



Universitat d'Alacant
Universidad de Alicante

Evaluating *Beauveria bassiana* on Red Palm
Weevil Field Management and Behaviour with
Acoustics and GIS

Johari Bin Jalinas



Tesis

Doctorales

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Universitat d'Alacant
Universidad de Alicante

Departament de Ciències del Mar i Biologia Aplicada
Departamento de Ciencias del Mar y Biología Aplicada

**“Evaluating *Beauveria bassiana* on Red Palm
Weevil Field Management and Behaviour
with Acoustics and GIS”**

Johari Jalinas

Tesis presentada para optar al grado de

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APLICADA

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Fdo.

Prof. Dr. Luis Vicente López Llorca

Alicante, 4 de noviembre de 2016

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“Verily, with hardship there is relief”. 94:6



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A mis padres,
a mi familia,
a Farrah Azwa y
a mi hija “Iman Solehah”

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Chapter 1

General Introduction



Chapter 1

General introduction

1.1 Palms

Palms are flowering plants in the monocots order of Arecales that belong to the family of Arecaceae (syn. Palmaceae). There are nearly 200 genera comprising 2400 species of palms which occur across the Neotropics, Africa, and Asia (Govaerts and Dransfield, 2005, Dransfield *et al.*, 2008). Palms only have apical growth. Their development is mostly restricted to its top stem, leaves, and inflorescences at the top and roots (Hodel, 2009). Besides, date palms are distinguished by their fruits and arrangement of leaves (Dransfield *et al.*, 2008; Snow, 2010).

Kingdom: Plantae

Order: Arecales

Family: Arecaceae, Syn. Palmae

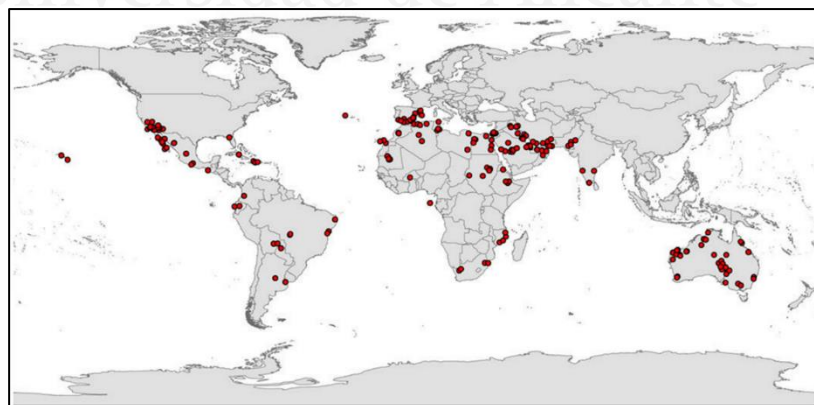


Figure 1.1. The current global distribution of date palm, *Phoenix dactylifera* (Shabani *et al.*, 2012).

Palms inhabit diverse habitat from rainforests (Gomez-Navarro *et al.*, 2009; Snow *et al.*, 2010) to deserts (Walther *et al.*, 2007). Palms display complex spatial patterns of

species distributions and diversity (Eiserhardt *et al.*, 2011). They have been important to humans throughout much of history and many species of palms are domesticated (Clement, 1992). For example, date palm (*Phoenix dactylifera* L.) is one of the oldest fruit crops grown in the arid regions of the Arabian Peninsula, North Africa, and the Middle East (Chao and Krueger, 2007). Besides, date palms are currently grown in many countries in the world (Figure 1.1).

Palms are among the best known and most extensively cultivated plant families for food and other uses (Kakn, 1988; Haynes and McLaughlin, 2000). For example in the tropical region of South East Asia, the palm species, *Arenga pinnata* provides a great number of food products and other uses that benefit local people (Mogea *et al.*, 1991). In addition, palms are also widely used in landscaping. They are easy to transplant even for large specimens because of the root system characteristics and the ability of their trunks to store water and carbohydrates (Hodel 2009). For people in cold climate regions, palms symbolize the tropics (Tomlinson, 2006) and vacations. In many historical cultures, palms were symbols for such ideas as victory, peace, and fertility. In addition, people from sub-Saharan Africa use at least 12 palms species during their spiritual rituals and in traditional medicine (Gruca *et al.*, 2014).

1.1.2 Economic Importance of Palms

Date (*Phoenix dactylifera* L.) and canary palms (*Phoenix canariensis* Chabaud) are of economic importance in Spain (Figure 1.2).

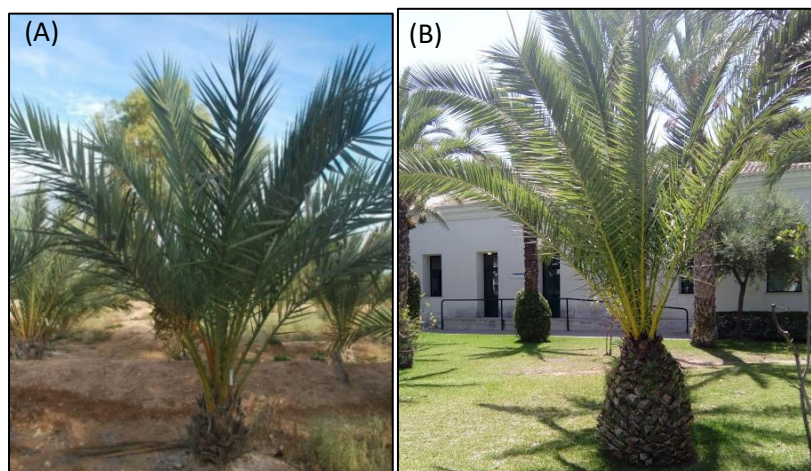


Figure 1.2. Palms in Province Alicante (SE Spain) (A) Date palms (*Phoenix dactylifera*) in Elche. (B) Canary palms (*Phoenix canariensis*) as the main ornamental palms in the University of Alicante.

These species of palms are most abundantly planted in the main tourist resorts at the Mediterranean coastal region from North to South of Spain (Cataluña-Valencia-Murcia-Andalucía) and also the main islands of Spain (Morici, 1998) (Canary and Balearic Islands) including the small island of Tabarca in the province of Alicante close to Alicante City (SE Spain) (Figure 1.3). Both species of palms are possessing sufficient cool or cold hardiness to be suitable for landscaping in subtropical areas including Mediterranean Basin's climate.



Figure 1.3. Date palms (*Phoenix dactylifera* L.) and canary palms (*Phoenix canariensis* Chabaud) are planted along the Spanish Mediterranean coastal region and Islands.

In the province of Alicante, date palms ornate the historic castles such as Castillo de Santa Barbara, Castillo de Altamira in Elche (Figure 1.4) and Castillo de Denia. Governmental buildings, beaches (Costa Blanca Beaches; from Denia to Torrevieja), Universities (University of Alicante and University Miguel Hernandez of Elche), Schools, Main public parks (El Palmeral, Alicante; Hort Del Cura and Municipal Park of Elche), zoological parks (Terra Natura Benidorm and Safari Elche) and main town streets (Ferry *et al.*, 2002) display palms. *Phoenix canariensis* is one of the most grown and appreciated ornamental plants in many bungalow houses and hotels in Alicante.



Figure 1.4. *Phoenix dactylifera* individuals in front of Altamira Castle in Elche.

The city of Elche (Elx) in Alicante Province (SE Spain) is a location of natural history for the date palms (*Phoenix dactylifera* L.). Date palms maintenance to the present represent a living testament to the historical Arabic Berber presence in Spain from the 8th to 17th Centuries (Ferry *et al.*, 2002). The date palms are most abundant and almost distributed throughout the city. Whole palms but especially leaves are used for religious traditional celebrations (Ferry *et al.*, 2002). *Phoenix dactylifera* palms are important for local food and drink industries in the Province of Alicante (Community of Valencia, SE

Spain). Date palms generate local industrial products such as fruits, drinks, and handcraft accessories. By-products from date palms are used in building structures such as roofing, animal feed, and also in several handcraft objects such as baskets and ropes (Figure 1.5). These products are important and part of symbolism for the city of Elche.



Figure 1.5. Exploitation of date palms fruits of *Phoenix dactyifera* (A). Date products (B) and drinks from local plantations (C). By-products from date palms are used in building structures (D) and handcraft accessories (E).

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1.1.3 Cultural Importance of Palms

Spain is the only country in Europe where the main monotheistic religions (Christian, Jews and Islam) coexisted. These three religions described the use of date palms in their holy books (Christian Bible, Jewish Torah and Muslim Quran). This points the cultural importance of palms. Accordingly, the historical date palm grove with more than 240,000 palms at Elche (SE Spain) was selected as a World Heritage Site by UNESCO. Moreover, the date palm grove at Orihuela, is the second largest in the Province Alicante has been declared as a Historic site by the Spanish Government (Giblin-Davis *et al.*, 2013).

The date fruits are suggested as healthy diets for Jews, Christians and Muslims community as well as nutrition practices. The etiolated ‘white’ palm leaves are used as a

religious symbol during major Easter Christian celebrations festivals. As the date palms are considered as “a tree of life”, many palm growers from local people in Community of Valencia produce white leaves from date palms by specific protocols for commercializing the demands of the yearly festival events (Figure 1.6). In addition, the demands of the white palm leaves in the city of palms (Elche) increase in the mid of August during the festival of El Misteri or “Festa d’Elx” which celebrates of the Ascension of the Virgin Mary in the mid of August. During the Holy Week the celebration on Palm Sunday, many people walk in the main streets in the city of Elche holding the white leaves as procession of Jesus triumphant. It is also common usage for Elche people collecting date fruits from public gardens.

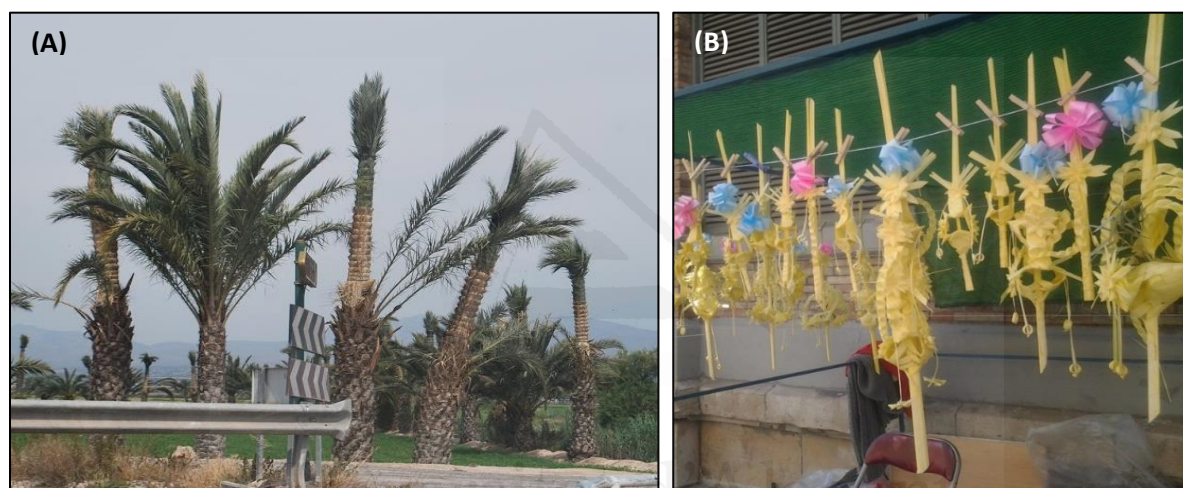


Figure 1.6. Process white leaf production from *Phoenix dactylifera* in Crevillente, Province Alicante (A) White leaves are made with different symbols are sold during the festival and religious celebrations in the city of Alicante (B).

Meanwhile, Jews and Muslims communities in Spain use date fruits as main ingredients in their daily meals. For example, Jewish community in Benidorm (Alicante) serve date fruits during the cultural and social events such as “Chanukah”. The eight day festival of light “Chanukah” begins on the eve of the 25th of the Jewish month of Kislev (December). They eat olives and date fruits to celebrate the triumph of light over darkness.

Muslim people which are mostly immigrants from North of Africa use date fruits as a special ingredients in making a special dish known as “Cous Cous”. This food is usually cooked on Friday (Holy day for Muslim people) at the restaurants in Alicante owned by Moroccan-Tunisian-Algerian Muslims. The demands of the date fruits from

those communities is increasing each year and highly demanded occurring during the fasting month (the holy month- Ramadhan). Muslim people eat date fruits before dawn as the preparation for fasting during the long daily light. Just after the sunset, they break their fasting by eating date fruits. Thus, the local products of date fruits which are available at the local markets are not sufficient and not enough to support the highly demands. Many date fruits are imported from Northern Africa countries such as Egypt, Algeria, Tunisia and Morocco. Recent insect infestations have affected date palms reduced the productions of the dates and date palm based products (El-Juhany, 2010).

1.2 The Red Palm Weevil, *Rhynchophorus ferrugineus*

Rhynchophorus ferrugineus (Olivier) (Coleoptera: Dryophthoridae) known as the red palm weevil (RPW) (Figure 1.7), causes significant damage to a broad spectrum of palms (Arecales: Arecaceae Syn. Palmae) worldwide. RPW weevil was accidentally introduced in Spain in 1996 (Barranco *et al.*, 1996). This invasive weevil has adapted well to the Mediterranean climate (EPPO 2007, EPPO 2008; Mizzi *et al.*, 2009) and has caused severe damage to its host plants (Manachini *et al.*, 2013). In Spain, RPW is a very important pest for date (*Phoenix datylifera* L.) and canary palms (*Phoenix canariensis* Chabaud) (Barranco *et al.*, 2000). There was also a report for RPW infestation to Fan palm (*Washingtonia filifera*) in Spain (Dembilio *et al.*, 2009). RPW damages palm in main touristic resources in Alicante Province (SE, Spain) and other locations in Southern Spain where has caused a negative impact in the landscape (Figure 1.8).

The invasion of RPW is especially dangerous in the city of Elche for the large number of palms present. RPW infestation should not being properly managed properly it would affect the historical palm grove declared world natural and history heritage by UNESCO (Ferry *et al.*, 2002) (Figure 1.9). Thus, RPW infested palms could lead to economic losses in the control managements as well as touristic attractions.

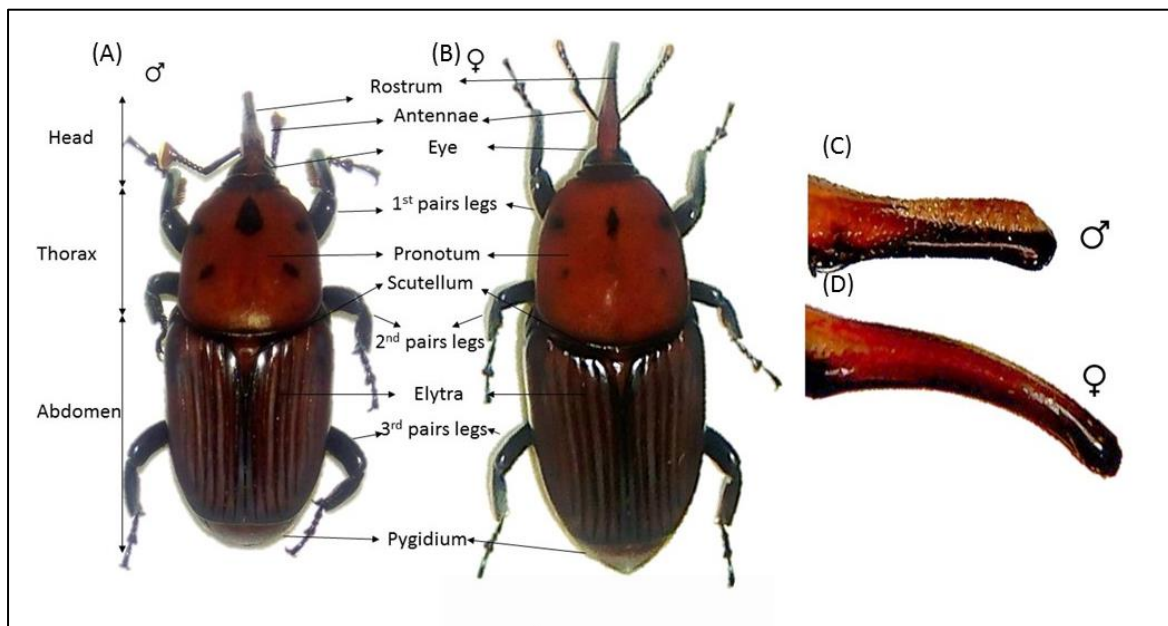


Figure 1.7. Male and female of *Rhynchophorus ferrugineus*. A) Male. Dorsal aspect. B) Female. Dorsal aspect C) Male rostrum. Lateral view. D) Female rostrum. Lateral view.

