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A first approach to the development of an innovative trapping system for *Gonipterus platensis* (Coleoptera: Curculionidae, Gonipterini)

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Abstract

Gonipterus platensis (Marelli 1926) is an important pest of eucalyptus trees worldwide. In Portugal, this defoliator weevil was reported for the first time in 1996 in the North region but, by 2003, it was already widespread to the whole territory (following the distribution of eucalyptus populations).

The main control method is the biological, using the egg-parasitoid *Anaphens nitens* (Girault 1928), but due to its inefficacy in high altitudes and regions with colder winters (North and Centre), it is necessary to find alternatives for controlling this insect. Chemical spraying was the complementary method applied, but its use decreased over the last years with the increase of the number of banned insecticides in certified forests and plantations. Genetic control has not reached the desirable parameters for quality of paper and tree growth, and it only represents a long-term solution with strong investment. Biotechnical control, contrary to genetic control, can present a sustainable short-term solution, and recent studies had already identified the potential volatiles responsible for host selection in *Gonipterus* species. However, it is still necessary to investigate their effect on the behaviour of the weevil.

In this study, we observed the behavioural responses of *G. platensis* to host volatiles (individually and combined) in wind tunnel assays, and identified the stronger attractive ones. Thereon we developed and tested different kits lure-trap in a severely attacked *Eucalyptus globulus* Labill. plantation in Penela (Coimbra, Portugal).

Ethyl phenylacetate and terpinolene were the most attractive compounds overall and the only ones that exhibited significant differences in intensity of response towards control. The combination of ethyl phenylacetate and alpha-pinene (ratio 1:1) had the best results from the combinations tested (third best overall). None of the kits lure-trap tested in the field assays proved efficient enough for constituting a new control method for *G. platensis.*

Our study emphasizes the important role of the ecological context (single vs combination; laboratory vs field conditions) in the behavioural activity of plant volatiles, and the role of efficient dispensers and traps in the success of biotechnical control methods.

Keywords: *Eucalyptus*, weevil, wind tunnel, volatiles, biotechnical control

Resumo

A importância económica do género *Eucalyptus* cresceu ao longo do século XX principalmente devido à sua utilização como matéria-prima na indústria papeleira.

Introduzido em Portugal por volta de 1829 com propósitos ornamentais, este género ocupa actualmente a maior área de floresta do território continental (26%; 812.000 ha). Esta expansão da área ocupada pelas populações de eucaliptos, principalmente de *Eucalyptus globulus* Labill., deveu-se à ausência inicial de pragas em conjunto com o já mencionado forte investimento da indústria papeleira. Esta espécie para além de uma grande plasticidade apresenta também uma elevada taxa de crescimento e excelente qualidade da pasta de papel produzida, tornando-a na espécie de eucalipto preferida para plantações.

Com a afirmação de Portugal como um dos maiores produtores de pasta de papel da Europa, urge a necessidade de uma melhor gestão das populações de eucalipto, nomeadamente em termos de gestão de pragas. Das várias espécies de insectos fitófagos que se alimentam de eucaliptos já introduzidas no nosso país, o gorgulho-do-eucalipto tem vindo a ganhar destaque pelos elevados danos económicos causados não só em Portugal mas à escala mundial.

O gorgulho-do-eucalipto, *Gonipterus platensis* (Marelli 1926), pertence ao recém-criado complexo de espécies crípticas "*Gonipterus scutellatus*", que reúne as espécies que anteriormente eram confundidas e aparecem erradamente referidas na bibliografia como *G. scutellatus*. *G. platensis é* originário da Tasmânia sendo actualmente a espécie com a distribuição mais vasta do complexo, afectando regiões da Austrália, Europa, América do Norte, América do Sul e potencialmente África. Detectado pela primeira vez em Portugal em 1996 na região do Norte, por volta de 2003 já se encontrava por todo o território português com especial incidência nas regiões Norte e Centro do país (devido à ineficácia dos métodos de controlo). Este insecto, tanto na fase larvar como na fase adulta, consome sobretudo as folhas causando uma diminuição da capacidade fotossintética da árvore e consequentemente do seu crescimento, o que por sua vez vai ter impactos a nível económico.

Em 1996, aquando da sua descoberta, foi criado um projecto nacional de monitorização e controlo de *G. platensis* e no ano seguinte iniciaram-se as introduções das primeiras populações do parasitóide de ovos *Anaphes nitens* (Girault 1928), originário da Austrália, nos focos iniciais. Essas introduções, tal como aconteceu em outros países, foram

altamente eficazes em baixas altitudes e regiões com climas amenos. Contudo em elevadas altitudes e regiões com invernos mais frios esta medida fracassou. Isto acontece devido a um desfasamento geográfico entre o parasitoide e o gorgulho evidenciado por condições microclimáticas desfavoráveis. O facto de o parasitoide ser originário de climas mais amenos (Austrália do Sul) e o gorgulho de climas mais frios (Tasmânia) faz com que o primeiro tenha dificuldade em adaptar-se a zonas ou alturas do ano com temperaturas mais baixas. O problema reside no facto que, em Portugal, as zonas onde o dano é significativo correspondem às regiões Norte e Centro do país que constituem cerca de um terço da área total de eucalipto.

Esta situação deu origem à necessidade de encontrar novos métodos que complementassem a acção do parasitoide. Apesar da sua inclusão em programas de Gestão Integrada de Pragas ser vista como último recurso, o controlo químico era a alternativa mais acessível. Contudo, com o aumento da legislação referente a pesticidas, que tem sido agravado pela recente crise mundial das populações de insectos polinizadores, o uso da maioria dos produtos foi proibido em florestas e plantações certificadas. Uma opção ainda em estudo é a introdução de duas outras espécies de parasitóides - *Anaphes tasmaniae* (Huber and Prinsloo 1990) and *Anaphes inexpectatus* (Huber and Prinsloo 1990) - provenientes da Tasmânia e que consequentemente apresentam uma resistência ao frio similar à do gorgulho. A produção de clones de *E. globulus*, novas espécies ou híbridos menos susceptíveis ao ataque do insecto também tem vindo a ser desenvolvida, mas até agora não se conseguiu encontrar nenhuma alternativa que tenha a mesma qualidade em termos de madeira e de floresta que *E. globulus*. Com a actual falta de opções, o controlo biotécnico emergiu como prioridade por apresentar soluções a curto-prazo e ser inócuo em termos ambientais. Até agora não há nenhum relato da existência de uma feromona, mas já existem estudos com voláteis de diferentes espécies de eucaliptos que sugerem quais podem ser responsáveis pela selecção de hospedeiro. Contudo, para que esses voláteis possam ser aplicados num programa de controlo, é necessário estudar as respostas comportamentais do gorgulho aos mesmos.

Na primeira fase do nosso estudo, três compostos com correlação significativa com a canópia afectada (terpinoleno, alfa-terpineol e alfa-felandreno), três compostos com fortes respostas em electroantenograma (benzil acetato, etilfenilacetato e 2-feniletanol) e dois compostos com elevada concentração nas folhas de *E. globulus* (alfa-pineno e 1,8-cineole) foram testados em túnel de vento de forma a analisar as respostas comportamentais que

induziam em insectos adultos de ambos os sexos. Para comparar as intensidades de resposta dos diferentes compostos foi utilizado um teste de Kruskal-Wallis, ao que se seguiu um procedimento de comparações múltiplas de forma a identificarmos quais os compostos que apresentavam diferenças significativas em relação ao controlo. Numa segunda fase, com uma duração de 3 semanas cada, foram realizados dois ensaios de campo numa população de *E. globulus* altamente afectada em Penela numa tentativa de encontrar um conjunto isco-armadilha eficaz na atração e captura do gorgulho. No primeiro ensaio foram utilizadas como isco placas celulósicas impregnadas com etilfenilacetato em conjunto com três diferentes tipos de armadilha: armadilha unitrap, armadilha multifunil de Lindgren e armadilha para o gorgulho do algodão. No segundo ensaio foram utilizados como isco recipientes cilíndricos de plástico poroso com a combinação de etilfenilacetato e alfa-pineno (num rácio de 1:1) e armadilhas tipo delta, colossus e do gorgulho do algodão modificadas para utilizar em conjunto com cola entomológica.

O objectivo principal de identificar voláteis presentes em *E. globulus* que induzam fortes respostas de atracção em insectos adultos *G. platensis* foi atingido. Os voláteis terpinoleno e etilfenilacetato tiveram as maiores intensidades de resposta demonstrando diferenças significativas em relação ao controlo, o que os torna nos compostos mais propícios a testes no campo. As combinações destes dois voláteis com alfa-pineno num rácio de 1:1 (e 1:1:1) foram menos atractivas que os seus componentes individualmente. A combinação com melhores resultados foi a de etilfenilacetato e alfa-pineno, num rácio de 1:1, que teve a terceira maior intensidade de resposta de todos os compostos, apesar de não ser significativamente diferente em relação ao controlo.

O segundo objectivo de encontrar um conjunto isco-armadilha que possa estar na origem de um novo método de controlo biotécnico para o gorgulho não foi atingido. No primeiro ensaio nenhum insecto foi capturado, apesar de dois terem sido recolhidos da superfície de duas armadilhas. Este resultado é justificado pela taxa de libertação, que parece ser insuficiente para se destacar em relação ao "ruído de fundo" ambiental, e ainda pela ineficácia das armadilhas em capturar o gorgulho. No segundo ensaio foram capturados seis insectos, o que apesar de ser uma melhoria continua insatisfatório para método de controlo. Apesar das armadilhas ainda não serem as mais adequadas, no segundo ensaio a possibilidade de fuga foi minimizada o que se reflectiu num melhor desempenho dos conjuntos isco-armadilha. Conclui-se então que a principal causa dos resultados insatisfatórios são os iscos

porque sendo constituídos unicamente por voláteis de eucalipto não conseguem sobrepor-se ao “ruído de fundo” produzido pelas próprias árvores, agravado pelo fato das taxas de libertação obtidas serem reduzidas.

O nosso estudo realça o importante papel do contexto ecológico (individual vs combinação; laboratório vs campo) na actividade comportamental de voláteis de plantas, e de difusores e armadilhas eficazes no sucesso de métodos de controlo biotécnico.

