevent eclosions), their application should be considered in areas where the use of insecticides is not an option, like wild life protected areas. However, more detailed studies about the methods of application for this fiber bands should be performed.

Considering all the work developed and the analyzed regions along the last years, it is clear that one of the most effective approaches is based on the correct and carefully management of the pine stands. In regions where an exhaustive management activity occurred along the year, like “Leiria” pine stand; the low levels of *M. galloprovincialis* populations associated with the establishment of a parasitoid guild are evident.

In conclusion, it is now comprehensively studied the parasitoid guild associated with the pine sawyer and the effects of the more common entomopathogenic fungi found in Portugal and associated with this beetle. Future samplings should focus on other natural enemies, such as bacteria or fungi, or even non-arthropod species such as birds and bats, which have been found to affect other *Monochamus* species elsewhere, and have not yet been studied in detail in Portugal. It is now possible to state that the basis for a biological control program against *M. galloprovincialis* populations started to be unveiled.

VII- DIRECTORS REPORT

Impact Factor Directors Report

Edmundo Manuel Rodrigues de Sousa and Pedro Miguel Pina Guerra Duarte Naves, directors of this dissertation presented by Ricardo Neves Petersen Silva reports about the impact factor of the publications that are part of this doctoral thesis.

The thesis here presented is comprehended by 5 research articles. From these five articles 4 of them are published in peer-reviewed journals indexed with impact factor. The fifth one is already submitted and waiting revision. Below is presented the Impact Factor (IF) of each journal on which the articles are published for the year of publication. It is also presented the 5-Year Impact Factor (5IF) when available.

IV.1 – Petersen-Silva, R., Naves, P., Sousa, E., Pujade-Villar, J., 2014. Rearing the Pine Sawyer *Monochamus galloprovincialis* (Olivier, 1795) (Coleoptera: Cerambycidae) on Artificial Diets. *Journal of the Entomological Research Society.* 16(1): 61-70

IF (2013): 0.347, **5IF**: 0.310. Located on the fourth quartile of Entomology

IV.2 – Petersen-Silva, R., Naves, P., Godinho, P., Sousa, E., Pujade-Villar, J., 2014. Distribution, hosts and parasitoids of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) in Portugal mainland. *Silva Lusitana*. 22(1): 67-82.

IF (2013): 0.0256 **5IF:** 0.050.

IV.3 – Petesen-Silva, R., Pujade-Villar, J., Naves, P., Sousa, E., Belokobylskij, S., 2012. Parasitoids of *Monochamus galloprovincialis* (Coleoptera, Cerambycidae), vector of the pine wood nematode, with identification key for the Palaearctic region. *Zookeys*. 251: 29-48.

IF: 0.917, **5IF**: 0.902. Located on the third quartile of Zoology

IV.4 – Petersen-Silva, R., Henriques, J., Inácio, J., Naves, P., Sousa, E., Pujade-Villar, J., 2014. Susceptibility of larvae and adults of *Monochamus galloprovincialis* to entomopathogenic fungi under controlled conditions. *International Journal of Pest Managemen*t. DOI: 10.1080/09670874.2015.1016472.

IF (2013): 0.750, **5IF**: 0.826. Located on the third quartile of Entomology

IV.5 – Petersen-Silva, R., Cardoso, M., Inácio, ML., Sousa, E., Naves, P. 2014. Effectiveness of fiber bands impregnated with Beauveria bassiana for the biological control of Monochamus galloprovincialis (Coleoptera: Cerambycidae) (Submitted to Biological Control)

IF: 1.873 **5IF**: 2.244. Located on the first quartile of Entomology

Thesis Directors

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Author Contribution Directors Report

Edmundo Manuel Rodrigues de Sousa and Pedro Miguel Pina Guerra Duarte Naves, directors of this dissertation presented by Ricardo Neves Petersen Silva reports about the contribution of the author for each of the publications that are part of this doctoral thesis.

IV.1 – Petersen-Silva, R., Naves, P., Sousa, E., Pujade-Villar, J., 2014. Rearing the Pine Sawyer *Monochamus galloprovincialis* (Olivier, 1795) (Coleoptera: Cerambycidae) on Artificial Diets. *Journal of the Entomological Research Society.* 16(1): 61-70

First author Contribution:

Ricardo carried the bibliographic review about techniques and artificial mediums available to the mass rearing of Cerambycidae species. Insect handling and laboratory mass rearing was conducted by the first author. The experiment design and development of the artificial mediums were conducted by Petersen-Silva, who also wrote the first version of the manuscript. Ricardo also helped on the data analysis

Contribution by the other authors:

Pedro Naves developed some of the used artificial mediums, helped on the laboratory handling and analyzed the statically relevant data. Pedro Naves also helped on the writing of the first versions of the manuscript. Edmundo Sousa and Juli Pujade Villar, as the other 2 authors reviewed the manuscript and gave relevant suggestions for its improvement.