Class: Insecta

Order: Coleoptera

Superfamily: Curculionidae (Laterille, 1802)

Family: Dryophthoridae (Schoenherr, 1825)

Genus: *Rhynchophorus* (Herbst, 1795)

Species: *Rhynchophorus ferrugineus* (Oliver, 1790)



Figure 1.8. Negative effect on the landscape of RPW infestations in Alicante Province (SE, Spain). A) Tabarca Island. B) Denia. C) Albufereta. Other locations in Southern Spain. D) Cartagena E) Cordoba F) Sevilla and G) Granada.



Figure 1.9. The red palm weevil threatens historic palms. A) General View of the Historical Palm Grove of Elche, UNESCO patrimony of humanity. B) Imperial date palm (ca. 170 years old) planted in Hort del Cura in Elche.

1.2.1 Life Cycle and Biology

The red palm weevil (RPW) is an example of holometabolous insect with complete metamorphosis which has egg, larva, pupa and adult stages (Esteban-Duran *et al.*, 1998; Giblin-Davis *et al.*, 2013) (Figure 1.10). Most RPW stages are spent inside the palm itself. RPW life span varies from 45 to 298 days (El-Mergaway and Al-Ajlan, 2011). This variation is due to humidity (Aldryhim and Khalil, 2003), host plant (Barranco *et al.*, 2000; Faleiro *et al.*, 2003) and temperature (Salama *et al.*, 2009; Peng *et al.*, 2016). *Rhynchophorus ferrugineus* also develops in winter if palm tissue remains intact at the peripheral part of trunks. The insect can complete 3 or 4 generations per year (Abe *et al.*, 2009).

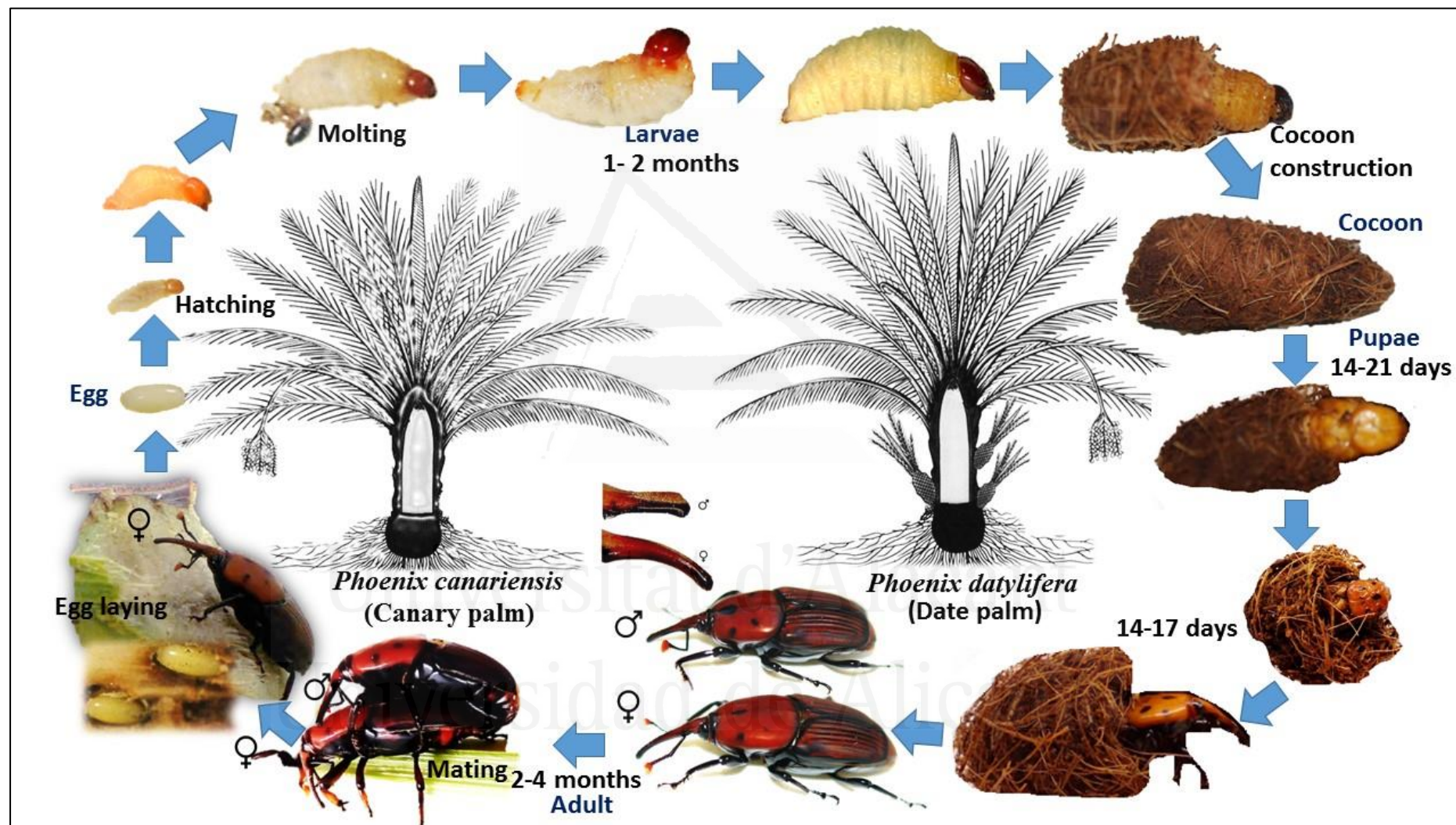


Figure 1.10. Life cycle of the red palm weevil, *Rhynchophorus ferrugineus*.

Each RPW female lays on average 338 ± 37 eggs (Salama *et al.*, 2009) on the date palm crown. The average size of an egg is 2.6 mm long and 1.1 mm wide. Eggs are laid in separate holes on the palm (Ince *et al.*, 2011). Eggs hatch within 2-5 days into legless larvae known as neonate larvae (Kaakeh *et al.*, 1997; Faleiro *et al.*, 2003). The larva is yellowish-white in color, with a brown head. The cuticular head skeleton used to chew food is hard (Moussian, 2010) (Figure 1.11). Lipids are the main component of the inner epicuticle, which permeate the pre-ecdysial cuticle (exocuticle) prior to sclerotization. Aliphatic polar and non-polar lipids in the epicuticle minimise the loss of body water (Hadley, 1981).

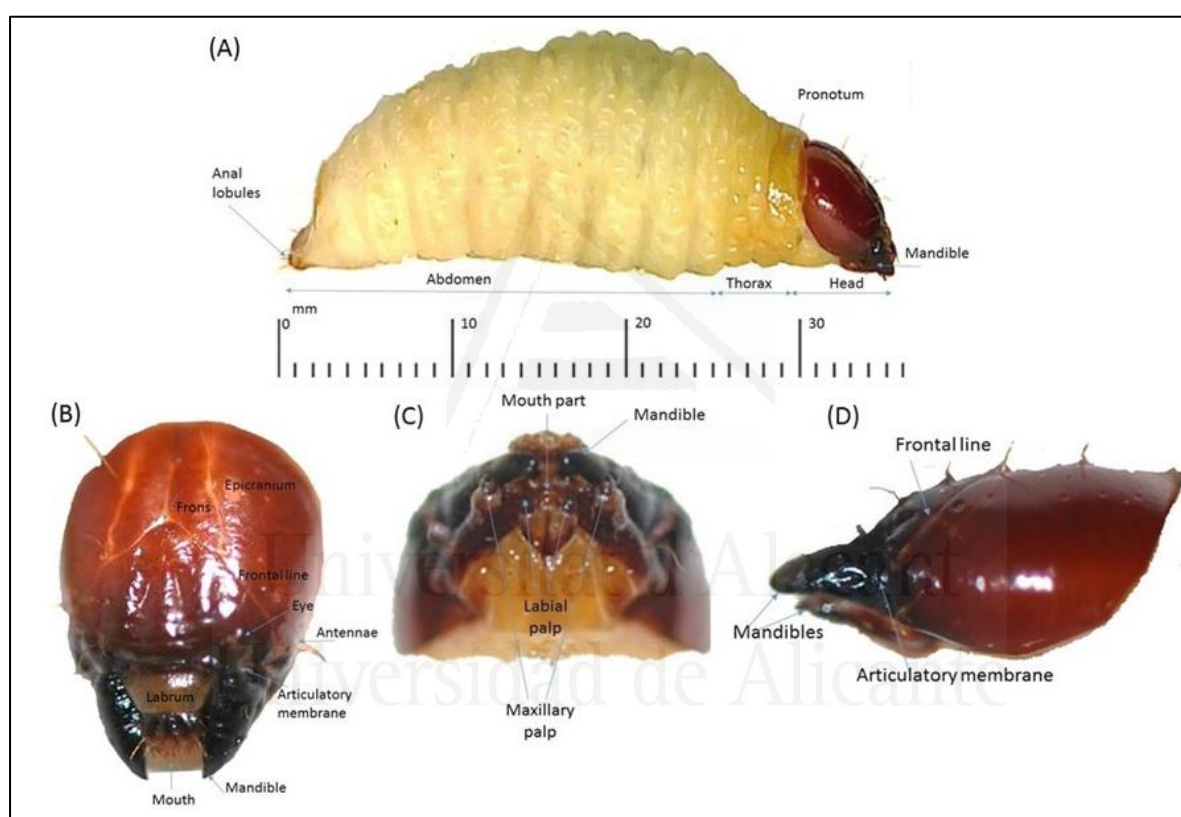


Figure 1.11. *Rhynchophorus ferrugineus* larvae general view (A). Head of a RPW larvae (B) dorsal view, (C) ventral view and (D) lateral view.

The movement of larvae is driven by peristaltic muscular contractions of the body and the assisted by head direction. RPW larvae mouthparts consist of strongly chitinized, mandibles and less heavily sclerotized, maxillae. Mandibles are shaped like pinchers with cutting surfaces and lined with teeth are used to grasp, crush, and cut palm tissues (EPPO, 2007) (Figure 1.12). Meanwhile, maxillae consists of mechanoreceptor and chemoreceptors which sense food (soft palm tissues and palm liquid) before ingestion (Nel

and De Villiers, 1988; Labandeira, 1997). RPW larvae bore tunnels inside the palms towards the crown and apical part of the palms (Trabzuni *et al.*, 2014). The apical meristem of palms is important for leaf and fruit development (Jalinas *et al.*, 2015). As a result, serious damage on all leaves in RPW infested palms could affect photosynthesis and soon the whole host palm growth.

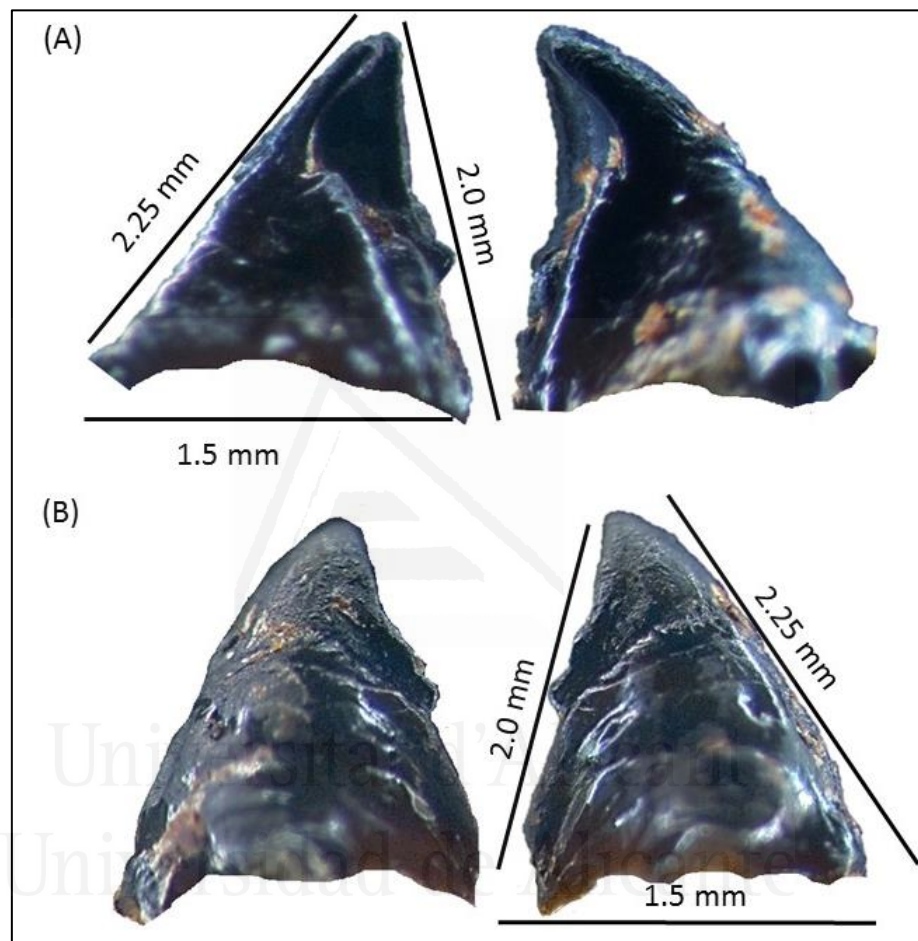


Figure 1.12. RPW larvae mandibles (A) dorsal view and (B) ventral view.

Even though the larval period is the longest inside the palms (varies from 1 to 3 months), RPW larvae do not only eat all the time. The growth of red palm weevil larvae is discontinued due to the sclerotized cuticular parts of the body which limits the expansion of body and head. After the formation of new cuticle of greater surface area, RPW larvae stop their feeding and moving activities. Subsequently, shedding of the old cuticle (ecdysis) takes place. This process is called molting. Several series of molting by RPW larvae happen

before reaching the perfect timing for the RPW pupae stage. Seven larval instars in the RPW larvae have been demonstrated in laboratory experiments (Jaya *et al.*, 2000).

When about to pupate, RPW larvae stop eating and construct a cocoon. They are of oval shape and made from palm fibres with an average length of 60 mm and a width of 30 mm. Head of RPW pupae is bent ventrally with long rostrum. Antennae and eyes are quite prominent. At the thoracic region, the wings and the six legs are developed (Figure 1.13). RPW pupae are first cream in colour. The emergence of RPW adults depend on the environmental temperature (Salama *et al.*, 2009). The pupation process takes an average of 14-21 days and then adult weevils emerge (Figure 1.14). The whole life cycle takes on average 4 months.

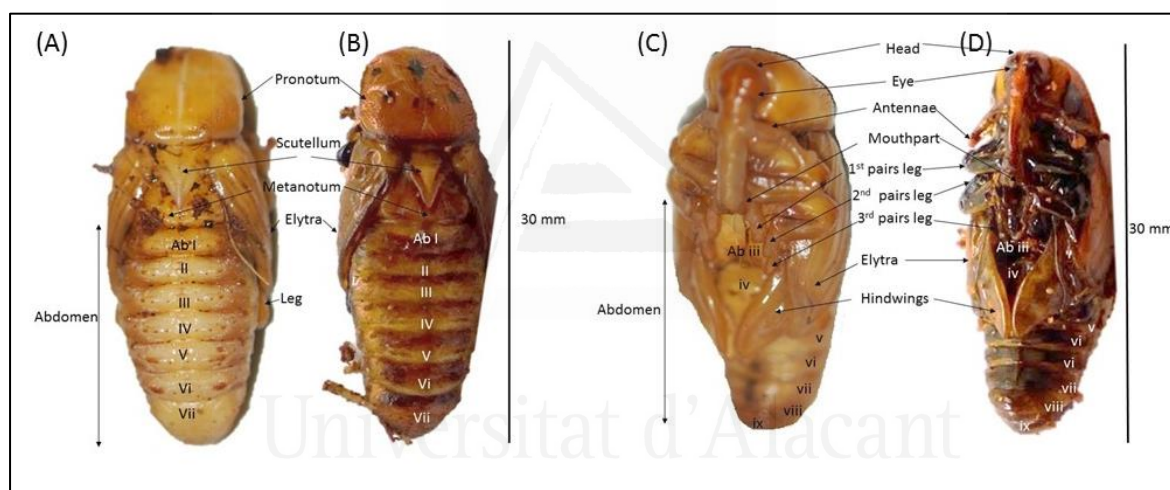


Figure 1.13. A *Rhynchophorus ferrugineus* pupae. Lateral aspect (A-B) and dorsal views (C-D).