Palavras-chave: *Eucalyptus*, gorgulho, túnel de vento, voláteis, controlo biotécnico

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1. Introduction

1.1. Eucalyptus – A Worldwide and Portuguese perspective

The species of the genus *Eucalyptus* gained a great economic importance throughout the 20th century, initially due to their use as ornamental trees but mainly as source of commercial cellulose fibre for paper pulp production (Valente et al. 2008; Paine et al. 2011). Eucalyptus trees are also important sources of pharmaceutical products (antiseptics, disinfectants, decongestants, diuretics, stimulants and balms), industrial oils and aromatic chemicals related with the production of perfumes and soaps (Doughty 2000). Some species of this genus have a huge plasticity and consequently an incredibly capacity of adjusting to a broader range of soil, water and slope conditions, outside of their native areas (Doughty 2000). These reasons led to their distribution expansion from their native range in Australia, Indonesia, the Philippines and New Guinea to the rest of the Asian and to the European, African and American continents, turning them in the most widely planted hardwood timber species (Paine et al. 2011).

Introduced in Portugal around 1829 for ornamental purposes, the genus *Eucalyptus* occupies nowadays the largest area of forest in the continental territory (26%; 812.000 ha) (Valente et al. 2008; ICNF 2013). The initial absence of significant pests and diseases followed by a great investment of the cellulose industry led to the expansion of eucalyptus plantations since the 1950's, mainly of *Eucalyptus globulus* Labill. (Tasmanian blue gum), which is a mid-size eucalypt (47 to 70 m high in Australia but can achieve greater heights in other countries) native to Tasmania and southern Victoria Province (Doughty 2000; Loch and Floyd 2001). Due to its capacity of growing in several soil types and adapting to different rainfall regimes, high growth rates and quality of the produced paper pulp, this species is the most popular eucalypt plantation species (Doughty 2000; Loch and Floyd 2001; Valente et al. 2008). Nowadays it is established worldwide with preponderance in climates with cool winters (e.g. Portugal, Spain, California, Chile and other areas with Mediterranean climate) (Skolmen and Ledig 1990; Loch and Floyd 2001).

In 2014, Portugal was the 3rd greatest European producer of paper pulp, with its sells availed in 2.235 million euros. Considering the huge area already occupied by *Eucalyptus* species, and the increasing demand (acquisition and consumption) for wood (6,4 and 2,4%

respectively, from 2013 to 2014), urges the need for a better management of the plantations, particularly in terms of integrated pest management (CELPA 2015).

Initially, as most species were grown from seeds, their predators did not go along with them, however, with the increase in area and the international commerce of eucalyptus, the dissemination of its pests and diseases was inevitable (Doughty 2000; Valente et al. 2008; Paine et al. 2011). *Ctenarytaina eucalypti* (Maskell 1890), which mainly damages eucalypts in nurseries, and *Phoracantha semipunctata* (Fabricius 1775), which caused severe mortality in dry areas during the last decades of 20th century, were the first eucalyptus pests detected in Portugal (Figo and Silva 1977; Figo 1981; Serrão and Bonifácio 1995). After that, several other insect species entered our country, which led to the current eleven species of herbivore arthropods: *Ctenarytaina eucalypti*; *Phoracantha semipunctata*; *Gonipterus platensis*; *Phoracantha recurva* (Newman 1840); *Ctenarytaina spatulata* (Taylor 1977); *Leptocybe invasa* (Fisher and LaSalle 2004); *Rhombacus eucalypti* (Ghosh and Chakrabarti 1987); *Ophelimus maskelli* (Ashmead 1900), *Glycaspis brimblecombei* (Moore 1964); *Blastopsylla occidentalis* (Taylor 1985) and *Thaumastocoris peregrinus* (Carpintero and Dellapé 2006) (Valente et al. 2008; Pérez-Otero et al. 2011; Garcia et al. 2013). We have also a long list of pathogenic agents of diseases (e.g. fungi of the genus *Mycosphaerella* that cause the eucalyptus spot disease) in our country (Valente et al. 2008).

One of the phytophagous insects with great economic importance not only in Portugal but also worldwide is *G. platensis* (Valente et al. 2008; Mapondera et al. 2012).

1.2. *Gonipterus platensis*

1.2.1. Taxonomic position and history of the nomenclature

Gonipterus scutellatus, commonly referred to as eucalyptus weevil or eucalyptus snout beetle, was used to describe the weevils from the Australo-Pacific tribe Gonipterini that caused severe damage to eucalyptus trees worldwide. Since the acceptance of *G. scutellatus* as the correct scientific designation, there has always been controversy involving its identification and the host species in different countries where it was introduced (Mapondera et al. 2012).

In 2012, through mitochondrial COI gene and male genitalia analysis, Mapondera and collaborators found 10 types of aedeagal sclerites that represent 10 distinct taxonomic (and

evolutionary) entities. Although these taxonomic entities are indistinguishable externally, they have differences in morphology and COI sequences.

Therefore, it was concluded that "*Gonipterus scutellatus*" as treated in literature constitutes a complex of at least 10 cryptic species. Of the several species names that have been associated with "*G. scutellatus*" only 5 of the 10 mentioned have been described and they correspond to *G. scutellatus* (Gyllenhal 1833), *G. notographus* (Boisduval 1835), *G. balteatus* (Pascoe 1870), *G. pulverulentus* (Lea 1897) and, the one relevant for our work, *G. platensis* (Marelli 1926). The undescribed species were coded as Gonipterus sp. n. 1-5 (Mapondera et al. 2012).

From this point on, we will distinguish in our work *G. scutellatus* sensu lato from *G. scutellatus* sensu stricto. The first is a reference to the complex (group of species) and/or all the works before the reclassification by Mapondera et al. (2012) that we cannot ascertain which species the authors are talking about, and the second when we are sure of the species used and referenced.

Despite not being very common in Tasmania, *G. platensis* is the species most often confused with *G. scutellatus* sensu stricto because it is also native and naturally endemic to there. Outside of Australia, *G. platensis* is the most widely distributed species of *G. scutellatus* complex (Mapondera et al. 2012).

Initially described as *Dacnirotatus platensis* by Marelli in 1926, this species was later recognized as belonging to the Australian genus *Gonipterus* Schoenherr by G.A.K. Marshall (Oberprieler and Caldara 2012). Later, in 1986, Wibmer and O'Brien defined it as a synonym of *G. scutellatus* (Mapondera et al. 2012).

The species of the genus *Gonipterus* belong to the following taxa: order Coleoptera; suborder Polyphaga; superfamily Curculionoidea; family Curculionidae; subfamily Gonipterinae; tribe Gonipterini (Oliveira 2006).

1.2.2. Distribution

Originally from Tasmania, *Gonipterus platensis* presence is now confirmed in New Zealand, Western Australia, southern South America (initially in Argentina, then spread to Brazil and ultimately to Chile), western North America (initially in California and then Hawaii),

western Europe (initially in Spain and then spread to Portugal), and potentially in South Africa (Echeverri et al. 2007; Mapondera et al. 2012).

In Portugal, *G. platensis* was reported for the first time in 1996 in the North (following the dispersion in Spain), where by that time it was restricted to the territory between the rivers Minho and Lima (Sousa and Ferreira 1996; Valente et al. 2004). However, by 2003 it was already widespread to the whole territory (following the distribution of eucalyptus trees), with major impacts in the North and Centre regions of the country (Valente et al. 2004).

The adult insect is a strong flier and can spread to long distances aided by the wind. Nonetheless, the main cause of dispersion is the international commerce and the transport as stowaway in eucalyptus logs, firewood, eucalyptus transplants from nurseries whose trees have egg capsules and larvae on the leaves or/and pupae in the soil in which they are rooted (Tooke 1953).

1.2.3. Ecology

With few exceptions, not proven scientifically, for almost 3 decades (since 1986 until 2012), not only *Gonipterus platensis* but also the previously mentioned species were wrongly considered as *Gonipterus scutellatus* in scientific studies (Mapondera et al. 2012). That led to a lack of data about the species that are now known to constitute the *G. scutellatus* complex. In these exceptional circumstances, general features can be considered transversal to all species. Mapondera et al. (2012) concluded that despite they are different species and have differences in morphology externally they are indistinguishable.

1.2.3.1. Life cycle

The life cycle of the beetle, as well as the life cycle of any terrestrial insect, is influenced by the climate (essentially by temperature but also by precipitation). Temperature has a direct effect on the activity period of adults, while precipitation, by affecting the soil moisture, has consequences on the development period of prepupae and pupae in the soil. These two components also affect the host plants of the weevil, indirectly affecting it too (Tooke 1953).

Females oviposit preferentially on the youngest/freshest leaves and have the capacity of delaying the oviposition when conditions are unsuitable (Tooke 1953). In Portugal, as well as in other regions with Mediterranean climate conditions, *G. platensis* presents two main

egg-laying periods: the first period in February-June (Spring-Summer) and the second in September-December (Autumn-Winter) (Sousa and Ferreira 1996; ICNF 2013).

Eggs have a subcylindrical form with rounded edges and measure in average 1 mm in length by 0.5 mm in width. Their colour vary from light yellow to golden (Tooke 1953; Mansilla and Pérez-Otero 1996; Sousa and Ferreira 1996). Eggs are deposited in rows inside of a capsule (oothecal) that it is basically a dark brown mass, constructed from excremental material (figure 1.a). Capsules can contain from 6 to 10 eggs and are laid on the upper leaf surface (Tooke 1953; Mansilla and Pérez-Otero 1996; Sousa and Ferreira 1996).

Young larvae eat their way out of the capsule and immediately start to feed on the soft surface of the leaf (epidermis and mesophyll), which makes them not easily detectable (Tooke 1953). Full-grown larva measures from 7.5 to 12 mm and is legless and smooth, has a black sclerotized head, a light yellow to greenish body, a faint dark median dorsal line and a heavy dark lateral stripe (figure 1.b) (Tooke 1953; Mansilla and Pérez-Otero 1996; Sousa and Ferreira 1996).

When larvae are ready to pupate, drop on the ground and form the pupal chamber 10-15 cm deep in the soil (Tooke 1953; Mansilla and Pérez-Otero 1996; Sousa and Ferreira 1996). This pupal chamber is made of particles of the soil cemented by a secretion of the larva (Tooke 1953). The pupa itself is yellowish-white and has structures and general body form similar to the adult (figure 1.c) (Tooke 1953; Sousa and Ferreira 1996).

The development of prepupae and pupae stages is highly dependent on precipitation. If they occur in desiccated soils, the period of development extends, but if they occur in extremely dry soils, the development ceases (Tooke 1953).

Adults are brown and have an elliptic form (figure 1.d). They have 7 to 9 mm in length, with females being significantly larger than males (Tooke 1953; Mansilla and Pérez-Otero 1996; Sousa and Ferreira 1996). During the colder months, the beetle finds shelter in rough bark of eucalyptus trees and only seeks for food on the warmest days (Tooke 1953). Adults are strong fliers and usually take flight during the heat of the day and during the midsummer months (Tooke 1953).