IV.2 – Petersen-Silva, R., Naves, P., Godinho, P., Sousa, E., Pujade-Villar, J., 2014. Distribution, hosts and parasitoids of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) in Portugal mainland. *Silva Lusitana*. 22(1): 67-82.

First author Contribution:

Ricardo performed a bibliographic review of all the parasitoid species associated with *M. galloprovincialis* and their distributions after the previous work. All *M. galloprovincialis* Portuguese distribution was also reviewed by Petersen-Silva. The first author also carried out the field and laboratory surveys that occurred in the past 4 years concerning the parasitoid survey. Parasitoid morphologic identifications were entirely made by Ricardo. The manuscript presented was also firstly written by Petersen-Silva.

Contribution by the other authors:

Pedro Naves and Edmundo Sousa performed field surveys that originated some of the presented data about *M. galloprovincialis* distribution. Laboratory identifications and handling previous to 2010 were conducted by Pedro Naves. Paulo Godinho developed the GIS map presented with minor assistance by Petersen-Silva and Pedro Naves. Edmundo Sousa, Pedro Naves, Paulo Godinho and Juli Pujade-Villar reviewed the manuscript.

IV.3 – Petesen-Silva, R., Pujade-Villar, J., Naves, P., Sousa, E., Belokobylskij, S., 2012. Parasitoids of *Monochamus galloprovincialis* (Coleoptera, Cerambycidae), vector of the pine wood nematode, with identification key for the Palaearctic region. *Zookeys*. 251: 29-48.

First author Contribution:

Ricardo performed a bibliographic review of all the parasitoid species associated with *M. galloprovincialis* and their distributions. All the field surveys and laboratory handling were conducted by Ricardo. All electronic microscope photography was also conducted by the author in the UB facilities. The morphologic identifications were primarily made by Ricardo. The manuscript presented was also firstly written by Petersen-Silva.

Contribution by the other authors:

Sergey Belokobylskij conducted the verification and collected all the additional information on the identification of parasitoids. He also created the identification key. Additional specimens present on the key that were not found on Portugal were also acquired by Dr. Sergey. Pedro Naves and Edmundo Sousa helped on the field design of the experiments and reviewed the manuscript. Pedro Naves also assisted on the laboratory handling and rearing of the specimens. Juli Pujade Villar reviewed the manuscript and helped on the electronic microscope photography.

IV.4 – Petersen-Silva, R., Inácio, L., Henriques, J., Naves, P., Sousa, E., Pujade-Villar, J., 2014. Susceptibility of larvae and adults of *Monochamus galloprovincialis* to entomopathogenic fungi under controlled conditions. International Journal of Pest Management. DOI: 10.1080/09670874.2015.1016472.

First author Contribution:

Ricardo performed a bibliographic review of entomopathogenic fungi associated with *M. galloprovincialis* and other close related species, together with application methods for these fungi. The experiment design, laboratory experiments and some data analysis were performed by Ricardo. The manuscript was firstly written by Petersen-Silva.

Contribution by the other authors:

Pedro Naves and Edmundo Sousa assisted in the entomological design of the experiment. Lurdes Inácio and Joana Henriques designed the fungi protocols and characterized/identified the fungi isolates. Pedro Naves conducted the statistical data analysis and helped on the document review. All the remaining authors reviewed the document

IV.5 – Petersen-Silva, R., Cardoso, M., Inácio, L., Sousa, E., Naves, P., 2014. Effectiveness of fiber bands impregnated with Beauveria bassiana for the biological control of Monochamus galloprovincialis (Coleoptera: Cerambycidae) (Submitted to Biological Control)

First author Contribution:

Ricardo performed the bibliographic review concerning new methods available for the use of entomopathogenic fungi against insect species to use in the field. All the insect handling and mass rearing was entirely conducted by the first author. The experiment design and development and some data analysis were conducted by Ricardo. The manuscript was firstly written by Petersen-Silva.

Contribution by the other authors:

Pedro Naves assisted in the entomological design of the experiment and on the statistical data analysis. Edmundo Sousa and Lurdes Inácio aid on the design of the assays that involved fungi development. Marina Cardoso helped on the re-isolation and morphological identification of the fungal isolates obtained from the dead insects and after-assay fiber bands. Marina Cardoso continuously assisted on the handling of the experiment both for insects and fungal isolated. All the authors reviewed the final manuscript.

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