After emerging from cocoons, RPW adults can fly long distances (Ávalos *et al.*, 2014). RPW males produce pheromones (Faleiro *et al.*, 1999; Abd-Allah and Al-Khatiri, 2005) to attract females for mating. This RPW aggregation increases with the synergy of the volatiles emitted by palm wounds (Gunawardena and Herath, 1995). The morphological identification of RPW is based on the anatomical structure of RPW adults (EPPO 2007; Wang *et al.*, 2009). Both female and male RPW are a reddish brown cylinder with a long rostrum; reddish brown dorsally, and ventrally their colour is dark brown. The average body size of an RPW male is 19-42 mm (length), 8-16mm (width). The average body size of females is 26- 40 mm (length), 10-16 mm (width) (EPPO 2007). The mouthparts are elongated in the form of a slender snout, which bears a pair of mandibles at the end (Figure 1.15A) and a pair of antennae near the base (Figure 15B). The easiest diagnostic

technique to differentiate male and female is based on snout characteristics (Prabhu and Patil *et al.*, 2009). RPW male snout has a pad of short brownish hairs whereas the RPW female is bare and a little longer (Figure 1.7C and Figure 1.7D).

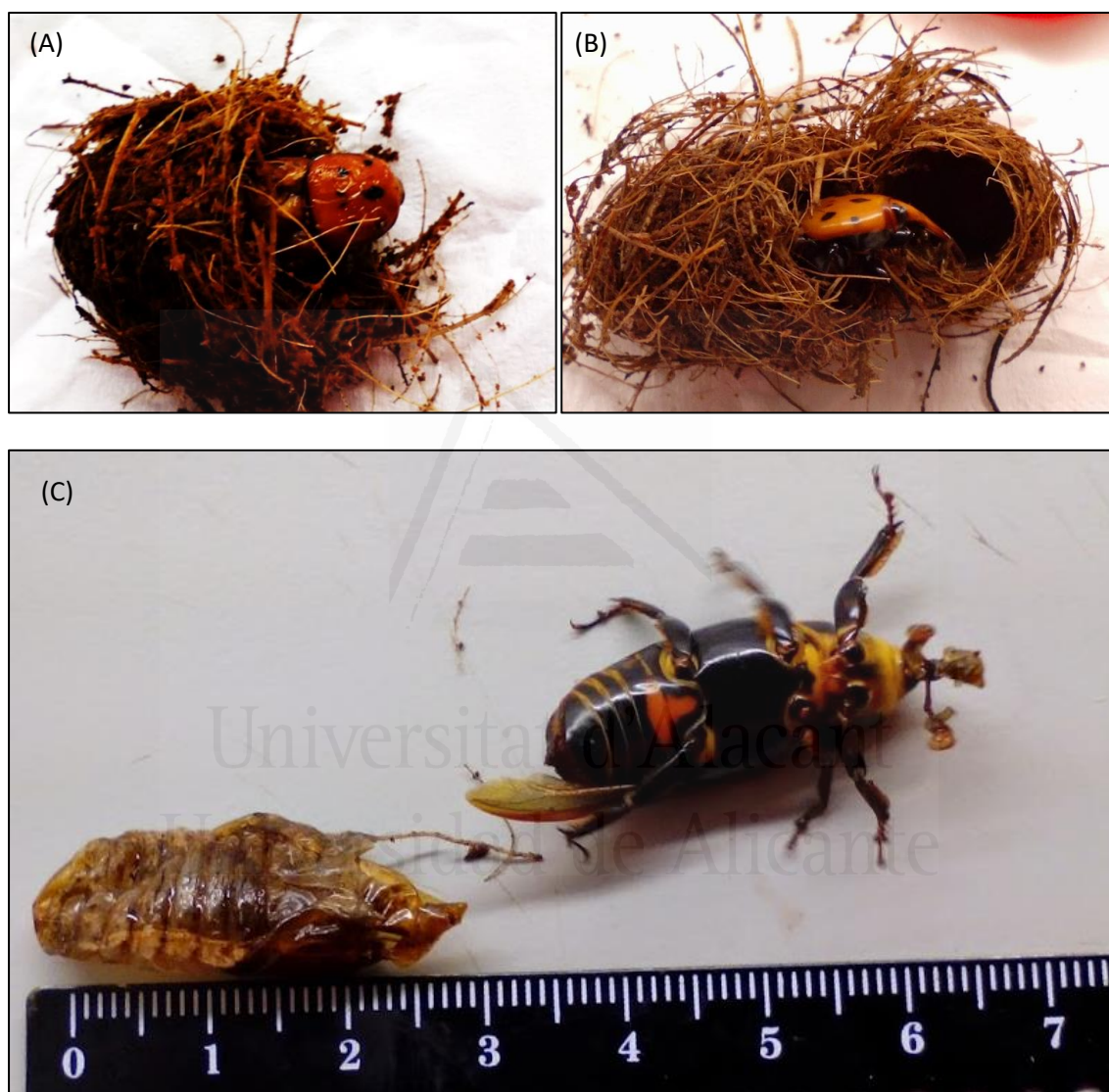


Figure 1.14. Transformation of RPW pupae to the adult phase (Metamorphosis) (A-B). C) The shedding of the old cuticle (eclosion) prior to emergence. Legs, wings and antennae starting to move.

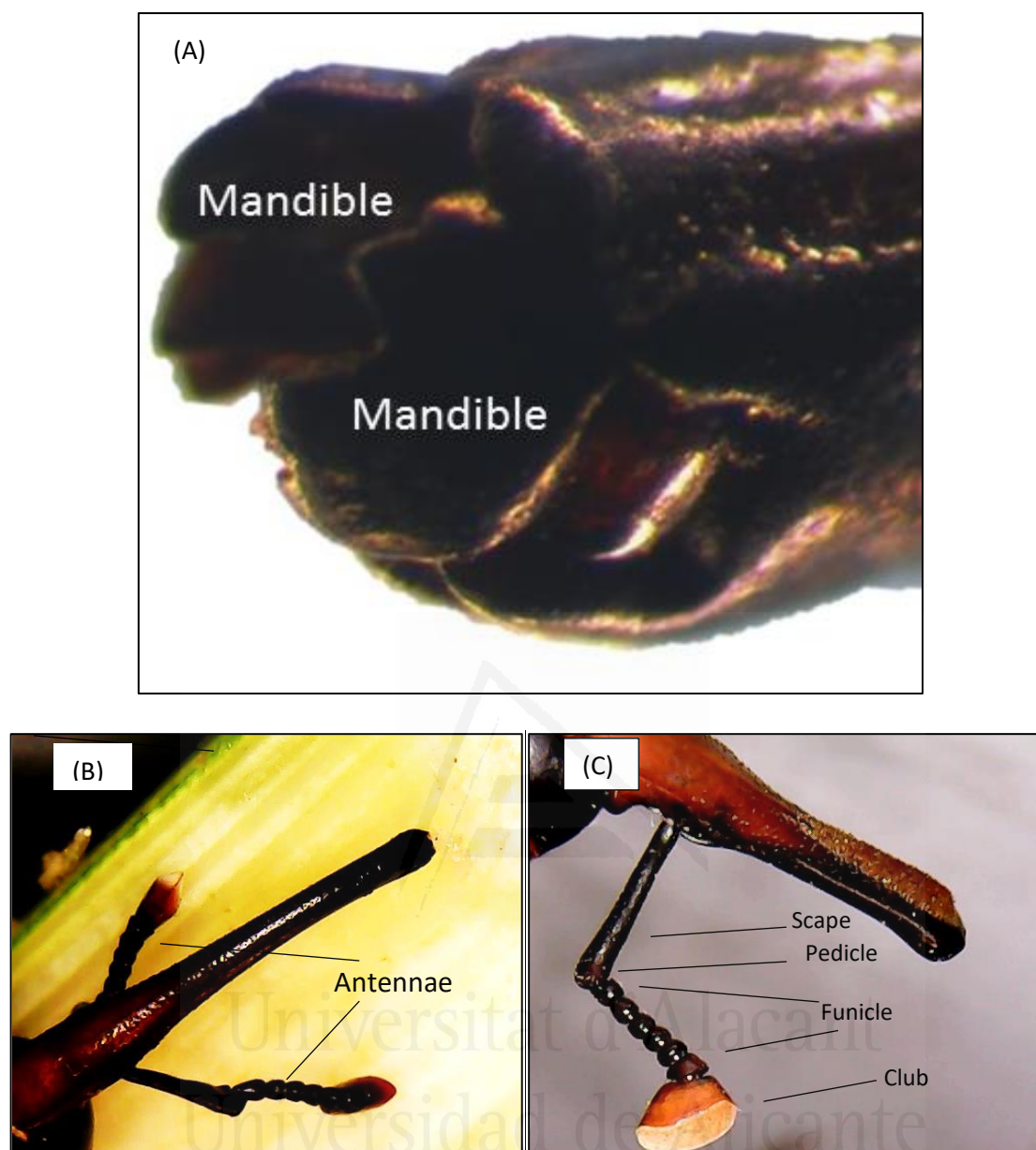


Figure 1.15. Red palm weevil morphology of mandibles and antennae. A) RPW adult mandibles. Lateral view. B) RPW antennae. General view. C) Morphological structure of antennae. Close-up.

1.2.2 Detection of RPW and monitoring of RPW the field

Detection of visual palm symptoms has been used to detect RPW infestation (Güerri-Agulló *et al.*, 2010) (Figure 1.16).

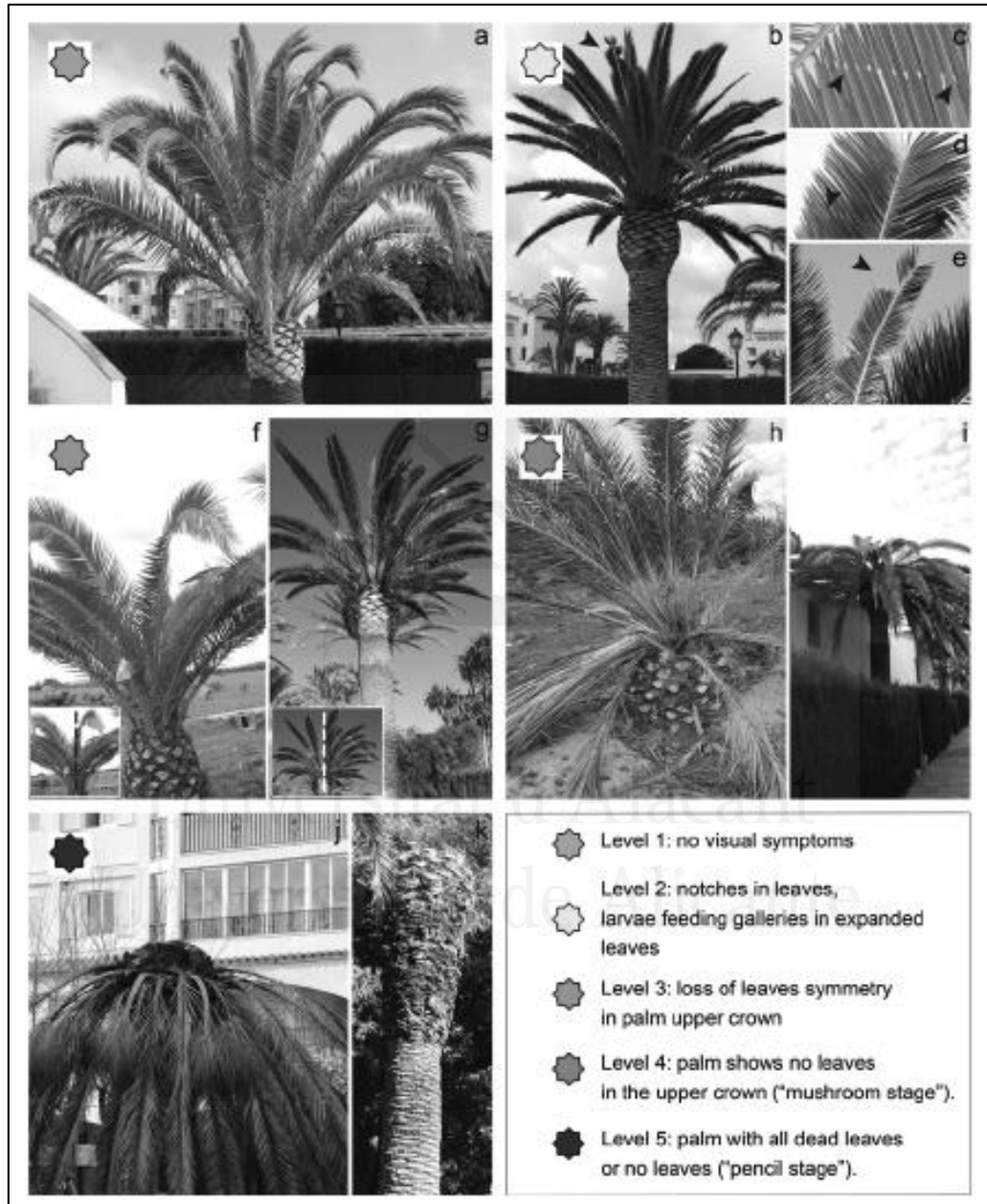


Figure 1.16. Visual scoring of RPW infestation in palms (Güerri-Agulló *et al.*, 2010)

Early damage of RPW on palms could be detected as palm leaf asymmetric growth due to tunnelling activities by RPW larvae near to the palm apical meristem. Moreover, heavy infestation by RPW larvae could stop development of young leaves in the palm meristem

area. Because of the hidden activities by RPW larvae in palm, early detection of palm RPW infestation is certainly difficult. In addition, emerging of damaged parts of immature leaves takes time. In consequence, this method is difficult to apply during early stages of infestation due to the concealed nature of RPW. However it is still applicable and relevant in the field for quickly assessment on the effect of treatments for managing RPW infested palms (Güerri-Agulló *et al.*, 2010).

RPW infested palms can also be detected using dogs for RPW smell detection. Such dogs are highly trained for recognizing the chemical signatures produced by plant feeding (Nakash *et al.*, 2000, Suma *et al.*, 2014). Another technique for detection of RPW infested palms is the use X-ray computed tomography (CT) scanning (Ma *et al.*, 2012). Thermal imaging has also been suggested to detect RPW infected palms (Golomb *et al.*, 2015).

1.2.2.1 Acoustic Detection

Acoustics is the study of sound, including its production, transmission, and effects (Crocker, 2008). Meanwhile, the development of acoustic technology such as acoustic detection equipment, software and hardware are carried out either by an acoustical engineers or personnel specializing in electrical engineering. Acoustic techniques have been used in many applications such as in medical (Xiang *et al.*, 2013), musical (Scheirer, 1998), food industrial (Watson *et al.*, 2012), environmental noise monitoring (Huang *et al.*, 2012) and animal behaviour studies (Fletcher, 2005). The study of sounds produced by living organisms is called bioacoustics (Fletcher, 2007, Gagliano *et al.*, 2012). These studies cover the understanding of all mechanical waves in gases, liquids, and solids including topics such as vibration, sound, ultrasound and infrasound (Crocker, 2008). A biologist who works in the field of acoustics is known as a bio-acoustician and is a specialized biologist in the field of Entomology which applies acoustics for detecting insect activity (Pollack 2000). Causal chain of acoustic emission signal analysis (Eitzen and Wadley, 1984) is paramount for the identification of target insects (Figure 1.17).