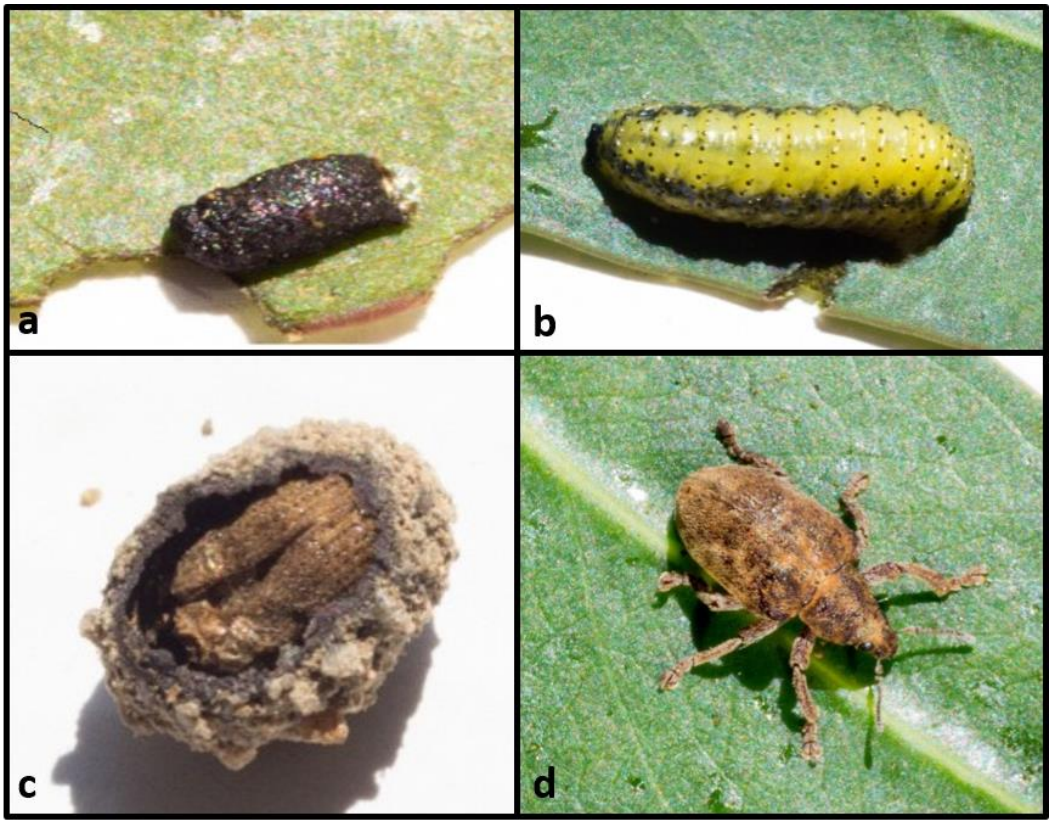

Figure 1. Life cycle of Gonipterus platensis: (a) oothecal; (b) larva; (c) pupa and pupal chamber; (d) adult insect (photos taken by Ana Raquel Reis - Altri).

1.2.3.2. Symptoms and damage of *Gonipterus platensis*

Gonipterus platensis can feed on several *Eucalyptus* species (despite having consequences in their development and performance), but it has a marked preference for *E. globulus* (Cordero-Rivera and Santolamazza-Carbone 2000).

Larvae feed only on the leaves while adults feed on the leaves and soft bark of young shoots (EPPO 2005). In the first larval stages, it feeds only on the epidermis but with the development it starts feeding on the parenchyma, originating holes in the leaves (figure 2.a) (Sousa and Ferreira 1996). Adults start by attacking the border of the leaves, leaving them with scalloped edges (figure 2.b) (Sousa and Ferreira 1996). In both cases (larvae and adults) leaves end up fully eaten, sometimes only remaining the central nervure (Mansilla and Pérez-Otero 1996; Sousa and Ferreira 1996).

Defoliations reduce the photosynthetic capacity of the tree and consequently its growth, and when consecutive may even kill it (figure 2.c) (Sousa and Ferreira 1996; EPPO 2005; Pinkard et al. 2006).

Figure 2. Damage in eucalyptus leaves produced by Gonipterus platensis: (a) larvae feeding; (b) adult feeding; (c) overall view of an eucalyptus plantation attacked in Barcelos (Braga, Portugal) with dead trees (photos taken by Carlos Valente - RAIZ).

1.2.4. Control methods of *Gonipterus platensis*

The choice for one or more control methods depends of general criteria like economical cost, environmental impacts, compatibility with cultures and other methods, or, in some situations like chemical control, of specific factors like proximity to public areas or watercourses (Tooke 1953; Hanks et al. 2000).

Tooke, in 1953, made one of the first and most extensive descriptions of control methods of *G. scutellatus* sensu lato.

1.2.4.1. Cultural control

The cultural measures proposed for controlling the weevil involved ploughing, cultivating, irrigation and fertilization. From those, ploughing was considered the more promising one but to be effective it needed to be practiced on a wide scale, so it was rapidly dropped (Tooke 1953; Richardson and Meakins 1986).

1.2.4.2. Physical control

The methods of physical control already tested consisted basically in pruning and burning. When applying these methods, it should be taken into account that the first is not beneficial in heavy infested areas and the second only should be used in extreme situations (Tooke 1953).

1.2.4.3. Biological control

Until 1927, the only orders described as affecting *G. scutellatus* sensu lato were Hymenoptera (e.g. *Anaphes nitens*) and Hemiptera (e.g. families Reduviidae and Pentatomidae), but only the first one proved to be effective enough to be applied as biological control method (Tooke 1953).

The introduction of the egg-parasitoid *Anaphes nitens* (Girault 1928) has proved to be the strongest measure of controlling *G. scutellatus* sensu lato and consequently the most applied worldwide (Oliveira 2006; Valente et al. 2010; Reis et al. 2012). In South Africa, it was such a success that was considered a rare example of biological control where an egg-parasitoid acting alone controlled a pest (DeBach and Rosen 1991). This successful campaign led to the introduction of this parasitoid in several countries, which proved to be widely effective (DeBach and Rosen 1991; Cordero-Rivera et al. 1999).

However, in some regions (e.g. regions of Galicia and Portugal), despite the number of releases of *A. nitens*, this agent did not have the same success and populations of *G. platensis* still cause considerable economic losses (Mapondera et al. 2012; Reis et al. 2012). Recent findings show that this control failure in some regions may be explained by a host-parasitoid (geographical) mismatch together with unfavourable microclimatic conditions. Whereas *A. nitens* is native from South Australia that is a region with warm to mild climate, *G. platensis* is native from Tasmania, which has a colder climate (Mapondera et al. 2012; Reis et al. 2012). This parasitoid shows marked difficulties in adapting to colder areas (e.g. high altitudes) or times of the year (e.g. winter) (Reis et al. 2012). While the precipitation do not seem to have direct influence in the parasitism rates of *A. nitens*, low temperatures (<10°C) constitute a limiting factor to the action of this agent. In Iberian Peninsula this temperatures occur

primarily during winter and early spring months, which matches with one of the periods of oviposition of *G. platensis* (Reis et al. 2012).

Nowadays two other species of *Anaphes* native of Tasmania – *A. tasmaniae* (Huber and Prinsloo 1990) and *A. inexpectatus* (Huber and Prinsloo 1990) - are under trial in Portugal. Unlike what happens with *A. nitens*, these two species show a similar cold tolerance to *G. platensis* (Valente et al. 2010).

1.2.4.4. Chemical control

Despite the inclusion of chemical methods in Integrated Pest Management programs it is seen as an ultimate alternative. With the deficient results of the biological control in some areas, chemical control has been explored as a complement to the action of *A. nitens* (Pérez-Otero et al. 2003; Santolamazza-Carbone and Ana-Magán 2004). Several natural and synthetic insecticides - azadirachtin, alpha-cypermethrin, *Bacillus thuringiensis* var. kurstaki (Berliner 1915), *Beauveria bassiana* (Bals.-Criv.) (Vuill. 1912), deltamethrin, ethofenprox, flufenoxuron, lufenuron, lambda-cyhalothrin and thiamethoxan - have been tested throughout the years to determinate their efficiency, mainly in terms of toxicity and/or selectivity (Pérez-Otero et al. 2003; Santolamazza-Carbone and Ana-Magán 2004; Echeverri-Molina and Santolamazza-Carbone 2010). The ideal insecticide should be effective against *G. platensis* but should not affect the parasitoid *A. nitens*, bees, or any other insect of the beneficial entomofauna, in order to be included in an IPM program (Pérez-Otero et al. 2003; Santolamazza-Carbone and Ana-Magán 2004).

Both the studies of Pérez-Otero et al. (2003) and Santolamazza-Carbonne and Fernandez Ana-Magán (2004) concluded that, despite not being the strongest insecticides, azadirachtin and flufenoxuron were effective against *G. platensis* and did not affect *A. nitens*, therefore are the most proper to include in an IPM programme. Later, Echeverri-Molina and Santolamazza-Carbone (2010) concluded that thiamethoxan was effective against *G. platensis* adults and compatible with biological control having low effects against beneficial arthropods and mammals. They also concluded that *B. bassiana* EC (a strain tested for the first time with *G. platensis*), contrary to previous experimentations of Pérez-Otero et al. (2003), was efficient against adult insects, and due to its low risk to humans and environment was the most promising product (tested by them) to promote on an IPM programme (Echeverri-Molina and Santolamazza-Carbone 2010). Both insecticides still need further tests with other life stages

(larvae and eggs), and the compatibility of *B. bassiana* with *A. nitens* still needs to be tested (Echeverri-Molina and Santolamazza-Carbone 2010).

The pesticide legislation of the European Union has evolved considerably over the years and is getting stricter with the recent pollinator populations crisis (Handford et al. 2015). Particularly in *Eucalyptus* species, the chemical treatment is not recommended due to the long flowering period that they present and the danger that it represents to the bees attracted by it (EPPO 2005). The Forest Stewardship Council already prohibited the insecticides with the active ingredients acetamiprid, flufenoxuron and thiacloprid in certified forests and plantations (FSC 2013, 2015). With the restriction of the use of pesticides of the neonicotinoids family, the European Commission prohibited the use of thiamethoxam in crops that attract bees (EC 2013). A recent study of Barbosa et al. (2015) showed that azadirachtin affects bumblebee's survival, reproduction and development even at lower doses than the maximum recommended for use.