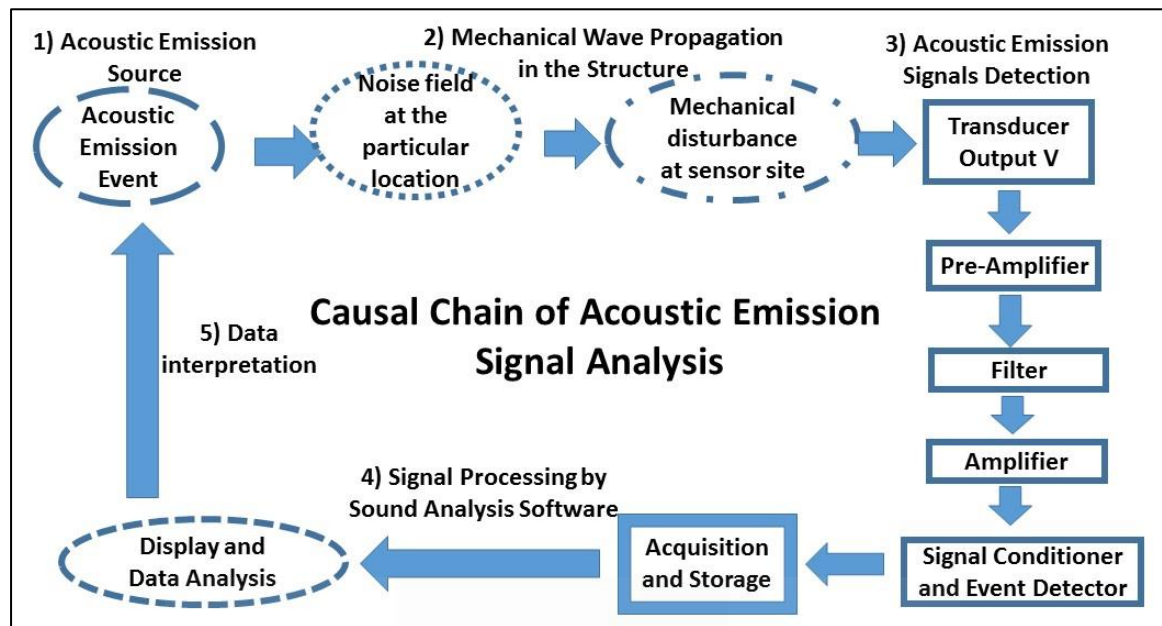


Figure 1.17. Causal chain of acoustic emission signal analysis steps. (Modified from Eitzen and Wadley, 1984)

Insects can generate sound during communication (Ganchev and Potamitis, 2007; Wang *et al.*, 2012), eating (Chesmore and Nellenbach, 2001), flight activity (Hamr and Bailey, 1985, Tsujiuchi *et al.*, 2007) and abdominal movements (Tsujiuchi *et al.*, 2007). Typically, insect feeding and movement sounds inside their host plants or substrates are low in intensity, and they attenuate quickly (Mankin *et al.*, 1996). The physical process of mechanical wave of pressure and displacement through a solid medium as a result of feeding and movement activities by insects is known as acoustic emission (AE) (Mindess, 2004) (Figure 1.18). This wave is an oscillation of matter that transfers energy through a solid material (Zhou and Ichchou, 2010).

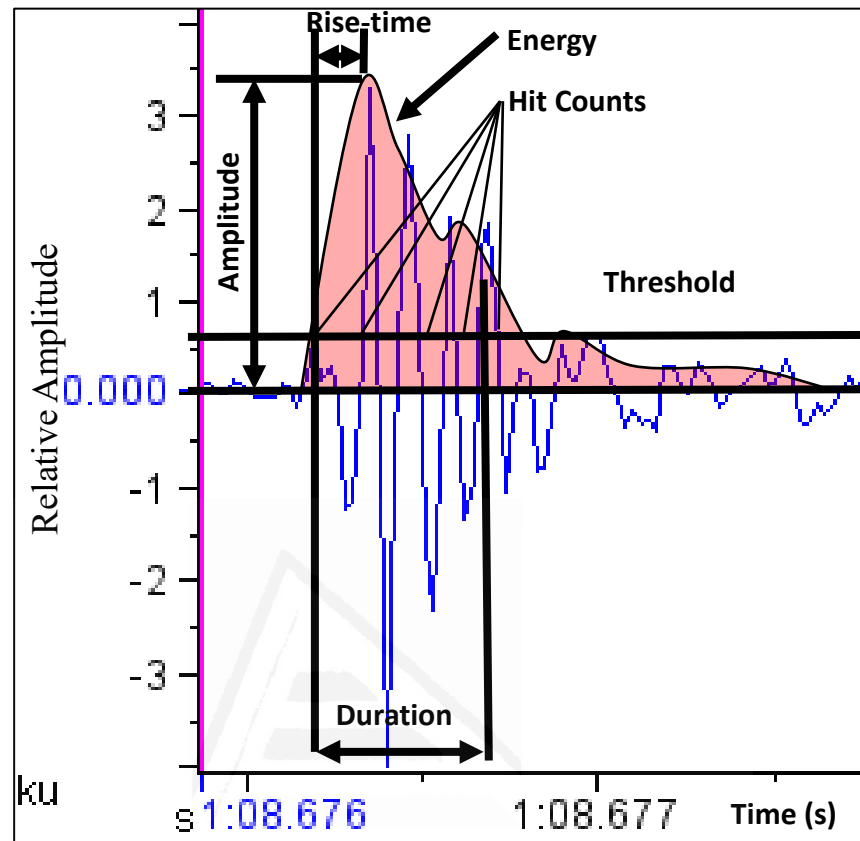


Figure 1.18. Acoustic emission features

This energy is detected by sensors (transducers) attached to the surface of the substrate and the sensors convert a mechanical disturbance to a voltage-time waveform that can be recorded and digitized (Beranek and Mellow, 2012). Recording of sound files could be back played and analysed in the sound analysis software for detecting the specific features of an acoustic signal such as an insect specific activity (Thode and Mellinger, 2010). Many acoustic sensors have been developed for detecting animal sounds (Nam and An, 2007).

Advances in signal processing and computer technology have introduced the possibility of automatically identifying insect species by acoustics. Research investigating techniques for automatically identifying Orthoptera with time domain signal processing and artificial neural networks are available (Chesmore and Nellenbach, 2001). The technique is widely applicable to insect pests especially those with hidden activities such as stored-product pests and urban pests (Mankin *et al.*, 2010).

Thus, applications of acoustics had been integrated in insect pest management strategies (Mankin and Gutiérrez, 2012). Detection of RPW sounds was found promising for early detection and monitoring of coconut tree (Siriwardena *et al.*, 2010; Martin *et al.*, 2015), canary palm (Herrick and Mankin, 2012; Jalinias *et al.*, 2015), date palm RPW infestations (Soroker *et al.*, 2004; Mankin, 2011a; Hetzroni *et al.*, 2016) (Figure 1.19). In addition, an acoustic sensor was developed and the sensor was specified for detecting RPW acoustic activity (Rach *et al.*, 2013). Portable acoustic devices built for detecting RPW infestations in the field are also available (Siriwardena *et al.*, 2010).

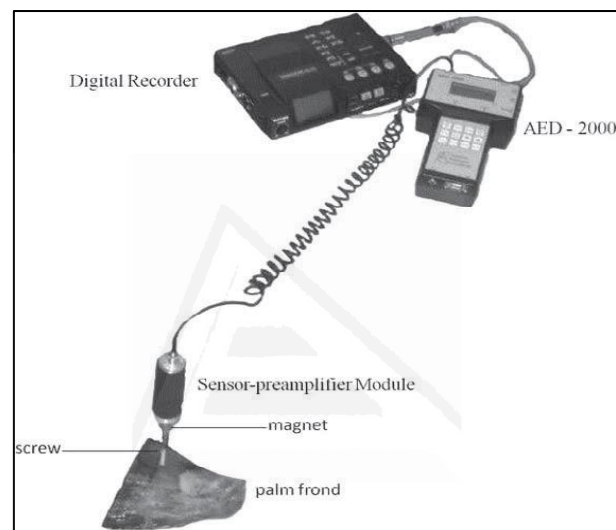


Figure 1.19. Experimental set-up for acoustic recording of *Rhynchophorus ferrugineus* early instar feeding in laboratory (Mankin *et al.*, 2012)

A mathematical method to automatically detect acoustic activity of RPW in palm offshoots (Potamitis *et al.*, 2009) and computer based programming (Hetzroni *et al.*, 2016, Potamitis *et al.*, 2009; Pinhas *et al.*, 2008) for detecting hidden activities of RPW were made available. New approach of signal analyses for detecting RPW have also been conducted (Mankin, 2011). Manual and automated acoustic RPW detection has been used to scan young palm trees (Hetzroni *et al.*, 2016). Acoustic signals have also been to evaluate the effect of chemical insecticide Imidacloprid against the termite, *Coptotermes formosanus* shiraki (Isoptera: Rhinotermitidae) (Osbrink and Cornelius, 2013).

1.2.2.2 GIS methods

Geographic information systems (GIS) are often described as "technology for storing, retrieving, processing and displaying spatial data (Dminic *et al.*, 2010). GIS represent more than a tool for spatial data handling. GIS offers the possibility of analysing the landscape in great detail on the basis of digital aerial photographs and satellite maps (Figure 1.20). The ability to overlay and analyse multiple spatial data layers provides the opportunity to explore the spatial nature of insect populations (Sciarretta and Trematerra, 2014).

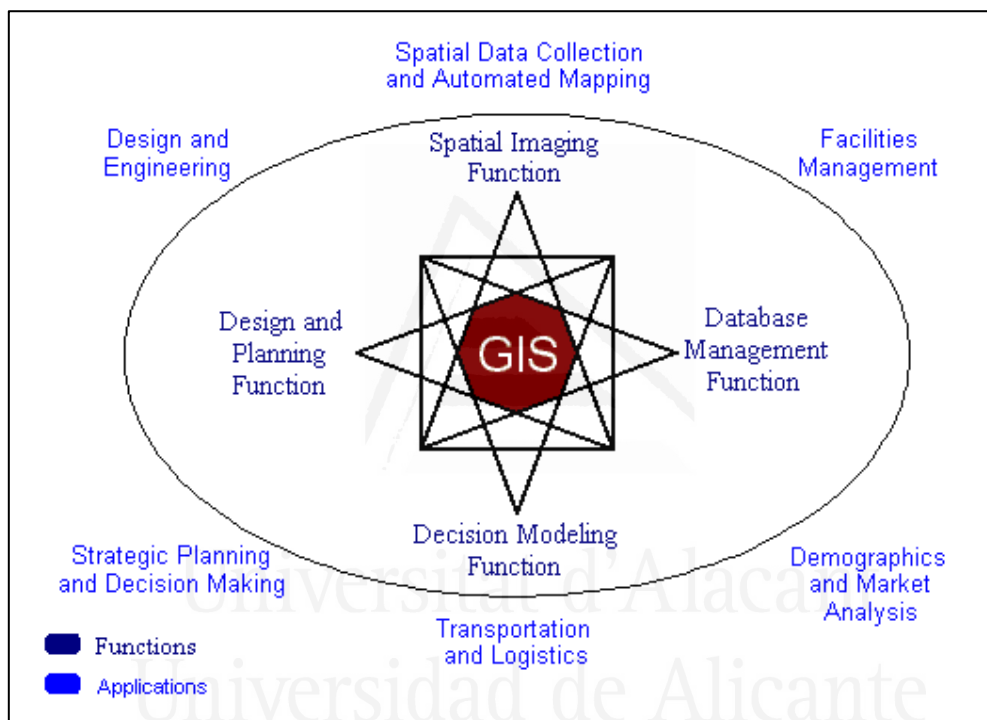


Figure 1.20. Geographic information systems (GIS) functions and applications (Mennecke *et al.*, 2000).

The rapid development of this technique has allowed more effective methods for detecting and monitoring insect pests, as well as understanding their spatial dynamics (Dminic *et al.*, 2010). In addition, geostatistical methods in GIS have also been used to characterize spatial variability in western (*Diabrotica virgifera virgifera* LeConte) and northern (*Diabrotica barberi* Smith & Lawrence) corn rootworm adult emergence patterns (Ellsbury *et al.*, 1998). Specific models such as Semivariograms in geostatistical methods of GIS could produce contour density maps of adult populations in the field, based on grid sampling of actual emerging adult populations (Ellsbury *et al.*, 1998). GIS has also been

used to estimate the short-term risk of forest insect outbreaks such as spatial-temporal spread behaviour of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae) (Bone *et al.*, 2013).

Processes that influence the spatial activity of insects include population growth (reproduction, mortality) and dispersal (immigration, colonisation and emigration). For example, aggregations of insects influencing the behaviour of other individuals through the emission of pheromones (Jiang *et al.*, 2002) or attraction towards plant volatiles (Carroll *et al.*, 2006).

Distribution patterns of insect pests documented at the field scale would have immense practical value through increased efficiency in sampling activities, more precise targeting of management activities and reduced operational costs for farmers and land managers. Geostatistical tools for the study of insect spatial distribution have been used in the integrated insect pest management of orchard and vineyard pests (Sciarretta and Trematerra, 2014).

1.3 Management and control

Education and research training are very important to tackle the issues of RPW invasion and infestation. Regular training and education concerning RPW infestation, prevention, early detection and curative methods for farmers or landscapers play an important role in managing RPW (<http://glenbiotech.es/servicios/cursos/>). Although, legislative control in Spain is very important especially the movement of planting materials between and within countries should be organized and controlled properly. Several techniques to control RPW are used in Spain such as RPW adult trapping by pheromone traps, biological control using fungi (Dembilio *et al.*, 2010a; Güerri-Agulló *et al.*, 2010 and 2011; Ricaño *et al.*, 2013, Jalinás *et al.*, 2015), and nematodes (Dembilio *et al.*, 2010b) and Chemical control (Cabello *et al.*, 1997; Llácer *et al.*, 2012; Dembilio *et al.*, 2010b and 2015). Palms in RPW most infested areas are mechanically removed to avoid pest spread (Figure 1.21).



Figure 1.21. Pulping of RPW infested palms.

1.3.1 Pheromone Traps

Insects such as the red palm weevil (RPW) rely on chemical signals called semiochemicals. They serve as a form of language that helps to mediate in their interactions. Semiochemicals can be divided into two groups based on who "sends" a message and who "receives" it: 1) **Pheromones** are chemical signals that carry information from one individual to another member of the same species. These include sex attractants, trail marking compounds, alarm substances, and many other intraspecific messages. 2) **Allelochemicals** are signals that travel from one animal to some member of a different species. These include defensive signals such as repellents, compounds used to locate suitable host plants, and a vast array of other substances that regulate interspecific behaviors.

Allelochemicals can be classified into three groups based on who "benefits" from the message:

1. **Allomones** benefit the producer by modifying the behaviour of the receiver such as a repellent or a defensive compound that deters predation.
2. **Kairomones** benefit the receiver but disadvantage the producer such as an odour that insects use to find its host.

3. **Synomones** benefit both sender and receiver such as plant volatiles that attract insect pollinators

RPWs locate palm trees by means of plant volatile cues and use an aggregation pheromone to coordinate a mass-attack (Antony *et al.*, 2016). Aggregation behaviour of RPW adults is caused by a pheromone emitted by RPW males which attracts both male and females (Abbas *et al.*, 2006). This aggregation is increased and synergized by the hostplant produced kairomone. Kairomones are chemical volatiles that attract RPW to host plants. Thus, RPW pheromones, ferrugineol (4-Methyl-5-nonanol) and ferrugineone (4-methyl-5-nonanone) have been identified and chemically synthesized and applied for RPW mass trapping in the field (Gunawardena and Herath, 1995).

Pheromone and food baits using palm petioles act synergistically for capturing RPW adults (Kaakeh *et al.*, 2001) (Figure 1.22). Aggregation pheromones are easy to handle, environmentally friendly, cheap, and safe to humans and mammals. However, their use is not an adequate tool to control RPW populations especially in zones of high infestations. In addition, RPW adult monitoring using pheromone traps in the palm plots in the city of Elche is restricted. This is because RPW from infested palms in the surroundings of Elche such as Santa Pola (South) and Crevillente (North) could migrate and invade the Historic Palm Grove with thousands of monumental palms.

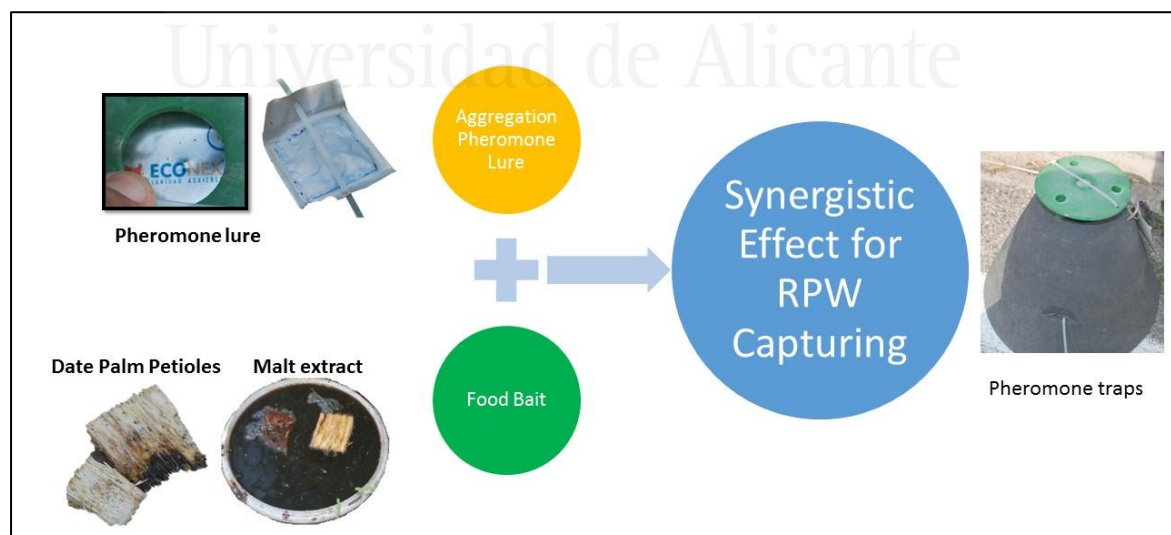


Figure 1.22. Example of pheromone and food baits used by Tragsa S.L for mass trapping of RPW adults in Elche (SE Spain).

1.3.2 Chemical treatments

Chemical control is the most common method to control RPW infestations in the host plants (Kaakeh, 2006). There are two main strategies for chemical control treatments which are: 1) curative (Dembilio *et al.*, 2015) 2) preventive treatments (Llácer *et al.*, 2012). Curative chemical treatments involve injection of insecticide in the trunk of RPW infested palms (Porcelli *et al.*, 2013) and spraying the palm (Figure 1.23). Meanwhile, preventive treatments only involve chemical spraying of the whole palms. Spraying and drenching of Imidacloprid (Confidor) oil dispersions (OD) as a drench in preventive and curative treatments against RPW is a common practise in *Phoenix canariensis*, *Washingtonia robusta*, *Washingtonia filifera* and *Trachycarpus fortunei* palms (Llácer *et al.*, 2012).



Figure 1.23. Chemical control of the RPW. A) Insecticide spraying on a canary palm. B) Injecting insecticides in the stem of date palm .

Source (A): http://www.algarvevida.com/nature/the_palm_tree_killer_en.

RPW infested old leaves and offshoots coming out from the trunk of palms are pruned. This creates wounds on the trunk and treated using high volume air compressor sprayer with the insecticide. Trunk injection of insecticides uses abamectin and imidacloprid applied by crown spray, stipe and frond injections with lethal doses to larvae of *R. ferrugineus* (Dembilio *et al.*, 2015) Several insecticides (diazinon, dimethoate, chlorpyrifos, carbaryl, oxamyl, carbofuran, imidacloprid, fipronil and methidathion) and application methods (soil application, trunk injections, sprays and a combination of trunk injections and sprays) have been tested on *R. ferrugineus* infesting *Phoenix canariensis* and

Phoenix dactylifera (Hernandez Marante *et al.*, 2003 and Porcelli *et al.*, 2013). Despite, chemical treatments show positive effects to control RPW infestations, the heavy usage of chemical insecticides could turn RPW resistant to these compounds.

Palms are frequently used as ornamentals in gardens and recreation areas. Therefore, large applications of chemicals specially spraying and drenching pose a threat to human health and the environment (non-targets) (Al-Samarie and Akela, 2011).

1.3.3 Biological control

Biological control is an important tool for ecologically friendly crop protection against pests that consists in using an organism (predator, parasitoid, or pathogen) to reduce the population density of its target pest. Biological control agents can be applied in classical biological control, augmentative biological control and conservative or natural control strategies (Batra, 1982; Hajek, 2004; Hajek and Delalibera, 2010).

1.3.3.1 Predators and parasitoids

Anisolabis maritima (Dermaptera: Carcinophoridae), has been recorded in Saudi Arabia as a predator on eggs and newly hatched larvae of the red palm weevil, *Rhynchophorus ferrugineus* (Al-Dosary, 2009). This potential biological control agent has not been tested in the field.

The parasitoid mite, *Rhynchopolipus rhynchophori* has also been tested on RPW. This parasitoid is able to suppress the population density of RPW within two weeks by sucking their body fluid in laboratory studies (Abdullah, 2009). Porcelli *et al.*, (2009) discovered two species of mites; *Centrouropoda almerodai* and *Uroubouella marginata* (Acari: Uropodina) on RPW in Malta island.

1.3.3.2 Entomopathogenic nematodes

Several isolates of entomopathogenic nematodes (EPN) infect adults of the Red Palm Weevil under both laboratory and field conditions (Elawad *et al.*, 2007; Saleh *et al.*, 2010). *Heterorhabditis bacteriophora* has displayed contagious distribution and caused

insect mortality (Salama and Abd-Elgawad *et al.*, 2010). In addition, the attraction behaviour of the EPNs *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* to the RPW under simulated natural conditions shows their infective potential (Santhi *et al.*, 2016).

1.3.3.2 Bacteria

Pseudomonas aeruginosa, a facultative pathogen infect the digestive tract of RPW larvae. This lead to mortality of RPW larvae in a laboratory test (Banerjee and Dangar, 1995). Several *Bacillus* species such as *Bacillus sphaericus*, *B. megaterium* and *B. laterosporus* have also been isolated from RPW infested area in Egypt and tested in the laboratory (Salama *et al.*, 2004). These bacterial isolates produce spherical endospores and crystalline endotoxin that can cause mortality on RPW larvae in the laboratory (Salama *et al.*, 2004). Isolates of *Bacillus thuringiensis* have also been tested on RPW in the laboratory (Mahmoud *et al.*, 2011)

1.4 Entomopathogenic fungi

Entomopathogenic fungi are the most abundant antagonists of insects. They were among the first organisms to be used for the biological control of pests. More than 700 species of fungi from around 90 genera are pathogenic to insects. Most are found within the Deuteromycetes (=mitosporic fungi) and Entomophthorales (Scorsetti *et al.*, 2007). Entomophthoralean fungi (Zygomycota: Zygomycetes) are a unique group of entomopathogens that cause natural epizootics among aphid populations (Wraight *et al.*, 1993; Vega and Kaya, 2012). *Beauveria bassiana* (Balsamo-Crivelli) and *Metarhizium anisopliae* (Metschnikoff) are two of the most important entomopathogenic fungi currently used against a wide range of arthropod, mainly insect (Vega and Kaya, 2012). Meanwhile, *Metarhizium acridum* is an entomopathogenic fungus which specifically infects and kills grasshoppers, locust and other insects in the Orthoptera (Guerrero-Guerra *et al.*, 2013).

B. bassiana is well characterized in respect to pathogenicity to several insects and they have been used as agents for the biological control of agriculture pests worldwide (Faria and Wright, 2001, Güerri-Agulló *et al.*, 2011; Vega and Kaya, 2012). *B. bassiana* is a common soil-borne fungus that occurs worldwide (Asensio *et al.*, 2005). It attacks a wide

range of both immature and adult insects. The extensive list of hosts includes such important pests as red palm weevils, white flies, aphids, grasshoppers, termites, Colorado potato beetle, Mexican bean beetle, boll weevil, cereal leaf beetle, bark beetles, lugus bugs, chinch bug, fire ants, European corn borer, codling moth and Douglas fir tussock moth (Zimmermann, 2007; Toledo *et al.*, 2007; Güerri-Agulló *et al.*, 2011; Vega and Kaya, 2012). There are many different strains of the fungus that exhibit considerable variation in virulence, pathogenicity and host range (Vega and Kaya, 2012; Ricaño *et al.*, 2013).

A broad range of *B. bassiana* strains have been isolated from a variety of insects worldwide that are used for agricultural purposes. The fungus infects RPW eggs, larvae and adults in the laboratory (Gindin *et al.*, 2006; Dembilio *et al.*, 2010a), semifield (Jalinas *et al.*, 2015) and field conditions (Güerri-Agulló *et al.*, 2011). Dried conidia of *Beauveria bassiana* solid formulation reduced the incidence of *R. ferrugineus* under field conditions in Spain (Güerri-Agulló *et al.*, 2011). An interesting feature of *Beauveria* is the high host specificity of some isolates. This thesis of PhD used *B. bassiana* 203 isolate (Figure 1.24). This isolate is from naturally infected RPW in Elche (SE Spain) (Güerri-Agulló *et al.*, 2010) which caused high mortality during the assessment of RPW adult semifield simulation for the field experiments in comparison to other *B. bassiana* isolates (Ricaño *et al.*, 2013). The fungi still grows actively on the solid formulation and persists for almost a year (Ricaño *et al.*, 2013). Numerous registered mycoinsecticide formulations based on *B. bassiana* are used for control of insect pests worldwide (Faria and Wraight, 2007).

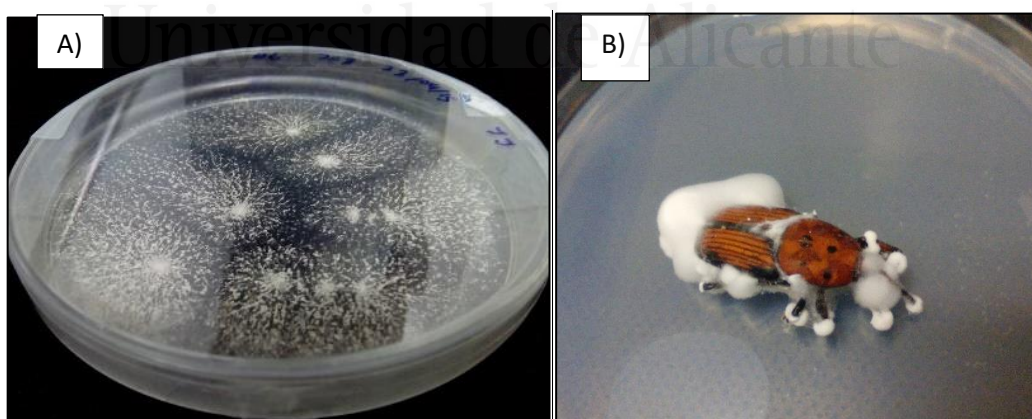


Figure 1.24. *B. bassiana* 203 isolates on CMA media (A). An infected RPW adult killed by the *B. bassiana* (B).

1.4.1 The life cycle of *B. bassiana* and mode of infection

The filamentous fungus *Beauveria bassiana* belongs to a class of insect pathogenic deuteromycetes (“imperfect” fungi/mitosporic fungi).

Kingdom: Fungi

Division: Ascomycota

Class: Sordariomycetes

Order: Hypocreales

Family: Clavicipitaceae

Genus: *Beauveria* (Vuill, 1912)

Species: *Beauveria bassiana* (Bals.-Criv.) Vuill

Beauveria bassiana has a dimorphic mode of growth and in the absence of the specific insect host, *B. bassiana* passes through an asexual vegetative life cycle that includes conidia germination, filamentous growth and sporulation (conidiophores). Molecular and cultural studies have provided insight regarding the phylogenetic position and reproductive biology of *B. bassiana*. An rDNA phylogeny by Sung *et al.*, (2001) supports a single evolutionary origin of *Beauveria* within the subfamily Cordycipitoideae of the Clavicipitaceae. *Cordyceps* species produce *Beauveria* anamorphs (asexual stage), clearly demonstrating that some *Beauveria* species are sexual such as *C. bassiana* (Li *et al.*, 2001; Sung *et al.*, 2006)

B. bassiana also colonize endophytically petioles date palms (*Phoenix dactylifera* L.) (Gómez-Vidal *et al.*, 2006) which is the main host for RPW. In the presence of its host insect for example RPW, *B. bassiana* switches to the pathogenic life cycle (Figure 1.25).

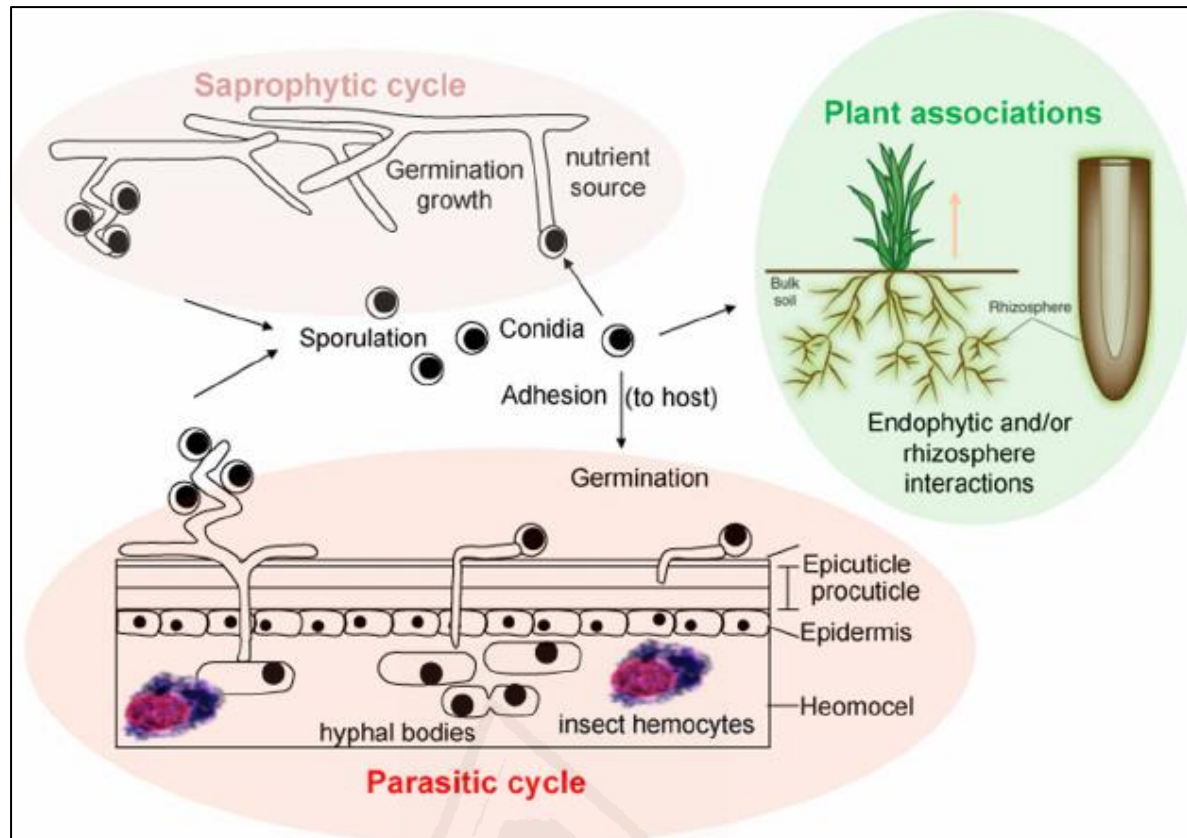


Figure 1.25. Overview of the multitrophic life cycle of *B. bassiana* (Ortiz-Urquiza *et al.*, 2015).

The mode of infection of RPW by *B. bassiana* has been studied in detail by Güerri-Agulló *et al.*, (2010) using Scanning Electron Microscopy (Figure 1.26). Insect cuticle is mainly composed of chitin and protein surrounded by wax, lipid layer or fatty acids (Moussian, 2010). The cuticle of insects functions both as an exoskeleton and as a barrier between the living tissues and the environment (Andersen, 1979; Noh *et al.*, 2016). Structural lipid is insoluble in organic solvents and it can only be liberated from cuticle by destructive oxidation (Wigglesworth, 1970).