1.2.4.5. Genetic control

Tooke, in 1953, proposed planting less susceptible species of eucalyptus in order to prevent the impacts of the eucalyptus weevil. Richardson and Meakins (1986) suggested a study of the chemicals that affect the palatability of eucalypts to *G. scutellatus* sensu lato followed by mass screening of native eucalypts to identify the most resistant genotypes. In South Africa, the effects of *G. scutellatus* sensu lato promoted this replacement of the susceptible species to more resistant ones (Tribe 2005). By 1916 the dominant species were *E. viminalis*, *E. globulus and E. maidenii,* that were also the most susceptible. However, nowadays almost 80% of all eucalyptus in South Africa belong to the more resistant species *E. grandis* (Tribe 2005).

1.2.4.6. Biotechnical control

- **A brief introduction of the use of semiochemicals in pest control**

Semiochemicals are chemical compounds (single substances or specific blends) emitted by an organism (plant or animal) that have effects on the behaviour of other individuals, affecting their activities such as feeding, mating or oviposition (Amaro 2003; Norin 2007; Heuskin et al. 2011). Depending if the emitter and the receptor of these signals are from

the same species (intraspecific) or from different species (interspecific), they are designated as pheromones or allelochemicals, respectively (Agelopoulos et al. 1999; Amaro 2003; Norin 2007; Heuskin et al. 2011). The mentioned categories are not mutually exclusive as the same compound can be both pheromone and allelochemical (Heuskin et al. 2011). According to their function, pheromones can be of different types, but the most common are sex, alarm, aggregation, host marking and trail pheromones (Amaro 2003; Heuskin et al. 2011). On the other hand, allelochemicals are divided in allomones (benefits the emitter), kairomones (benefits the receptor) and synomones (benefits both the emitter and the receptor), depending of the organism that is beneficed by its action (Amaro 2003; Heuskin et al. 2011).

In the particular case of pests, these substances provide information about the chemical ecology and behaviour of the insect, which can be very useful for its control (Agelopoulos et al. 1999). Usually they are used in lures for attracting and capturing the pest (Agelopoulos et al. 1999; Norin 2007). However, before the use of a semiochemical is effective, several processes need to be fulfilled (Agelopoulos et al. 1999). First, the source of the volatile emissions has to be identified, to then the compounds be extracted. Of that extracting process results a complex blend that needs to be analysed in order to select the single volatiles with the desired response. Normally this selection is done with the assistance of electrophysiological recording techniques (Agelopoulos et al. 1999). For example, through gas chromatography coupled with electroantenography and gas chromatography coupled to mass spectrometry, single volatiles with physiological activity can be distinguished from the other volatiles of the blend (Agelopoulos et al. 1999; Bouwer 2010). Nevertheless, electrophysiological activity only demonstrates that the insect is sensible to a determined substance. It does not give information about its behavioural effect or the concentrations or combinations at which it is efficient (Evans and Allen-Williams 1992; Agelopoulos et al. 1999; Ansebo et al. 2004).

To obtain that kind of information is necessary to perform laboratory bioassays in olfactometer or wind tunnel (Agelopoulos et al. 1999; Bouwer 2010). Only after those assays, the compounds with promising results can be tested in the field to, if successful, be applied in a control method and commercialized (Agelopoulos et al. 1999).

- **Semiochemicals in *Gonipterus platensis* control**

In 1953, Tooke tried to correlate the host preference of *G. scutellatus* sensu lato with the oil composition of different *Eucalyptus* species but did not have much success. Nevertheless, he concluded that the majority of preferred hosts had 1,8-cineole in their essential oils (Tooke 1953).

Fifty years later, in a study using *E. amygdalina*, *E. risdonii* and their interspecific hybrids, Dungey and Potts (in contradiction to what Tooke stated) concluded that 1,8-cineole content did not seem to have influence on *G. scutellatus* sensu lato oviposition (Dungey and Potts 2003).

Branco and collaborators (2010), by comparing the main emissions of terpenes from leaves of high susceptible (YG015, VG061 and MB043) and low susceptible (VR1277 and VT005) genotypes of *E. globulus,* identified the volatiles related with eucalyptus susceptibility/resistance to *G. platensis* attack. Then by analysing the correlation between percentage of canopy affected (for each genotype) and volatile emissions, they determined the main volatiles responsible for *E. globulus* susceptibility to *G. platensis*. Alpha-phelandrene and terpinolene showed significant differences between high and low susceptible genotypes and had significant positive correlations with the percentage of canopy affected. Although alpha-terpineol did not have significant differences between high and low susceptible genotypes, it had positive correlation with percentage of canopy affected.

Bouwer (2010), by gas chromatography coupled to electroantennography and gas chromatography coupled to mass spectrometry, using crushed leaves of three *Eucalyptus* species (two susceptible hosts, *E. globulus* and *E. viminalis*, and one non-host, *E. citriodora*), identified individual host volatiles to which females of *Gonipterus* sp. 2 (undescribed species of the *G. scutellatus* complex) showed electrophysiological responses. Green leaf volatiles ((Z)-3-hexen-1-ol, (E)-2-hexenal and (Z)-3-hexenyl acetate) produced the greatest response, followed by aromatic compounds (2-phenylethanol, benzyl acetate and ethyl phenylacetate) (Bouwer 2010). Terpenes, such as 1,8-cineol (confirming Tooke's results), γ-terpinene, α-pinene and β-pinene, had the weakest responses from the ones mentioned above (Tooke 1953; Bouwer et al. 2014). The majority of the volatiles that proved to be antennaly active to Gonipterus sp. 2 are also active for other phytophagous and predatory insects (exception made of benzyl acetate and ethyl phenylacetate), which makes them not specific enough to be used in specific mass trapping experiments (Bouwer 2010; Bower et al. 2014).

However, none of the mentioned compounds has already been tested in behavioural assays with *G. platensis.*

1.2.4.7. Control of *Gonipterus platensis* in Portugal

In 1996, a national project for monitoring and controlling *G. platensis* was created. The first populations of *A. nitens* (imported from Spain) were released in 1997 in the area where the weevil was initially detected. From 1998 to 2000, annual releases of parasitoids, originating from a rearing facility created in our country, were made. By the end of 2000, *A. nitens* was established in almost all the regions damaged by *G. platensis*. However, in 2001 and 2002 new releases had to be made in areas with low or no parasitoid populations (Valente et al. 2004; Valente et al. 2014). Summarily, this measure has been successful in low altitudes and mild climates but it is not efficient in high altitudes and regions with colder winters (Center and North). The average crown defoliation is reduced to almost 0% in altitudes below 450m, with parasitism reaching values close to 100%. On the other hand, with the increase of the altitude the average crown defoliation increases too, reaching medium values higher than 75% for altitudes higher than 700m (Valente et al. 2004; Reis et al. 2012). Unfortunately almost one third (28%) of the total area of eucalyptus populations is in the Center and North of the country, above 450 m, where the damage is substantial (Reis et al. 2008; Reis et al. 2012).

In those areas, where conditions are not suitable for an effective control by the parasitoid, was necessary to found other methods that could complement its action. In short-term, the suggested alternative was the use of the anti-chitin commercial product Cascade (flufenoxuron, BASF) but its use was prohibited by the Forest Stewardship Council (FSC) (Evangelista and Valente 2008; Valente et al. 2014). Since 2010, two other neocotinoid insecticides – Calypso (thiacloprid, BAYER) and Epik (acetamiprid, SIPCAM) – proved effective against larvae and adults of *G. platensis* and safe to bees and *A. nitens,* after laboratory bioassays conducted at INIAV (Bonifácio, com. Pess.; Valente et al. 2014). However, in 2015, FSC banned their use in certified forests and plantations (FSC 2015). In midterm, two Tasmanian species, *A. tasmaniae* and *A. inexpectatus*, that have similar cold tolerance to *G. platensis,* are also under trial in Portugal but laboratory rearing proved more difficult than to *A. nitens* and small populations released failed to persist in the field (Valente et al. 2010; Valente et al. 2014). In long-term, the main objective is to produce less susceptible *E. globulus* clones, *Eucalyptus* species or hybrid. By now, the best option found was *Eucalyptus nitens,*

that proved to be less attacked than *E. globulus,* but it does not have qualities (in terms of forest and wood) of the last one (Valente et al. 2014).

With the actual lack of solutions, the search for a biotechnical control method gained relevance and became a priority in the Control Plan for the insect *G. platensis* (eucalyptus snout beetle) (ICNF 2014). Without any report on the existence of pheromones, allelochemichals, namely kairomones present the best option to use as lure in traps for the capture of the weevil. Branco and collaborators (2010) and Bouwer (2010) already studied plant volatiles that may be responsible for host selection/preference in *Gonipterus* species. Nevertheless, it is crucial to test them in wind tunnel and field assays before suggesting their incorporation in population monitoring and control programs (Agelopoulos et al. 1999; Bouwer 2010).

1.3. Objectives

The main goal of our work is to identify volatiles present in *Eucalyptus globulus* that induce strong attraction responses for their future use in a lure for *G. platensis* adults.

Our second goal is to develop and test different dispensers and traps alongside with the selected compounds in order to obtain a primary kit lure-trap for capturing *G. platensis.*

1.4. Framing the theme in Ecology and Environmental Management

The investigation of the plant volatiles responsible for host selection in *Gonipterus platensis* contributed not only to the knowledge of this species ecology and behaviour, but also to the development of lures and traps aiming its capture.

Our results are important contributions to the advance of an innovative biotechnical control method that can be included in future Integrated Pest Management programs and therefore contribute to a better management of the eucalyptus populations worldwide.

2. Material and Methods

2.1. Insects

Gonipterus platensis adults for wind tunnel bioassays were captured in a severely attacked *Eucalyptus globulus* population at Arouca, Portugal (40°56' 05''N; 08°12' 27''W) between 20/02/2015 and 19/06/2015, by RAIZ research team led by Dr. Carlos Valente. They were mailed to INIAV (Oeiras, Portugal), where they were weighted, sexed (according to Rosado-Neto and Marques 1996) and placed individually in cylindrical recipients of 7.7 cm high and 5.3 cm in diameter. Weevils were kept in laboratory at room temperature of 23.2 ± 2.7 °C on a 13:30 h ± 1 h photoperiod and a diet of *Eucalyptus* leaves.

Since there was no way of determinate the age of the weevils and it was beneficial for our work to use freshly captured ones, their longevity was registered after entering the laboratory as a method to evaluate its health condition at the moment they were tested.

2.2. Plant volatiles

Eucaliptus globulus volatiles were selected based in the studies of Branco and collaborators (2010) and Bouwer (2010). The criteria used were: existence of significant positive correlation with the percentage of canopy affected, strong electroantennogram responses (prioritizing the species specific volatiles) or high percentage (concentration) in *E. globulus* leaves. The eucalyptus volatiles selected for our work are listed in table 1.

Table 1. Volatile compounds from Eucalyptus leaves tested as attractants for Gonipterus platensis adults.

Compound	Chemical purity (%)	Source[d]
Alpha-pinene[a]	95	SEDQ
1,8-cineole[a]	99.66	Sigma-Aldrich
Terpinolene[b]	85	Sigma-Aldrich
Alpha-terpineol[b]	90	Sigma-Aldrich
Alpha-phellandrene[b]	98.2	Sigma-Aldrich
Benzyl acetate[c]	≥99.7	Sigma-Aldrich
Ethyl phenylacetate[c]	98	Sigma-Aldrich
2-phenylethanol[c]	99	Sigma-Aldrich

[a] Sociedad Española de Desarrollos Químicos S.L., Barberà del Vallès, Barcelona, Spain; Sigma-Aldrich Química, S.L., Sintra, Lisbon, Portugal. [b] High percentage in *E. globulus* leaves. [c] Significant positive correlation with mean percentage of canopy affected. [d] Strong electroantennogram responses.

All the compounds were kept, as recommended by the provider, in a fresh and dry place until use.

2.3. Wind tunnel bioassays

The behavioural responses of adult weevils to the selected volatiles were evaluated in nonchoice tests. Observations were carried out in an acrylic glass wind tunnel that was 200 cm long, 80 cm high and 80 cm wide (figure 3). Air was pulled by a fan in the basis of the tunnel, purified by a set of filters - one HEPA HMZ9 MINIPAK-H absolute filter, one HEPA HCN9 MULTICEL-H absolute filter, one charcoal-coated Multi-carb FCA1 filter and one PRE-FIL MCZ primary filter (F.C.R. S.p.A., Cinisello Balsamo, Lombardy, Italy) - and redirected to the tunnel at a selected speed of 0.4 m/s. In the direction of the exit of the tunnel was an independent purifying unity with another set of air filters in order to prevent the saturation of chemicals in the closed room where the tunnel is installed. Illumination was provided by six fluorescent bulbs mounted 150 cm above the wind tunnel, giving a light intensity of 224 lux. A KIMO CTV200 sensor (KIMO S.A., Marne-la-Vallée, Île-de-France, France) fixed in the pole closest to the downwind end of the tunnel measured the temperature and air velocity. Room temperature was registered using a HOBO U23 Pro v2 Temperature Data Logger (Onset Computer Corporation, Bourne, Massachusetts, USA).

Figure 3. Wind tunnel used in the behavioural bioassays (photo taken by the author).

A platform of 170 cm long and 10 cm wide was placed inside the wind tunnel to provide a physical connection between the origin (initial position of the insect) and the source of the odour facilitating their walking movement and the registration of their responses by the observer. The platform was divided by five equally spaced points: origin (O), ¼, ½, ¾ and source (S) (figure 4).

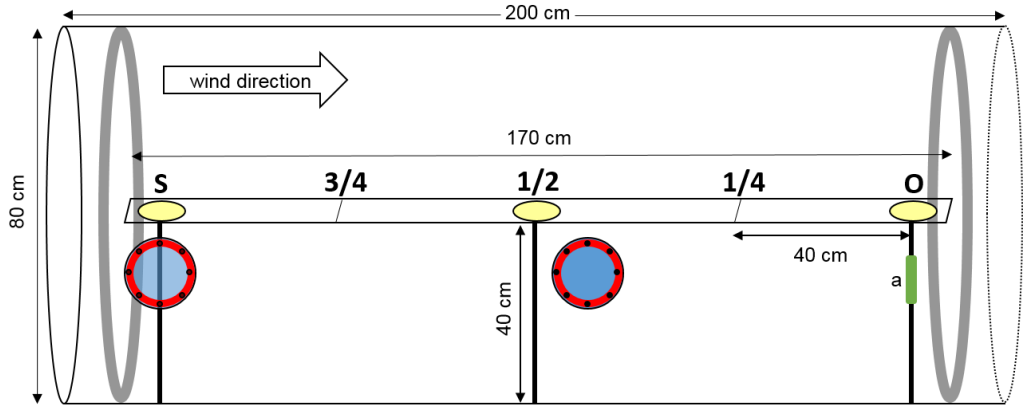

Figure 4. Schematic drawing of the wind tunnel and platform used in the study; a - KIMO CTV200 sensor.

For each compound, 30 insects (15 males and 15 females) were tested. The assays were performed after a 3-day fast and each individual was only tested once.

The period for which the weevils should fast before being tested was defined by a preliminary assay that compared the responses of 30 weevils (15 males and 15 females) to alpha-pinene maintained in three different diets - 10 fed (5M/5F), 10 after a 3-day fast (5M/5F) and 10 after a 7-day fast (5M/5F). The diet that promoted the best results was selected for the main assays with different compounds.

In the beginning of each assay a selected weevil was brought from the laboratory rearing room to the wind tunnel room, and there from the recipient to an arena (watch glass with 8,5 cm of diameter and 1cm high), placed in the downwind end of the platform where it was allowed to acclimate to the wind tunnel.

Each assay had a maximum duration of 15 minutes, starting with 5 minutes of acclimatisation to the wind tunnel conditions and 10 minutes of exposure to the odours. All the assays were conducted during the photophase.

Using a Draeger CH25301 air current test tube (Draeger Safety UK Ltd., Blyth, Northumberland, England) was observed that a continuous and undisturbed odour plume reached the insect in the start position. Strips of Whatman no. 1 filter paper (Whatman

International Ltd., Maidstone, Kent, United Kingdom) with 2cm x 1cm were loaded with 10 µl of compound (except for control) and hanged in a wire structure at the upwind end of the platform.

The insect's response was recorded as the time (in seconds) they spent in each of the four areas/sections of the platform. To quantify the behavioural responses of the insects was defined that the intensity of response corresponded to the sum of the periods spent in each section multiplied by the weight assigned to the respective section. In order to enhance the time spent in the areas closer to the odour source and some behaviours considered relevant, we attributed a weight scale present in table 2.

Table 2. Weights assigned to the different areas of the platform for the Gonipterus platensis intensity of response quantification.

Weight	**Section/behaviour**
1	Origin - ¼
2	¼ - ½
3	½ - ¾
4	¾ - Odour source area
5	Reach the odour source area
6	Touch the source
1/2	Stand still at the origin all the assay
1/10	Fly away from odor source

The assay was over when:

a) The insect touched/grabbed the odour source (filter-paper strip);

b) The insect reached the odour source area without touching it;

c) The insect flied off the platform to the surface of the wind tunnel;

d) The insect flied to the tunnel exit;

e) The maximum duration (10 minutes) was reached without occurring any of the previous situations.

In the situations a) and b) the remaining time was considered as spent in the odour source area.

Considering that volatiles with significant strong responses are present in *E. globulus* leaves in low percentage, and in order to detect a possible synergism between compounds with contrasting percentages, we combined them with a more common but still strongly attractive volatile (alpha-pinene). The combinations tested are described in table 3.

Table 3. Designations, components and respective ratios of the combinations used in the study.

Designation	Components	Ratio
C1	ethyl phenylacetate and alpha-pinene	1:1
C2	terpinolene and alpha-pinene	1:1
C3	ethyl phenylacetate, terpinolene and alpha-pinene	1:1:1

The selection of the compounds for the field assays was made primarily following the criteria of the higher attractive power (greater intensity of response) and secondarily the economic viability of those compounds for later use in monitoring and mass trapping programs.

2.4. Lure release rate measurement

To assess the release rates of the selected lures to be tested in field assays was used an environmental chamber Fitoclima S600 (Aralab, Sintra, Lisbon, Portugal) at a constant temperature of 25 °C. A kit of 6 cellulose plates (3 cm x 4cm) and a cylindrical plastic recipient (7 cm high and 2.5 cm in diameter) were tested with 9 and 25 ml of ethyl phenylacetate, respectively. The cellulose plates were provided by Witasek PflanzenSchutz GmbH (Feldkirchen in Kärnten, Carinthia, Austria). The plastic recipients were identical to the ones used in Galloprotect Plus for alpha-pinene (Sociedad Española de Desarrollos Químicos S.L., Barberà del Vallès, Barcelona, Spain). Release rate was estimated using a gravimetric method. Before starting the assay, we measured the tare weight of each dispenser. In the assay, the lures were weighed after 24, 48, 72 and 96 hours.

2.5. Field bioassays

The field trials took place between 5/8/2015 and 29/9/2015 in a severely attacked *E. globulus* plantation in Penela, Coimbra, Portugal (40°0' 49''N; 08°15' 14''W). For these trials were used two compounds: ethyl phenylacetate and alpha-pinene, individually and combined (C1).

The first assay occurred from 4/8/2015 to 25/8/2015. The traps used were: Lindgren multiple funnel trap, unitrap/bucket funnel trap and boll weevil trap (figure 5). The lure used was a kit of six cellulose plates impregnated with a total of 9 ml of ethyl phenylacetate.

Figure 5. Traps used in the first field bioassay to trap Gonipterus platensis: (a) Lindgren multiple funnel trap, (b) unitrap and (c) boll weevil trap (photos taken by Luís Mota – RAIZ).

The second assay occurred from 8/9/2015 to 29/9/2015. The traps used were: colossus trap, delta trap and a modified version of the boll weevil trap with Temoocid entomological glue (Impex Europa S.L., Vilagarcía de Arousa, Pontevedra, Spain) (figure 6). The lure used was a kit of two plastic recipients, each with 25 ml of ethyl phenylacetate or alpha-pinene (C1).