Fungal pathogenesis mainly starts with the secretion of cuticle degrading enzymes. Known cuticle degrading enzymes are chitinases, proteases and lipases. *B. bassiana* for instance produces extracellular enzymes able to degrade to the major components of insect cuticle, e.g., endoproteases, aminopeptidases, carboxypeptidases, lipases, esterases, chitinases, and N-acetylglucosaminidases (Leger *et al.*, 1986; Pedrini *et al.*, 2007)

Conidia germinate on the surface of the cuticle and hyphal tubes penetrate insect's integument directly and differentiating appressoria (Clarkson and Charnley, 1996; Güerri-Agulló *et al.*, 2010) (Figure 1.26h). These are specialised structures for ensuring adhesion of fungi to the host. They are common to fungal pathogens of plants such as *Magnaporthe oryzae* (Geoghegan and Gurr, 2016) and animals such as nematophagous fungi (Lopez-Llorca and Claughner, 1990) or entomopathogenic fungi like *B. bassiana* (Kumar *et al.*, 1999). Host penetration by entomopathogenic fungi requires the secretion of cuticle-degrading enzymes such as chitinases (El-Sayed *et al.*, 1993) and proteases (Dias *et al.*, 2008; Bye and Charnley, 2008). During the conidia germination and penetration of insect cuticle layer, entomopathogenic fungi produce proteases (Dias *et al.*, 2008; Bye *et al.*, 2008) and chitinases (El-Sayed *et al.*, 1993). When having penetrated the cuticle, the fungus alters its growth morphology and produces hyphal bodies, which circulate in the haemolymph and proliferate by budding (Clarkson and Charnley, 1996; Vega and Kaya, 2012). These secondary metabolic peptides of entomogenous fungi, such as destruxins and efraeptins, are considered to be important virulence determinants (Bandani *et al.*, 2000). They are believed to suppress the host's immune system. The exact mechanism(s) have not been fully explained but studies show that destruxins, inhibit haemocyte activity. The death of the insect results from a combination of factors: mechanical damage resulting from tissue invasion, depletion of nutrient resources and toxicosis (Bandani, 2008).

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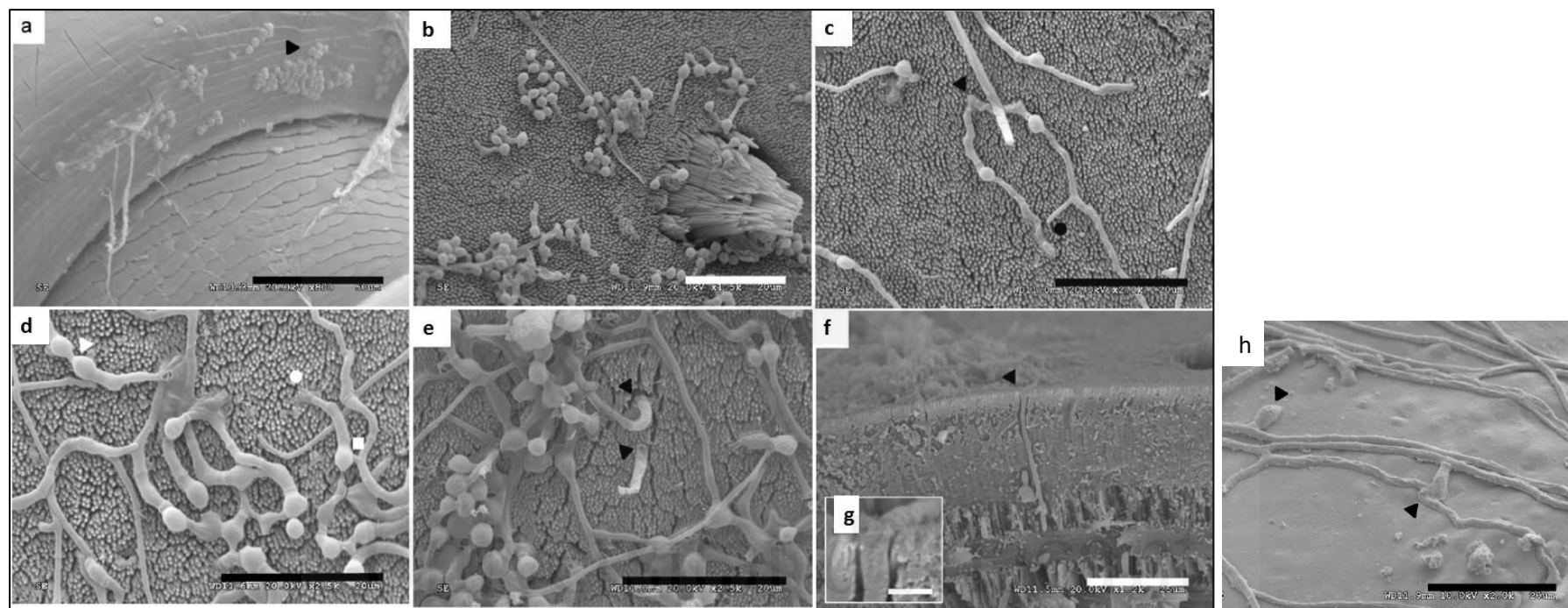


Figure 1.26. Process of infection by *B. bassiana*. a) Ungerminated conidia. Bar= 50 μ m. b) Germinated conidia (“germlings”) of *B. bassiana* on a *R. ferrugineus* cuticle zone with cuticle ornamentations. Bar = 20 μ m. c) *B. bassiana* hyphal fusion (arrow head) and attempted hyphal anastomosis (circle) on *R. ferrugineus* cuticle. Bar = 20 μ m. d) Close-up of germlings showing conidial fusion (arrowhead), conidium-hypha fusion (solid square), and appressorium adhering to cuticle (solid circle). Bar = 20 μ m. e) Hyphae penetrating (arrow heads) *R. ferrugineus* cuticle. f) A hypha (arrowhead) penetrating the cuticle 1 DAI. Bar = 25 μ m. (g) Detail of penetration point. Bar = 2 μ m. h) Close-up showing two intercalary appressoria (arrows). Bar =20 μ m (from Güerri-Agulló *et al.*, 2010).

1.4.2 Volatile organic compounds (VOCs)

Volatile organic compounds (VOCs) are carbon based solids and liquids that enter the gas phase by vaporizing at 20°C and 0.01 kPa (Pagans *et al.*, 2006). VOCs appear as intermediate and end products of various metabolic pathways and belong to numerous structural classes such as mono- and sesquiterpenes, alcohols, ketones, lactones, esters (Korpi *et al.*, 2009). Fungi produce various volatiles (Crespo *et al.*, 2006; Müller *et al.*, 2013) involved in communication with living environment. Volatile organic compounds (VOCs) could mediate for instance in fungal-insect interactions (Yanagawa *et al.*, 2009).

1.4.3 Avoidance of fungi by insects

Fungal VOCs also are being investigated for their insecticidal and repellent activities. For example, the VOCs produced by *Muscodor* spp., including nitrosoamide, have been shown to kill insects (Strobel *et al.*, 2010). *Muscodor vitigenus* produces naphthalene, formerly used in “mothballs”, and functions as an effective insect repellent (Daisy *et al.*, 2002). In addition, VOC profiles have been correlated with the potential of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* to reduce termite populations (Hussain *et al.*, 2010a). The volatile profile of the virulent fungi contained n-tetradecane and alkenes, while the non-virulent ones contained many branched alkanes (Hussain *et al.*, 2010b).

The ability of insects to detect and respond to entomopathogenic fungi within the order Hymenoptera has been widely assessed. There are reports of avoidance of fungi by species within the Coleoptera (Ormond *et al.*, 2011), Isoptera (Yanagawa *et al.*, 2009), Hemiptera (Meyling and Pell, 2006) and Orthoptera (Thompson *et al.*, 2007) (Figure 1.27). In addition, VOCs from fungi show neurotoxicity effects in *Drosophila melanogaster* (Inamdar *et al.*, 2010). A potential function of fungal VOCs in the interactions between *B. bassiana* and RPW is discussed in the **Chapter 4** of this PhD thesis manuscript.

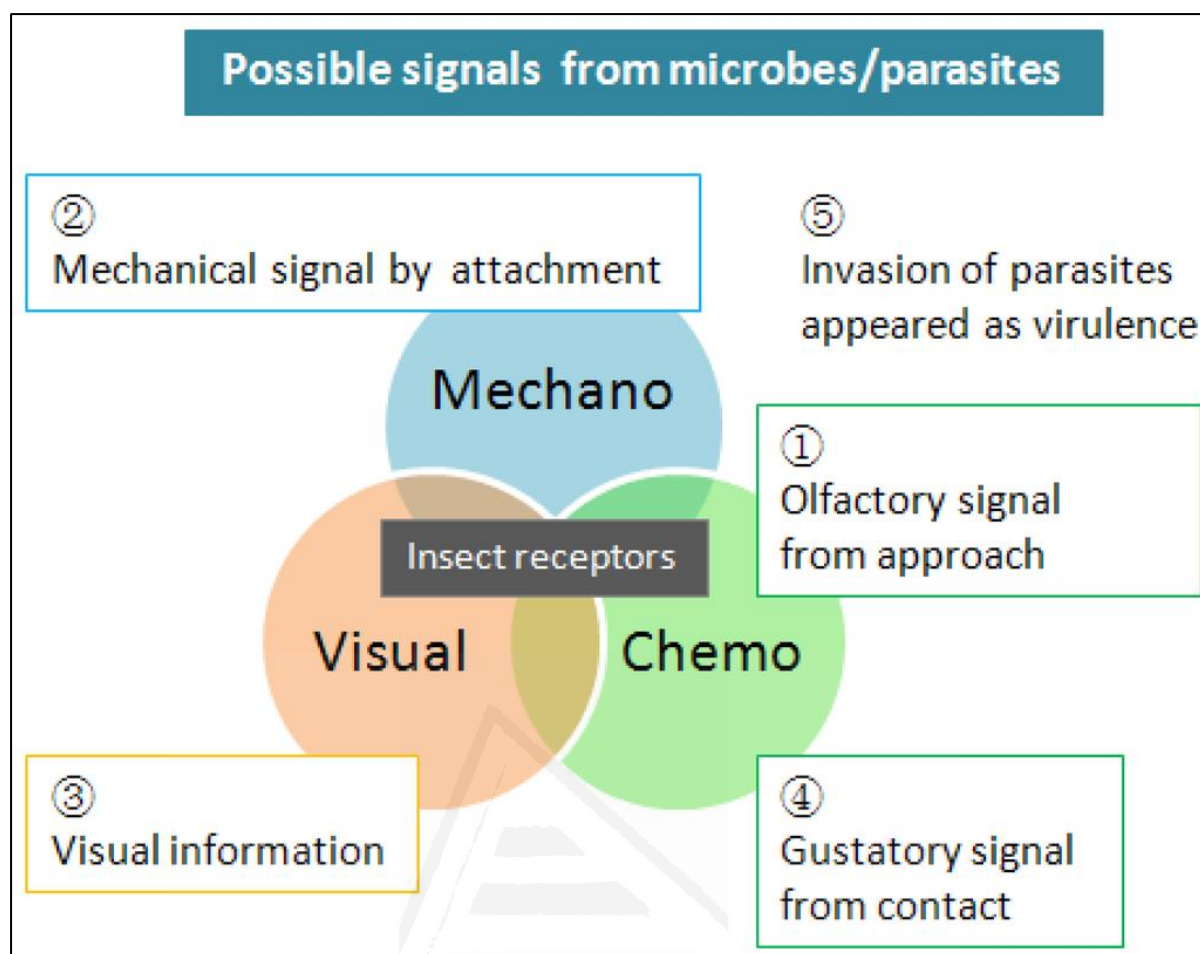


Figure 1.27. Host insects probably find an invasion of pathogens by smell or behavior of infected individuals (Zhukovskaya *et al.*, 2013).

1.4.4 VOCs detection

The increasing scientific interest in the biochemistry, physiology, ecology and atmospheric chemistry of fungal VOCs has led to the development of a variety of systems for the collection and analysis of fungal volatiles. There are many techniques for trapping VOCs. Liquid absorption is a common technique for enriching compounds in reactive liquids like solutions of dinitrophenylhydrazine (DNPH) (for aldehydes), acetyl acetone (for formaldehyde) or aqueous carbonate solutions (for organic acids). These procedures combine trapping and derivatization of the target compound. Solid sorbents play an important role for the trapping of VOCs (Table 1). They overcome some serious disadvantages of liquid absorbents for trapping VOCs (Salthammer and Uhde, 2010).

Table 1.1. Properties of some solid sorbents for trapping fungi VOCs (modified from Salthammer and Uhde *et al.*, 2010).

Type	Structure	Surface area (m ² /g)	Products	Desorption
Inorganic	Silica gels	1–30	Volasphere, Florisil	Solvent
	Molecular sieves	500–800		Solvent
	Aluminum oxides	~300	Alumina F1	Solvent
Carbon based	Activated Charcoal	800–1200		Solvent
	Carbon molecular Sieves	400–1200	Carbosieve, Ambersorb, Spherosorb, Carboxen	Solvent/ Thermal
	Graphitized carbon blacks	12–100	Carbotrap, Carbopack, Carbograph	Thermal
Porous polymers	Styrene, divinylbenzene or polyvinylpyrrolidone polymers	300–800	Porapak Q/N, Chromosorb 106/102,	Thermal/ solvent
	Phenylphenylene oxide polymers	20–35	Tenax	Thermal
	PU-Foams			Solvent

Gas Chromatography- Mass Spectrometry (GC-MS), due to its powerful separation and highly sensitive detection capabilities, is the main method for the detection and analysis of fungal VOCs (Matysik *et al.*, 2009). VOCs can then be identified using a library or database of mass spectra, or by comparison of retention times and spectra with those of known standards.

1.5 Aims of this work

Biological control agents such as mycopesticides are the most important component in integrated pest management (IPM) for reducing RPW infestation or incidence. Our knowledge on the effects of *B. bassiana* to control red palm weevil (RPW) invasion and infestation in the field has progressively been improved. Thus, the use of chemical insecticides for control RPW infestations must be reduced. Although the effects of *B. bassiana* on RPW palm damage have been assessed using visual palm damage, the effects of *B. bassiana* on RPW larval activities inside the host palms still remain unknown.

The general aim of this PhD thesis is to understand the effects of *B. bassiana* on RPW larval behaviour inside RPW infested palms using acoustic detection. RPW is the main threat to palms. This has economic, cultural and historical consequences in European and Worldwide Natural Heritage and resources. Therefore, the aim of this thesis is also to explore acoustics to monitor the spatial and temporal activity of the RPW in restricted areas for using pheromones traps such as the city of Elche. In addition, the potential of VOCs from *B. bassiana* has also been investigated to find alternative ways to protect the palms from RPW attack.

Summarising, the specific aims of the present work are the following:

1. Improve early acoustic detection of RPW larvae in infested host palms (*Phoenix canariensis* and *Phoenix dactylifera*)
2. Explore the potential of acoustic methods to assess the progress of *B. bassiana* infection of RPW larvae within host palms.
3. Evaluate acoustics methods to assess the effect of imidacloprid on RPW activity in the field
4. Evaluate the RPW management treatments in the field using GIS methods based on RPW acoustic activity
5. To identify VOCs from entomopathogenic fungi as new sustainable tools for controlling RPW infestations.

This PhD Thesis is based on three research works.

Chapter 2. Johari Jalinas, Berenice Güerri-Agulló, R. W. Mankin, R. Lòpez-Follana and Luis Vicente Lòpez-Llorca. 2015. Acoustic assessment of *Beauveria bassiana* (Hypocreales: Clavicipitaceae) effects on *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) larval activity and mortality. *Journal of Economic Entomology*. 1–10. DOI: 10.1093/jee/tov023

Chapter 3. Johari Jalinas, Berenice Güerri-Agulló, R. W. Mankin Jose Emilio Martínez-Pérez, and Luis Vicente Lòpez-Llorca. monitoring red palm weevil field management using acoustics and GIS. Manuscript submitted.

Chapter 4. Johari Jalinas, Frutos C. Marhuenda-Egea, and Luis Vicente Lòpez-Llorca. *Beauveria bassiana* (Hypocreales:Clavicipitaceae) volatile organic compounds (VOCs) repel *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) females. Manuscript in preparation.