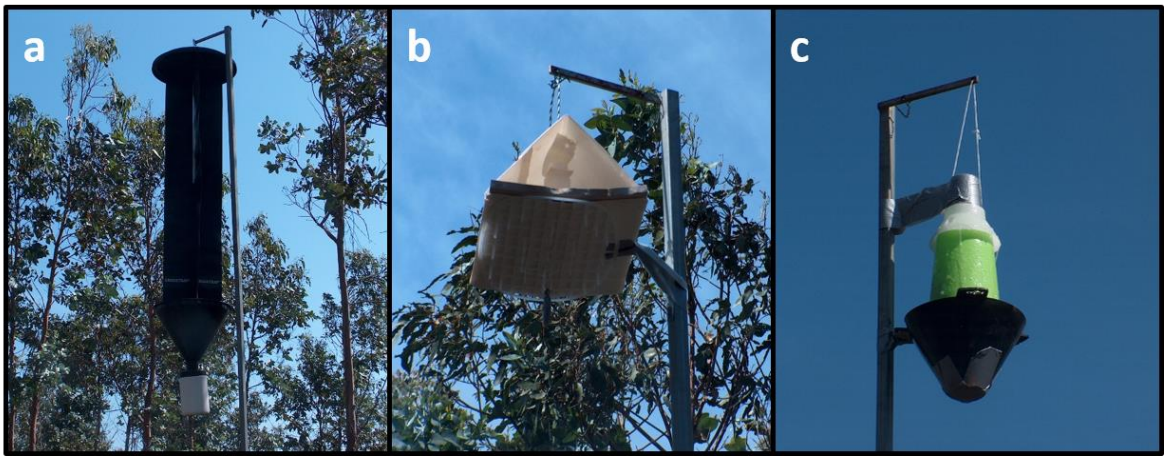

Figure 6. Traps used in the second field bioassay to trap Gonipterus platensis: (a) colossus trap, (b) delta trap and (c) a modified version of boll weevil trap (photos taken by Luís Mota – RAIZ).

Both assays had a duration of 3 weeks with rotation of the traps and collection of the captured insects at the end of each week in order to minimize the trap placement effect.

Before the first and second assays *G. platensis* populations were monitored, searching for traces (attacked leaves, faeces and egg capsules) and adult individuals, to evaluate the viability of starting the assay.

In all assays, nine traps, three of three different types were hanged/fixed in metal poles (at approximately 3 m high) and disposed following a latin square experimental design with approximately 50 m distance between trap (figure 7).

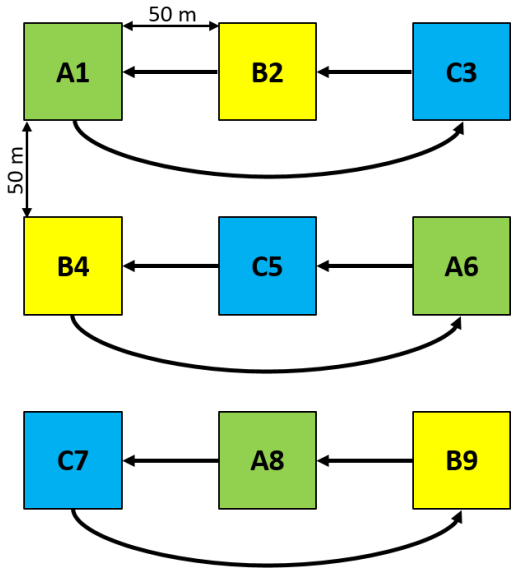

Figura 7. Placement of the traps following a latin square experimental design. A, B and C correspond to different types of trap. Arrows indicate weekly rotation scheme.

2.6. Boll weevil trap wind tunnel bioassays

Due to the characteristic design of the boll weevil trap and unexpected lack of captures in the first field bioassay, wind tunnel bioassays were performed to observe the viability of this trap for *G. platensis*, where 10 insects (5 males and 5 females) were tested following the same methodology and in the conditions previously described. A kit of six cellulose plates impregnated with 9 ml of ethyl phenylacetate was used as lure. Due to the trap's size and odour plume dispersal, only the upper components of the trap (funnel and capture chamber) were used. The experiment was divided in two parts. The first part consisted in the recording of the movement of the insect in the direction of the trap and had a maximum duration of 10 minutes. The second part consisted in the analysis of the weevil's behaviour after establishing contact with the trap. This part ended when the insect entered the chamber of the trap (capture) or abandoned the area of the trap showing disinterest towards it.

2.7. Statistical analysis

Behavioural (nonchoice tests in the wind tunnel) data did not presented normality or homogeneity of variances so nonparametric procedures were used. Kruskal-Wallis ANOVA was used in two situations: compare *G. platensis* intensities of response kept under different diets, and to different compounds. In the last, the Kruskal-Wallis ANOVA was followed by a multiple comparisons of mean ranks (for all groups) procedure in order to identify which compounds showed statistically significant differences relatively to control.

Differences in responses between sexes were analysed with Mann-Whitney U tests.

Spearman's rank correlation coefficient was used to assess the temperature and longevity influence in the behavioural responses.

A significant level of 0,05 was considered for all statistical tests.

All statistical computing was carried out using data analysis software STATISTICA 12 (Dell Inc., Austin, Texas, USA, 2013).

3. Results

3.1. Preliminary wind tunnel assay

Overall results of the preliminary wind tunnel assay are presented in table 4.

Table 4 . Summary table of the preliminary wind tunnel assay results with Gonipterus platensis kept in different feeding diets (n = 10).

Diet	Temperature (°C)	Intensity of response (mean ± s.d.)
Fed	19.4 ± 1.3	708.3 ± 465.9
3-day fast	19.7 ± 1.6	1195.6 ± 1016.3
7-day fast	20.2 ± 0.8	858.6 ± 642.5

Insects submitted to the 3-day fast had, in average, higher intensity of response than 7-day fast and fed insects. Temperature inside the wind tunnel did not seem to affect the intensity of response of the insects. In general, as we can observe in figure 8, males had higher intensity of response than females.

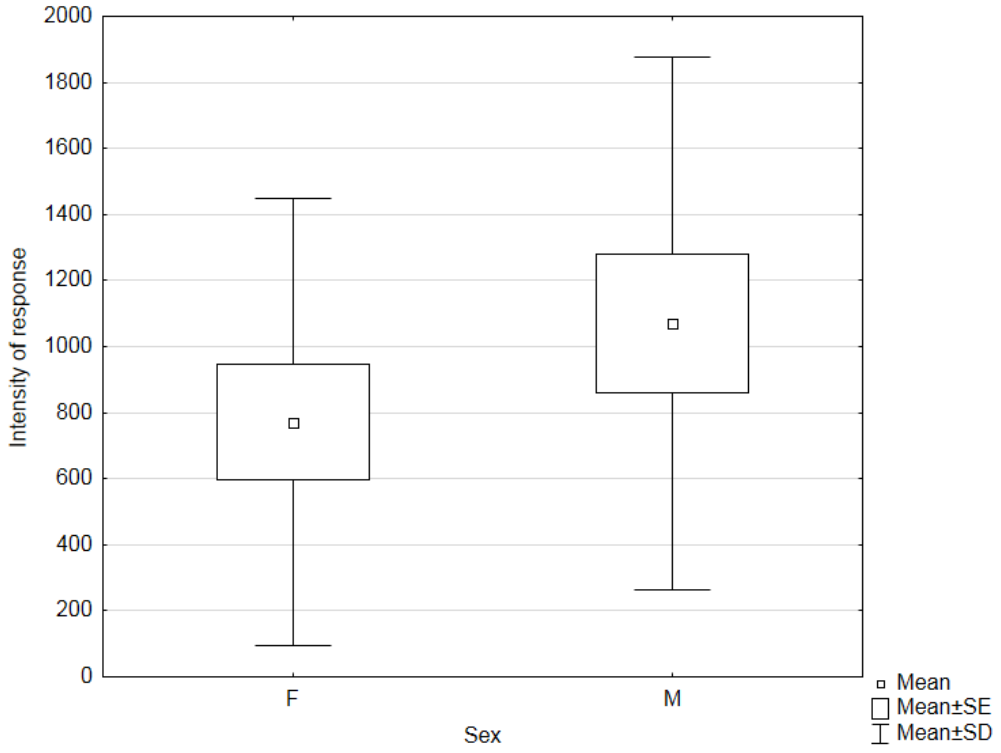

Figure 8. Boxplot of Gonipterus platensis adults intensity of response by sex in preliminary wind tunnel bioassays (n = 10).

Despite the previously mentioned observations, there were no significant differences in intensity of response between insects kept under different diets (Kruskal-Wallis ANOVA, $H_{(2, 30)} = 4.0998$; $P = 0.1287$). Intensity of response was not correlate with temperature ($r = 0.0015$, $P = 0.9939$) and did not differ significantly between sexes (Mann-Whitney U test, $P = 0.3615$).

3.2. Main wind tunnel assays

The results of the main wind tunnel assays for single volatiles are summarized in table 5.

Table 5. Summary table of the main wind tunnel assays with Gonipterus platensis (n = 30).

Compound	Temperature (°C)	Longevity (days)	Intensity of response (mean ± s.d.)
Control	22.0 ± 0.9	24.6 ± 32.4	925.3 ± 928.3
Alpha-phellandrene	23.0 ± 1.0	27.7 ± 28.3	1711.2 ± 1081
Ethyl phenylacetate	23.4 ± 0.5	34.0 ± 25.7	2234.8 ± 749.3
2-phenylethanol	23.6 ± 1.0	34.2 ± 26.3	1691.7 ± 1078.1
Terpinolene	23.5 ± 0.6	33.7 ± 29.2	2031.3 ± 853.5
Benzyl acetate	23.3 ± 0.8	26.9 ± 22.4	1412.6 ± 1054.4
Alpha-terpineol	23.4 ± 0.7	26.9 ± 22.2	1511.2 ± 1142.5
1,8-cineol	23.8 ± 0.6	41.6 ± 23.3	1720.1 ± 1213.7
Alpha-pinene	24.1 ± 0.6	22.0 ± 19.7	1740.6 ± 1220.2
C1	25.6 ± 0.8	16.0 ± 19.4	1967.7 ± 912.7
C2	25.6 ± 0.8	14.6 ± 14.7	1302.6 ± 1065.2
C3	26.5 ± 0.7	16.6 ± 15.8	1636.8 ± 1059.2

All the compounds tested had higher intensity of response than control.

Ethyl phenylacetate had the strongest responses of the single volatiles, followed by terpinolene and alpha-pinene. Combination C1 (ethyl phenylacetate and alpha-pinene, ratio 1:1) had the strongest responses of the combinations tested, followed by C3 (ethyl phenylacetate, terpinolene and alpha-pinene, ratio 1:1:1) and C2 (terpinolene and alpha-pinene, ratio 1:1). Ethyl phenylacetate had the higher responses overall, followed by terpinolene and C1 as evidenced in figure 9.