Chapter 2

Acoustic Assessment of *B. bassiana* (Hypocreales: Clavicipitaceae) Effects on *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) Larval Activity and Mortality



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Chapter 2

Acoustic Assessment of *B. bassiana* (Hypocreales: Clavicipitaceae) Effects on *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) Larval Activity and Mortality

2.1 Summary

Rhynchophorus ferrugineus (Olivier) (Coleoptera: Dryophthoridae) is an economically important pest of palm trees in the subtropics. *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Clavicipitaceae), has been shown to be pathogenic against *R. ferrugineus* in laboratory and field studies. However, because they remain inside the trunks until adulthood, the slowing of feeding and increases in mortality of internally feeding *R. ferrugineus* larvae over time after *B. bassiana* treatment has not been established. To explore the potential of acoustic methods to assess treatment effects, sound impulses produced by untreated, 10^4 , and 10^6 conidia ml^{-1} *B. bassiana*-treated larvae in palms were recorded for 23 d, after which the palms were dissected and the larvae examined. Analyses were performed to identify trains of impulses with characteristic patterns (bursts) produced frequently by moving and feeding larvae but only rarely (3–8% of the larval rate) by interfering background noise or tree vibrations. The rates of bursts, the counts of larval impulses per burst, and the rates of impulses in bursts decreased significantly over time in both *B. bassiana* treatments but not in the control. This supports a hypothesis that larvae had briefer movement and feeding bouts as they became weaker after infection, which reduced the counts of larval impulses per burst, the rates of bursts, and the rates of impulses in bursts. There is considerable potential for use of acoustic methods as tools for nondestructive assessment of effects of biological control treatments against internally feeding insect pests.

2.2 Introduction

Rhynchophorus ferrugineus (Olivier) (Coleoptera: Dryophthoridae), the red palm weevil, causes significant damage to a wide range of palm species worldwide. In Spain, *R. ferrugineus* is an important pest of date, *Phoenix dactylifera* L. (Arecaceae) and canary palms (*P. canariensis* Chabaud; Ferry *et al.*, 2002; EPPO 2008). Adults can be monitored and trapped with pheromone–food attractant baits, but the larvae feed hidden inside the trunks, making it difficult to detect and control (Fiaboe *et al.*, 2011).

In Spain and other Mediterranean countries where infestation is prevalent in urban areas, there is a strong emphasis on the development of integrated pest management strategies based on chemical treatments, pheromone traps (Faleiro and Chellapan, 1999), and biological control such as entomopathogenic fungi (Shah and Pell, 2003; Gindin *et al.*, 2006; Dembilio *et al.*, 2010a; Güerri-Agulló *et al.*, 2011). The most commonly used control treatments are insecticides such as Diazinon, Imidacloprid, and Phosmet (Abbas, 2010). However, heavy use of chemical treatments causes environmental damage and harms nontarget organisms, and also leads to the development of insecticide resistance. Pheromone traps are excellent monitoring devices but capture only adults, leaving the more harmful larvae to destroy the trunk and emerge later. Moreover, the potential for adults to be attracted to palm trees located near pheromone traps (Roda *et al.*, 2011) and the capability of adults to escape from dry or nearly dry traps (Fiaboe *et al.*, 2011) suggests that pheromone traps may be more useful monitoring or control tools in commercial palm areas than in zones with highly valuable historic palms like Elche-Alicante, Spain. Consequently, there remains interest in development of additional alternatives such as entomopathogenic fungi for *R. ferrugineus* management.

Beauveria bassiana (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) is an entomopathogenic fungus that has been shown to cause *R. ferrugineus* mortality in the laboratory (Gindin *et al.*, 2006; Güerri-Agulló *et al.*, 2010; Ricaño *et al.*, 2013). A solid formulation of *B. bassiana* isolate Bb 203 dusted around the crowns, as well as the upper stems and petioles of palm trees, was demonstrated to reduce *R. ferrugineus* populations in southeastern Spain palm groves (Güerri-Agulló *et al.*, 2011). Some of the field treatment effects could be assessed by observations of visible differences in damage in treated and

untreated palms, but the effects on larvae hidden inside the palms could not be observed directly.

Exploring the potential of other methods to assess *B. bassiana* treatment effects in field environments, we hypothesized that comparisons of the temporal and spectral patterns of sounds produced by larvae inside palms might reveal significant differences between signals produced by fungus-treated and untreated larvae. Previous laboratory studies reported that fungus treated larvae often produce weaker movements or eat for shorter intervals than untreated larvae (Ekesi, 2001; Nussenbaum and Lecuona, 2012). In addition, several previous studies, including Hussein *et al.*, (2010), Gutiérrez *et al.*, (2010), and Rach *et al.*, (2013), have demonstrated that *R. ferrugineus* larvae can be detected acoustically. Temporal and spectral pattern analyses of sounds produced by insect larvae in trees have been applied to distinguish such sounds from background noise in laboratory and field environments (Mankin *et al.*, 2008a;b, Mankin, 2011). Large insect larvae moving and feeding in trees produce trains (groups) of brief, broadband sound impulses separated by intervals <25ms that can be identified by specialized software. Trains with characteristic patterns that contain >6 and <200 impulses (designated as bursts) are known to be reliable indicators that insects are present within the sensor detection range (Mankin *et al.*, 2008a). We hypothesized that *B. bassiana* treatment effects on larvae might be reflected in a slower rate of bursts or a smaller count of larval impulses per burst. In addition, treatment effects might be reflected in a smaller count of “burst impulses” per recording, where burst impulse refers to impulses occurring only within and not outside a burst. To consider whether sublethal and lethal effects might be identifiable, we conducted experiments with 30-d-old larvae at two different treatment levels, about two and four orders of magnitude below levels known to cause 100% mortality in previous studies (Dembilio *et al.*, 2010a; Ricaño *et al.*, 2013).

2.3 Materials and Methods

2.3.1 Entomopathogenic Fungi

The *B. bassiana* strain used in the experiment, *Bb* 203, was isolated from naturally infected *R. ferrugineus* adults in southeast Spain (Daimès, Elche; CBS 121097; Güerri-Agulló *et al.*, 2010) and is maintained in the fungal collection of the Glen Biotech and Department

of Plant Pathology, University of Alicante. The fungus was kept in darkness at 4°C on corn meal agar (CMA; BBL Sparks, MD). A solid formulation of *B. bassiana* was prepared according to Güerri-Agulló *et al.*, (2010). Conidial suspensions were obtained by shaking 2 g of 15-d-old solid formulation in 20 ml of 0.2% Tween 80. Conidial concentration was determined using a Neubauer hemocytometer and subsequently adjusted to 10^8 conidia ml⁻¹. A series dilution was made to 10^6 conidia ml⁻¹ (10^6 treatment) and 10^4 conidia ml⁻¹ (10^4 treatment). Sterile distilled water containing 0.2% Tween 80 was used as control.

2.3.2 Palms

Assays were performed on 5-yr-old potted *P. canariensis* obtained from an officially inspected nursery (Elche, Alicante, Spain) and certified free of *R. ferrugineus* infestation. They were maintained in a greenhouse on the University of Alicante campus. Palms were watered twice weekly and kept inside independent cylindrical, wire cloth cages to avoid accidental additional infestation. A 6 by 60-mm screw (PZ3, Standers, Leroy Merlin, Lezennes, France) was inserted near the base of the palm for use as a signal waveguide. The palm then was prepared for artificial infestation by drilling two 30-mm-diameter by 6-cm holes into the palm at opposite ends of a diameter. Three test palms used as no larva controls were prepared for artificial infestation but were not inoculated with larvae.

2.3.3 Insects

Adult *R. ferrugineus* were collected in Elche using bucket traps baited with 4-methy-5 nonanol and 4-methyl-5 nonanone (Kaakeh *et al.*, 2001). Insects were maintained in the laboratory in an incubator at $25 \pm 0.5^\circ\text{C}$ in darkness. Plastic boxes (40 by 30 by 21 cm) were set with a folded piece of moistened filter paper containing thin green apple slices that were replaced three times per week. Adults from the stock colony were sexed by visual inspection of their snouts (Prabhu and Patil 2009). Adults were bred in pairs in individual 100-ml specimen bottles (Deltalab, Barcelona, Spain) using green apple as both food and oviposition substrate. To maintain the humidity of rearing containers, 20 by 5cm filter paper (Refe 1510, Filtros Anioia S. A., Barcelona, Spain) was wet with distilled water and placed into the specimen bottle.

After 2 d, eggs were collected from both apple and paper. The eggs were placed in a sterile 9-cm-diameter plastic Petri dish with an artificial diet substrate (Alarcón *et al.*, 2002). Egg hatching was recorded daily for up to 6 d. Emerged larvae were individually transferred to 20-ml coulter tubes containing 10 ml of artificial diet, and after 15 d, they were transferred into specimen bottles containing 50 ml of artificial diet, which was replaced every 15 d.

Larvae were inoculated using a dipping technique. With the larval head held by hand, the abdomen was dipped into the *B. bassiana* conidial suspension and then placed into a coulter tube and transferred into a hole previously drilled into a test palm. The hole was plugged with the coulter tube to prevent larval escape.

Larvae from the 30-d age group were randomly selected into groups of 16 for controls, which were placed in pairs into 8 palms. Twenty larvae exposed to the 10^4 treatment were placed in pairs into 10 palms, and 10 exposed to the 10^6 treatment were placed into 5 palms.

The palms were dissected 25 d after artificial infestation to check the status of larvae and palm damage. Visual symptoms of infestation, insect status (alive, dead, or absent) and stage (larva, pupa, or adult), and damage (tunnel-distance and number of petioles invaded by larvae per palm) were recorded.

2.3.4 Acoustic Recordings.

Beginning on the first day after infestation, 180-s recordings of acoustic signals from each test palm were made between 1000 and 1900 hours on 14 different days over a 23-d period. A sensor–preamplifier module (model SP-1 L, Acoustic Emission Consulting [AEC] Inc., Sacramento, CA) was connected by a magnetic attachment to the signal waveguide screw at the base of each palm. The signals were fed from the sensor module through an amplifier (AED-2010, AEC Inc. Sacramento, CA) to a digital audio recorder (model HD-P2, Tascam, Montebello, CA) at a 44.1-kHz digitization rate. The recording procedures were similar to those described by Dosunmu *et al.*, (2014).

To avoid high levels of ventilation fan and other background noises present in the greenhouse, the palm in each recording was moved temporarily to a nearby storage cabin

and then returned to the greenhouse. In addition, the signals were monitored with headphones as they were being collected to avoid faulty readings or periods of loud background noise. Nevertheless, because the cabin was not fully soundproofed, periods of vehicular noise (car, train, helicopter, etc.), wind, and bird calls were detected on different occasions, and signal processing was conducted to discriminate such background noises from larval sound impulses, as described in the next section. To assist in these analyses, background sounds were recorded from each test palm on the day its waveguide screw was inserted and before its petioles were drilled for larval placement.

2.3.5 Signal Processing.

Insect movement and feeding sounds typically are produced as trains (groups) of 3–30-ms impulses with similar, distinctive spectral patterns (Mankin *et al.*, 2011). Mean spectra (profiles) of such sounds can be used to help distinguish insect sounds from background noises. However, because the spectra of insect-produced sounds are modified as the signals travel through tree structures (Mankin *et al.*, 2008a,b), the larval sound impulses in this study could not be distinguished from background noises reliably by their spectra alone without also considering the temporal pattern of the impulses. Therefore, we conducted a two-stage spectral and temporal pattern assessment process developed in Mankin *et al.*, (2008b) to provide a more reliable indicator whether signals detected in the recordings were larval sounds or incidental background noise.

In the first stage of the assessment procedure, recordings were prescreened using the Raven 1.3 software sound analysis program (Charif *et al.*, 2008) to survey the different types of signals that had been recorded and locate intervals that contained groups of sound impulses without confounding background noise (Mankin *et al.*, 2011). Signals in each recording were examined whenever their amplitudes exceeded an amplitude threshold (Mankin *et al.*, 2008b) set just above the background noise. Signals that were not composed of groups of brief impulses were discarded from further analysis.

Four types of sound impulses with distinctive spectral features were identified during the prescreening of multiple recordings collected over the duration of the experiment. Mean spectral averages (profiles) of the four frequently occurring types of larval sound impulses were constructed using the DAVIS insect signal analysis program (Mankin *et al.*, 2008b), as described in the Results section. Next, the sound impulses in

each recording were least-squares matched by DAVIS against each of the four profiles and were assigned to the profile type of best fit. Impulses that failed to match within a total least-squares difference of 40 dB between 1 and 15 kHz were discarded as a background noise (Mankin *et al.*, 2008b).

2.3.6 Data Analysis

In the final stage of analysis, DAVIS classified impulse trains containing >6 and <200 profile matching impulses, as larval bursts in each recording, based on the high likelihood that they were produced by larvae and not by extraneous background sounds (Mankin *et al.*, 2008a,b; Dosunmu *et al.*, 2014; Herrick and Mankin, 2012). The burst type was assigned as the type of which the plurality of impulses in the burst had been classified. The times and types of each burst and the count of impulses in each burst (burst impulses) were saved in a spreadsheet for statistical analyses. Multiple bursts of several different types were observed in recordings from most of the infested palms. An overall rate of bursts was calculated by summing the numbers of bursts of each type and dividing by the recording duration. Mean counts of larval impulses per burst were calculated by dividing the total counts of impulses in bursts by the number of bursts in the recording. Overall rates of burst impulses were calculated by summing the numbers of impulses of each type in the recording and dividing by the recording duration. The overall rates of bursts, the counts of larval impulses per burst, and the overall rates of larval impulses occurring in bursts were tested as potential indicators of larval activity in subsequent statistical analyses. Two-tailed, paired t-tests were conducted, palm by palm, to compare mean rates of bursts, mean number larval impulses per burst, and mean rates of burst impulses at the end of the study, 22–23 d after treatment, against the mean rates 15 d previously, when the rates were near overall mean values.

2.4 Results

2.4.1 Larval Sound Impulse Characteristics.

The larvae in each palm produced sound impulses with a broad range of amplitudes, spectral features, and temporal patterns. An example showing a typical range of signals is

a 2-s section of recording from a control palm with untreated larvae (Figure 2.1). The example has two impulse trains, one with 35 impulses and the other with 10.

Four profiles that characterized the spectra of the most commonly observed larval impulses were constructed from mean spectra of groups of distinctive impulses collected on the first day of recording. A low frequency (LowF) profile contained a prominent low frequency peak near 1.9 kHz (Figure 2.2). The profile was constructed as a mean spectrum from 145 impulses collected in a palm infested with 10^4 treatment larvae. A low-midrange frequency profile contained a peak near 2.0 kHz and an additional peak at 6.7 kHz (Low-MidF in Figure 2.2). The Low-MidF profile was constructed as a mean spectrum from 1,289 impulses collected in a palm containing untreated control larvae. A high-frequency profile contained a high-amplitude peak near 10.8 kHz (HighF in Figure 2.2), and a broadband profile included numerous peaks from 2.0 to 12.5 kHz (Broadband in Figure 2.2). The HighF profile was constructed as a mean spectrum from 66 impulses collected in a palm containing 10^4 treatment larvae. The Broadband profile was constructed as a mean spectrum of 994 impulses collected from a third palm with 10^4 treatment larvae.

The four profiles were applied by DAVIS matching analysis subroutines (Mankin *et al.*, 2011) to classify each sound impulse in the experiment. Impulses that matched closely with a larval profile were classified as larval impulses, and trains containing >6 and <200 larval impulses were classified as bursts, used previously as indicators of the likelihood of insect presence (Mankin *et al.*, 2008a,b).

Both trains marked in Figure 2.1 were classified as bursts according to the spectral and temporal pattern analyses of their impulses. Each train contained impulses matching more than one profile type. It should be noted that impulses occurring outside the bursts were discarded from analysis because the lack of a temporal pattern provided less certainty that they had been produced by larvae rather than background noise sources.

2.4.2 Effects of *B. bassiana* Treatments on Larval Activity.

The overall mean rates of bursts, mean counts of impulses per burst, and mean rates of burst impulses varied considerably among treatments over time after treatment (Figure 2.3). The mean rate of bursts in the untreated control varied in a narrow range between 0.25 and 0.5 bursts s^{-1} , while the rates in both the 10^4 and 10^6 treatment decreased <0.3 bursts s^{-1} after

the first week of testing (Figure 2.3A). At least one burst was detected from each larva-infested palm on each day of testing, in conformance with previous findings that the occurrence of bursts in a recording is strongly correlated with the presence of active insects nearby the sensor.