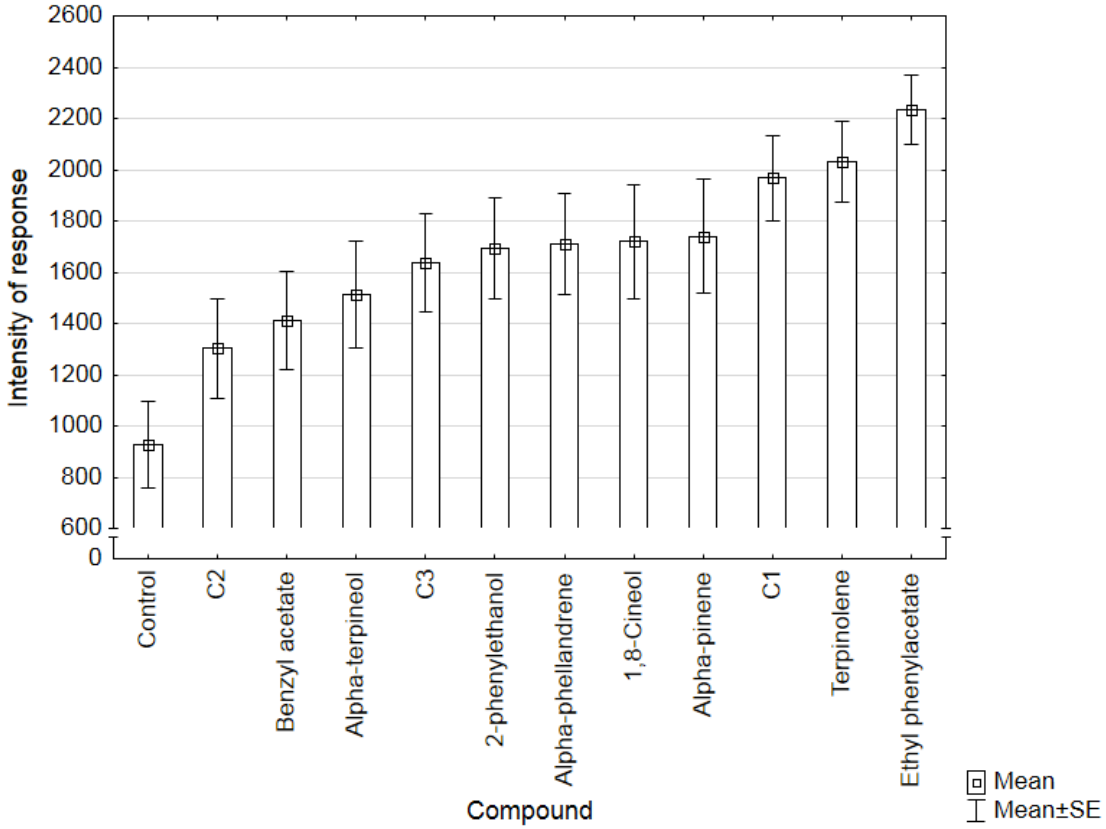

Figura 9. Mean intensity of response of Gonipterus platensis adults for each compound and control in main wind tunnel bioassays (n = 30).

In general, males had higher intensities of response than females (figure 10).

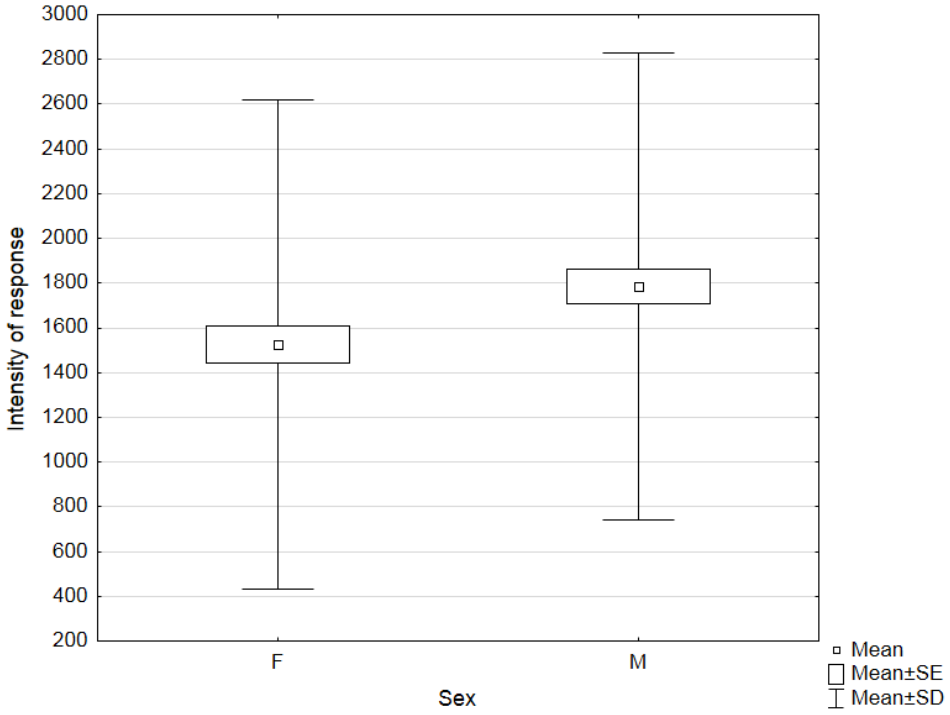

Figure 10. Boxplot of the intensity of response of Gonipterus platensis adults by sex in main wind tunnel bioassays (n = 30).

Intensity of response was not correlated neither with longevity (r = 0.0683, P = 0.1958) nor temperature (r = 0.1010, P = 0.0556).

There were significant differences in intensity of response between the tested compounds in the main wind tunnel assays (Kruskal-Wallis ANOVA, $H_{(11, 360)} = 31.9863$; P = 0.0008). The two single volatiles with strongest responses, ethyl phenylacetate (P = 0.0001) and terpinolene (P = 0.0294), showed significant differences towards the control. Despite C1 was the combination with better results the difference to control was not significant (P = 0.0675).

The overal differences of intensity of response between sexes were significant (Mann-Whitney U test, P = 0.0016), and the compounds with statistically significant differences between sexes were benzyl acetate (Mann-Whitney U test, P = 0.0421), 1,8-cineole (Mann-Whitney U test, P = 0.0225) and C1 (Mann-Whitney U test, P = 0.0381), with males outscoring females (figure 11).

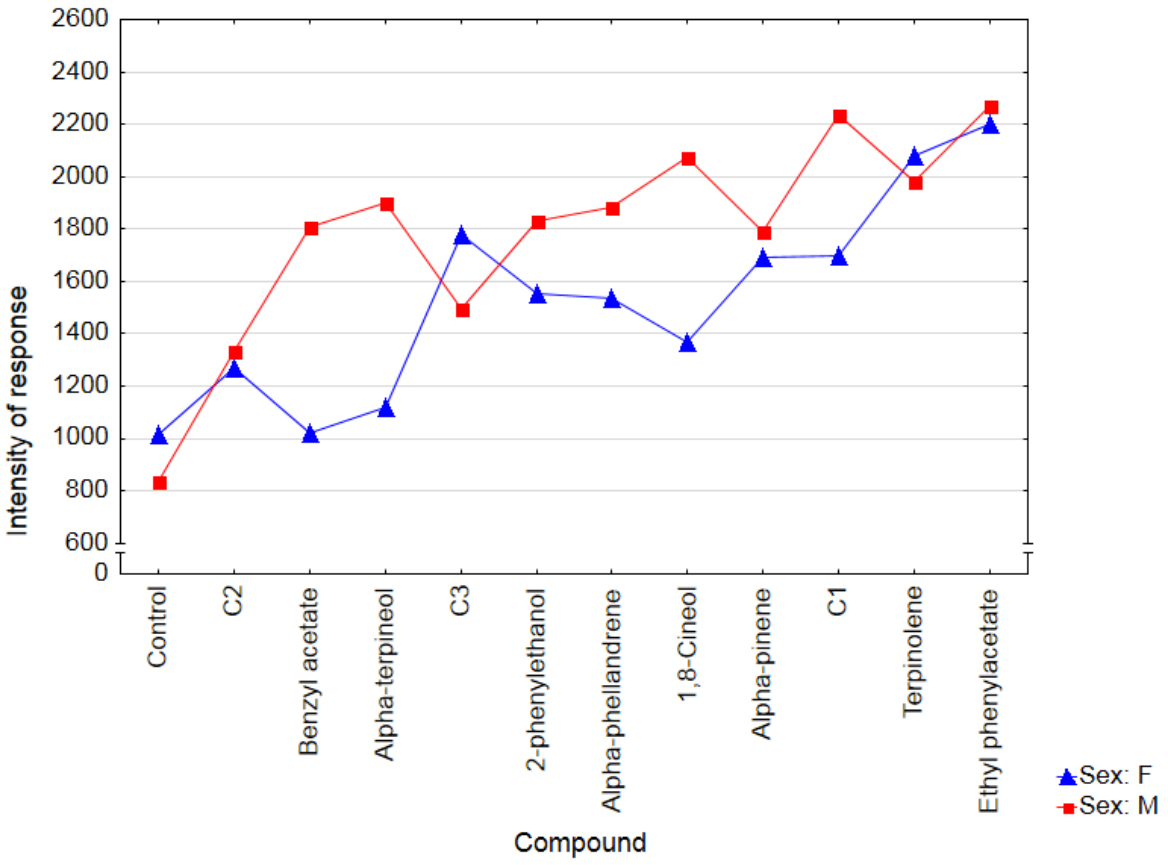

Figure 11. Mean intensity of response of Gonipterus platensis by sex for each compound in main wind tunnel bioassays (n = 30).

3.2. Release rate calculation for field display

The release rates estimated for the different dispensers are presented in table 6.

Table 6. Release rates (g/day and g/min) of the different dispensers for ethyl phenylacetate.

	Release rate	
Dispenser	**g/day**	**g/min**
Cellulose plates	0.1081	7.5069×10^{-5}
Cylindrical plastic recipient	0.0244	1.6968×10^{-5}

The kit of six cellulose plates had a higher release rate than the cylindrical plastic recipient for ethyl phenylacetate.

The release rate of alpha-pinene in the cylindrical plastic recipient is 0.5 g/day (3.4722×10^{-4} g/min) (Sanchez-Husillos et al. 2015).

3.3. First field bioassay

Before the field bioassay, we clearly observed the presence and abundance of *Gonipterus platensis* adults in the sampled area.

The results of the first field assay are summarized in table 7.

Table 7. Gonipterus platensis adults collected in the first field bioassay.

		Insects collected per trap type		
Date (dd/mm/yyyy)	**Temperature (°C)**	**Lindgren**	**Unitrap**	**Boll weevil**
4/8/2015-11/8/2015	18.0 ± 2,6	1	1	0
11/8/2015-18/8/2015	18.3 ± 1.5	0	0	0
18/8/2015-25/8/2015	18.9 ± 1.5	0	0	0

In this assay, no insect was captured during the three weeks of experimentation. Nonetheless, the two insects registered in table 7 were collected from the surface of the traps: one from the Lindgren trap and another from the unitrap.

3.4. Boll weevil trap wind tunnel bioassay

From the twenty adult weevils tested, 75% (eight females and seven males) did not reach the trap or the area close to it and 25% (three males and two females) reached the trap. These five weevils explored the area around and the trap itself but ended up by moving away from it after a less than a minute, never getting inside the capture chamber.

3.5. Second field biossay

Before the field bioassay, we clearly observed the presence and abundance of *Gonipterus platensis* adults in the sampled area.

The results of the second field bioassay are summarized in table 8.

Table 8. Gonipterus platensis adults captured in the second field bioassay.

		Insects captured per trap type		
Date (dd/mm/yyyy)	**Temperature (°C)**	**Colossus**	**Delta**	**Boll weevil modified**
8/9/2015-15/9/2015	17,2 ± 0,7	1	0	1
15/9/2015-22/9/2015	16,9 ± 1.5[a]	0[b]	0[b]	1
22/9/2015-29/9/2015	17,3 ± 1.4[a]	2	1	0
a – Presence of precipitation; b – One trap felt during the week				

In this assay, six insects were captured during the three weeks of experimentation. In the first week two insects were captured: one by a colossus trap and other by a modified boll weevil trap. In the second week, one insect was captured by a modified boll weevil trap. In this visit, two traps (one colossus and one delta trap) were found lying on the ground probably due to the strong winds felt during the previous days. In the third and final week, three insects were captured: two by colossus traps and one by a delta trap. It must be taken into account that the last two weeks were also characterized by the presence of precipitation, which decreases insects activity and therefore affected negatively the lures and traps performances.

4. Discussion

It has been proved that the physiological state of the insect affects its responsiveness to host plant volatiles (Bruce and Pickett 2011). Several studies with different species of beetles shown that a period of starvation increases the response of the insect to host odour in both frequency and strength (Visser 1976; Bartelt et al. 1990; Prokopy et al. 1995; Knolhoff et al. 2006). The choice of the deprivation period was made aiming the maximization of the weevil's response to host odours. In our study, *Gonipterus platensis* adults submitted to a period of starvation did not have significantly different intensities of response from the ones fed. Nonetheless, 3-day fast had better results than the other diets so we decided to apply it on our methodology.

The results achieved with the wind tunnel bioassays confirmed Branco and collaborators (2010) conclusion that terpinolene was responsible for eucalyptus susceptibility to *G. platensis*. They also revealed that the strong antennal responses to ethyl phenylacetate described in Bouwer (2010) and Bouwer et al. (2014) corresponded to high attractiveness. However, this did not happen for the other compounds mentioned in their studies, namely 2-phenylethanol, benzyl acetate, 1,8-cineol and alpha-pinene. 2-phenylethanol and benzyl acetate had high antennal responses but low attractiveness, and 1,8-cineol and alpha-pinene had lower antennal responses but higher attractiveness. Electroantennography results cannot be interpreted as predictions of the behavioural responses of the insect to the tested volatile compounds (Altuzar et al. 2007). A study comparing antennal and behaviour responses of female codling moths (*Cydia pomonella*) to host and non-host plant volatiles, concluded that high antennal responses are not necessarily synonym of high attractiveness, as well as lower antennal responses are not an indicator of low attractiveness because the tested compound may have a behavioural role (Ansebo et al. 2004). Evans and Allen-Williams (1992) in a behavioural study (in olfactometer) with cabbage seed weevil (*Ceutorhynchus assimilis*) and host volatiles stated that a strong antennal response does not mean that the compound is even attractive. A repellent compound may be present in the host but at a dose so low that its effect is neutralized by other compounds of the host odour (Evans and Allen-Williams 1992).

Although it is proven that insects have stronger behavioural responses to volatile's combination than to isolated compounds (Bruce and Pickett 2011), in our results that was not

obvious. Our three combinations (C1, C2, C3 at a ratio of 1:1, 1:1 and 1:1:1 respectively) had worse results than two of their three components (ethyl phenylacetate and terpinolene) when presented isolated to *G. platensis*. This may be due to the component's ratio (natural ratios are highly attractive, while unnatural are not) of the components, which plays an important role in host odour recognition (Altuzar et al. 2007; Webster et al. 2010; Bruce and Pickett 2011). The C1 combination (ethyl phenylacetate and alpha-pinene) had the best results followed by C3 (ethyl phenylacetate, terpinolene and alpha-pinene) and C2 (terpinolene and alpha-pinene) with the worst results. A first analysis of these results may suggest that the presence of terpinolene in equal dose as the other components makes the combination less attractive. Therefore our attempt in finding a synergism between compounds with strong attraction responses but contrasting percentages in the constitution of the *Eucalyptus globulus* leaves (alpha-pinene has a relative high percentage compared with terpinolene and ethyl phenylacetate) failed (at the tested ratios). An interesting result were the significant differences in intensity of response to C1 between males and females, in opposition to what happened with the components individually, ethyl phenylacetate and alpha-pinene. This may be explained by the complex behavioural activity of volatiles involving several factors (context, ratio and dose), or may also reveal sexual dimorphism in the perception and behavioural response to plant volatiles as happens with other beetles (Evans and Allen-Williams 1992; Altuzar et al. 2007). Nonetheless, further research is necessary to understand the cause of this difference.

In summary, from the volatiles tested individually, only ethyl phenylacetate (best performance individually and overall) and terpinolene (second best individually and overall) justified the inclusion in the field bioassays. From the combinations, C1 (ethyl phenylacetate and alpha-pinene; ratio 1:1) had the best results (third best overall) despite not being significantly different from control and having significant different responses between male and female insects.

Although, by the time of the assays, it was possible to confirm the abundance of *Gonipterus platensis* adults in the selected *Eucalyptus globulus* plantation, our results in both field assays did not allowed us to take any conclusions about which kit of lure and trap is the most adequate for a trapping system. None of the traps of the first field bioassay captured any insect, however, two insects were collected on two traps in the first visit, which may indicate that insects were attracted to the traps but were not captured. Studies throughout

the years showed unitrap is an efficient alternative to capture weevils (e.g. sweetpotato weevil, *Cylas formicarius,* and Asian palm weevil, *Rhynchophorus ferrugineus*) (Jansson et al. 1992; Hallett et al. 1999; Reddy et al. 2012; Reddy et al. 2014), so our results were unexpected. Lindgren multiple-funnel trap was already expected to have poor performances, as they had in other studies with weevils (e.g. pales weevil, *Hylobius pales* and pitch-eating weevil, *Pachylobius picivorus*) (Miller and Crowe 2011). Despite boll weevil trap had a poor performance, in line to what happened in a previous study by Jannsson et al. (1992) for sweetpotato weevil, due to the behaviour observed in laboratory we expected to have some captures. That is why, after the first field bioassay, we tested the boll weevil trap in wind tunnel assays to observe the reaction of our weevil to it. In fact, no insect was able to enter the capture chamber of the trap and so we concluded that, despite the design was promising, it would not work for *G. platensis*.

The field assays failure is also related to the background odour from the eucalypts and the tested lures low release rates. Recent studies showed that discrepancy of behaviour between laboratory and field bioassays may be explained by the interaction with background odour (Knudsen et al. 2008; Schröder and Hilker 2008). The background odour of the surrounding vegetation can blur the stimuli of a single source (plant or, in our case, lure). Therefore, the volatile that proved attractive in the absence of the masking odour cannot elicit the expected behavioural response when in presence of it (Knudsen et al. 2008; Schröder and Hilker 2008). Which leads us to the possibility that our release rate might not be sufficiently high to simulate the host and overlap the background odour (Sweeney et al. 2006; Knudsen et al. 2008; Schröder and Hilker 2008).

With that into account and the grabbing capacity of the weevil we decided to use traps that are constituted by materials that difficult/minimize that capacity (colossus traps with Teflon coated panels) and traps with entomological glue (delta and modified boll weevil traps) which reduces the uncertainty associated with insect escapes. In addition, a new dispenser (cylindrical plastic recipients) was used with the C1 combination since it has already been used successfully with alpha-pinene in field trials (Sanchez-Husillos et al. 2015). In the second assay, despite the lower temperatures and precipitation felt in the last two weeks, the results improved. Delta trap seemed not to be the best option for weevils as Reddy et al. (2012) observed in an experiment with the sweetpotato weevil. Colossus and the modified version of boll weevil trap are good alternatives for future field tests.

Despite the number of captures increased, it is still not enough to constitute a viable biotechnical control method. Although there is still a huge margin for improvement in terms of traps, what seemed to be the main problem and should be the priority is the development of a stronger (more attractive) lure that can simulate the natural conditions and compete with the host trees.

5. Conclusion

The main goal of identifying host volatiles to which *Gonipterus platensis* adults are strongly attracted was achieved. Ethyl phenylacetate had the strongest attraction responses overall followed by terpinolene and combination C1 (ethyl phenylaacetate and alpha-pinene, ratio 1:1), which had the strongest responses from tested combinations. Only terpinolene and ethyl phenylacetate had significant differences in intensity of response towards control. At the ratios tested, the combinations did not presented better results than their components isolate, contrary to what was expected.

The second goal of obtaining a primary kit lure-trap viable for capturing *G. platensis* was not achieved, although important contributions for future development can be taken from the field assays. Despite the improvement of the results in the second assay, none of the kits lure-trap tested proved efficient enough to constitute a biotechnical control method. Nevertheless, considering this was the first world attempt to trap this weevil, results can be considered encouraging to continue the research efforts in this control measure.

We therefore suggest the test of more volatiles of the host species of *G. platensis* and more combinations, varying both the number of compounds and ratio. Different wind tunnel/olfactometer methodologies can be used like, for example, exposing the insect to blends (3 to 10 compounds) in non-choice tests and then remove components to discover which are essential for attracting the weevil and which are not, or compare different blends in choice tests to evaluate if exists preference between them (Bruce and Pickett 2011). Nonetheless, the key for a successful lure would be the discovery of a pheromone to use alongside host volatiles, which needs lower release rates to grant similar results (Byers et al. 1985; Schröder and Hilker 2008). In a second instance is important to develop slow-release dispensers for the selected semiochemicals. These dispensers can release a concentration capable of affecting the insect behaviour during the period of its ocurrence (Heuskin et al. 2011). The final step should be the adaptation of existent weevil's traps or development of new traps specific for *G. platenis*, which should take into account our behavioural observations. Based on the results achieved the best model will be a trap with a surface that takes advantage of its grabbing capacity and guides the weevil to a capture chamber constituted by materials (e.g. Teflon) or in a shape that prevents the insect to escape.

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