2.4.2.1 Background Noise Trains.

The no-larva treatment served as a control, and provided an estimate of the mean rate of occurrence of background noise trains misclassified as larval bursts by the signal analysis procedure. With uninoculated palms, bursts were detected at rates of 0.01667 ± 0.0064 bursts s^{-1} in recordings obtained over a 12-d period, slightly below the rate of 0.02 bursts s^{-1} suggested by Mankin *et al.*, (2008a) as a threshold for low likelihood of infestation. As noted in Mankin *et al.*, (2011), short broadband impulses can be produced by extraneous tapping or other percussive noises and by stress or mechanically induced acoustic emissions inside the palm. The $0.01667 s^{-1}$ mean rate of bursts in the no-larva control is ~ 3.3 – 6.7% of the $0.25 - 0.5$ bursts s^{-1} mean rate of bursts in the untreated control, which can be used as an estimate of the fraction of background noise trains misclassified as larval bursts in this experiment.

An alternative estimate of the fraction of misclassified larval bursts can be obtained from the result that, in a random sample of five recordings, 49 of the 1,470 (3.3%) trains initially classified as bursts by temporal pattern analysis were discarded by the spectral pattern analysis because $<50\%$ of the impulses in those trains matched within a total least-squares difference of 40 dB of any larval profile. If the rate of noise impulses that matched one of the four profiles was the same order of magnitude as the rate of noise impulses that failed to match a profile, $\sim 3\%$ the larval bursts plotted in Figure 2.3A potentially are misclassified background noise trains, and this estimate is the same order of magnitude as the fraction of misclassifications estimated from the no-larva control.

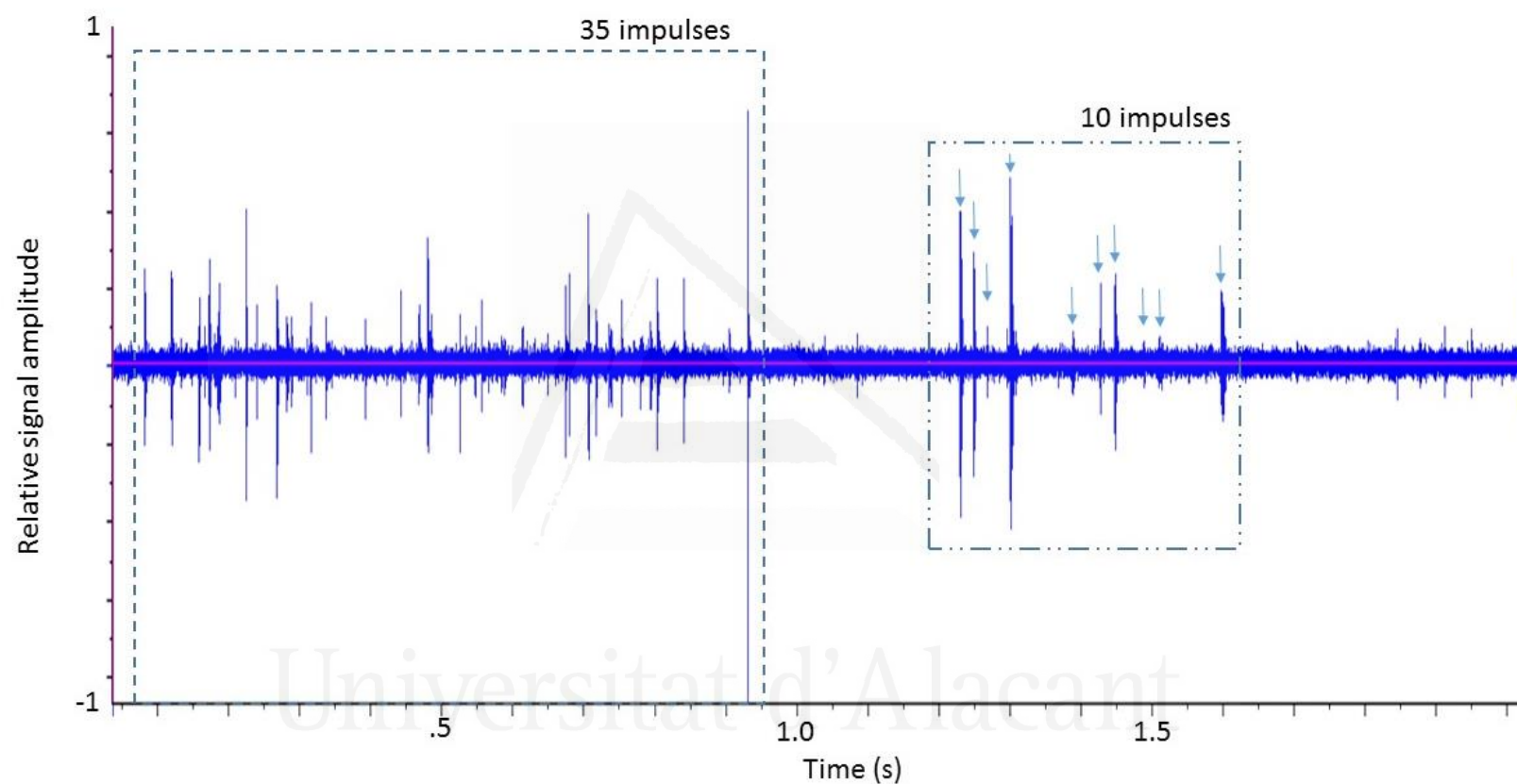


Figure 2.1. Example of typical amplitudes and temporal patterns of impulses recorded from a control palm with untreated larvae. Two impulse trains separated by a quiet interval of 30 ms are indicated by boxes, one with 35 impulses (dashed line), and one with 10 impulses (dash-dot-dotted line). Impulses are marked with arrows in the second train (10 impulses).

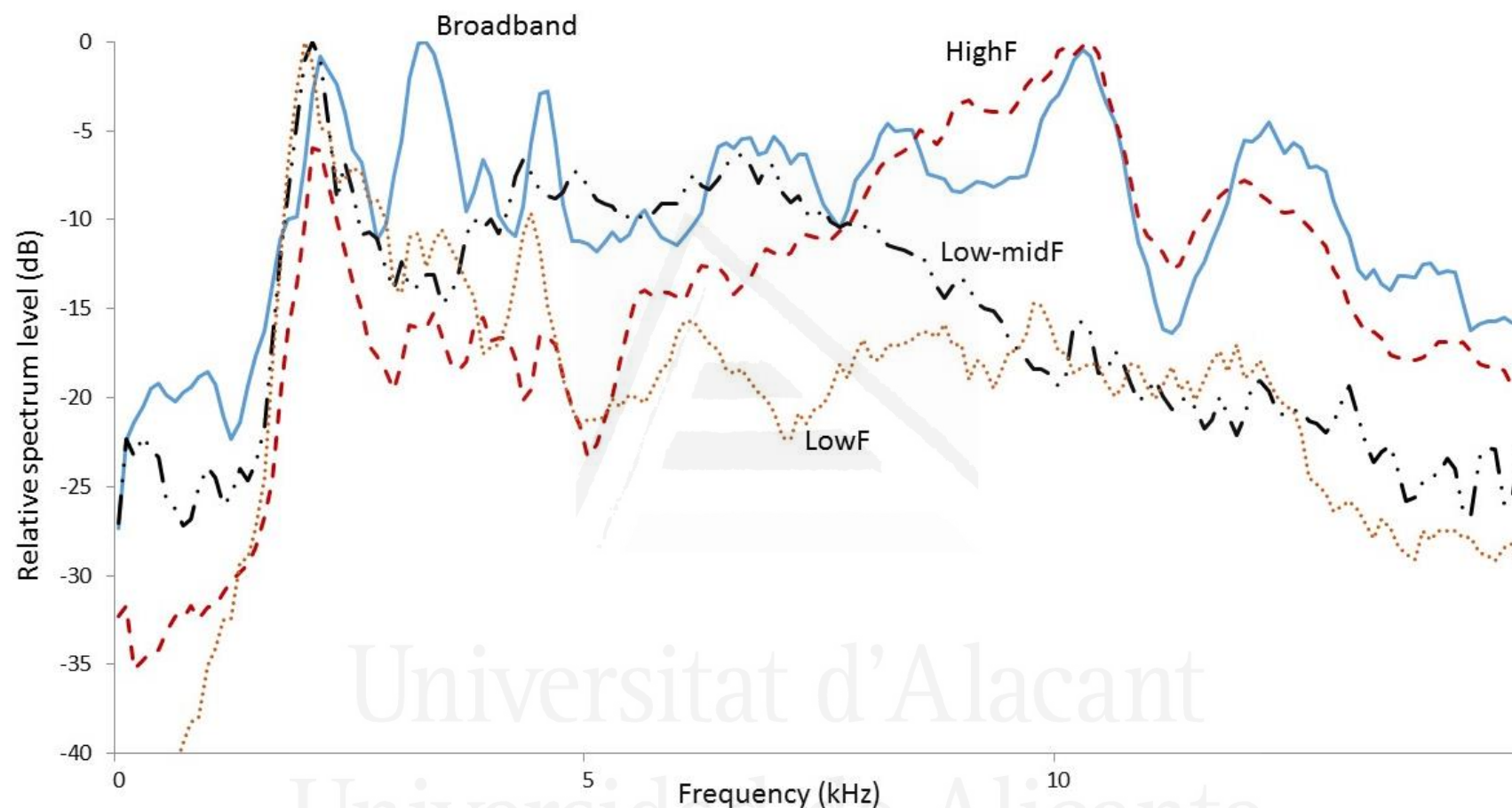


Figure 2.2 Spectral profiles of four distinctive types of larval sound impulses detected in palms: Broadband (with energy over a broad range of frequencies), solid line; LowF (energy primarily near 1.9 kHz), dotted line; Low-midF (energy near 2.0 kHz and second broadband peak from 4.3–6.7 kHz), dash-dot-dotted line; and HighF (energy near 2.0 kHz and second broadband peak from 8–10.8 kHz), dashed line. Horizontal axis indicates frequency in kHz and vertical axis indicates relative spectrum amplitude in dB.

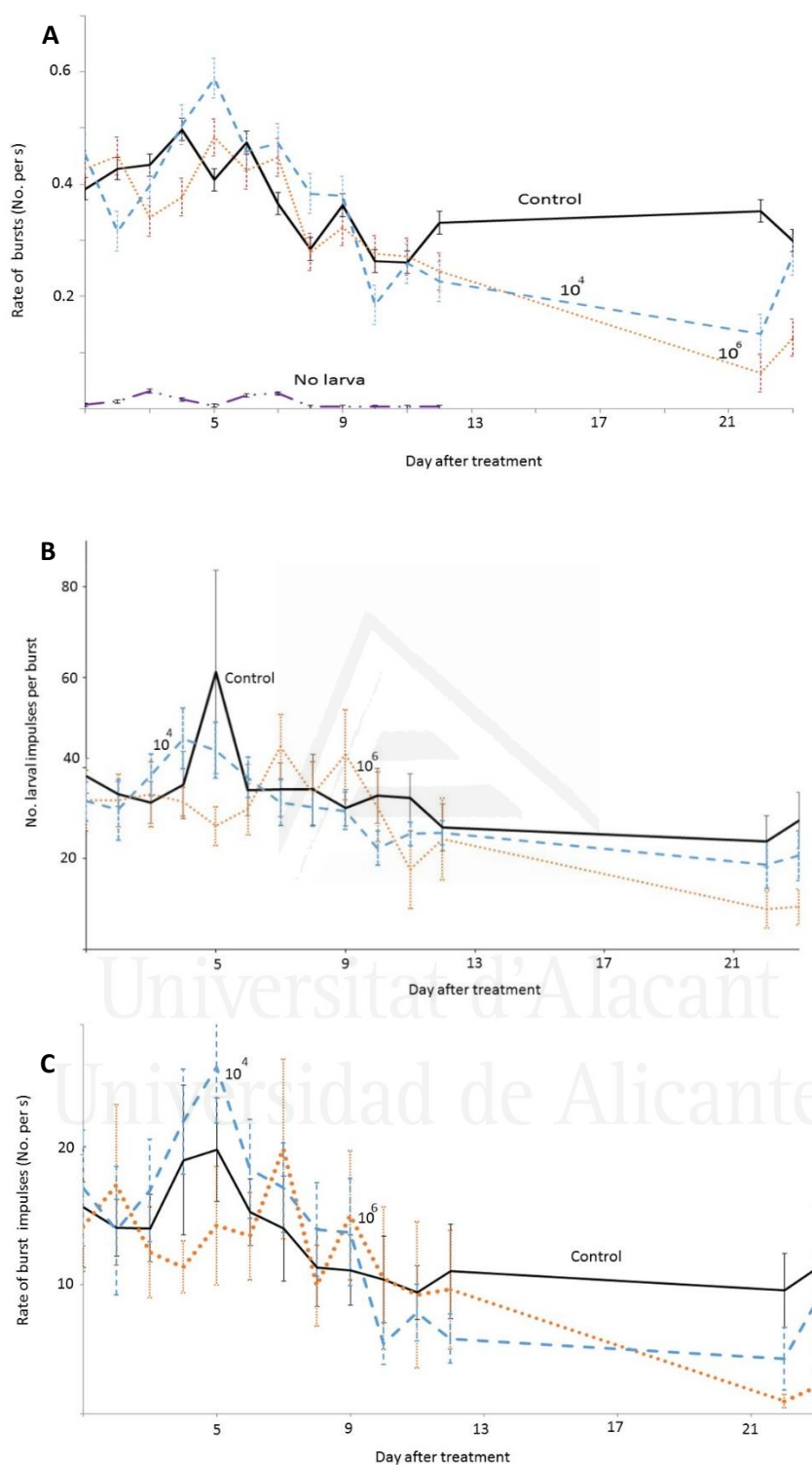


Figure 2.3. Mean \pm SEM of (A) rates of burst, (B) number of larval impulses per burst, and (C) rates of bursts impulses detected on different days after treatment with different doses of *B. bassiana*: control treatment, solid line; 10^4 treatment, dashed line; and 10^6 treatment, dotted line; no larva (background noise control), dash-dot-dotted line. SEs are shown by vertical bars, horizontal axis indicates day after treatment.

2.4.2.2 Analysis of Variance.

Based on the trends observed in Figure 2.3 for the effects of different treatments over the duration of the experiment, analysis of variance was conducted on the effects of treatment, time after treatment (day), and palm on burst rate, count of larval impulses per burst, and rate of burst impulses, considering also the interaction between treatment and time after treatment. Significant differences were found for treatment, palm, and time after treatment, but not for the interaction between treatment and time after treatment (Table 2.1).

Table 2.1. Analysis of variance of effects of treatment, palm, day, and the interaction between treatment and day on rates of bursts ($n = 359$ observations), No. impulses per burst ($n = 322$), and rates of burst impulses ($n = 338$)

Parameter	df	<i>F</i>	<i>P</i>
Bursts:			
Treatment	3	34.12	<0.0001
Palm	22	2.48	0.0004
Day	13	5.16	0.0001
Treatment*Day	38	0.78	0.817
Impulses / burst:			
Treatment	2	9.66	< 0.0001
Palm	20	5.09	< 0.0001
Day	13	4.76	< 0.0001
Treatment x Day	26	1.11	0.3364
Burst impulses:			
Treatment	3	10.67	< 0.0001
Palm	22	3.39	< 0.0001
Day	13	5.81	< 0.0001
Treatment x Day	31	0.72	0.859

Inspection of Figure 2.3 revealed no clear trends in the mean rates of bursts, the count of larval impulses per burst, and the mean rates of burst impulses in the 10^4 and 10^6 treatments during the first week after treatment, but decreases in these values appeared by the last two days of testing (days 22 and 23). Paired t-tests were conducted (Tables 2.2–2.4) to test the hypothesis that the three parameters had decreased at the end of testing from their values 15 d previously (days 7–8). In all of these comparisons, significant decreases were found for parameter values in the 10^4 and 10^6 treatments, but not in the untreated controls.

Table 2.2 Mean (\pm SEM) rates of bursts 22-23 d after treatment in 30d-age-group compared to same tree 15 d previously

Treatment	Change in No. bursts s^{-1}	df	<i>t</i>	<i>P</i>
10^6	-0.268 ± 0.076	9	3.55	0.0062
10^4	-0.225 ± 0.060	19	3.75	0.0014
Control	0.0007 ± 0.053	15	-0.01	0.9898

Table 2.3. Mean (\pm SEM) change in Nos. of larval impulses per burst 22-23 d after treatment in 30d-age-group compared to same palm 15 d previously

Treatment	Change in No. impulses / burst	df	<i>t</i>	<i>P</i>
10^6	-30.390 ± 4.982	9	6.10	0.0002
10^4	-12.212 ± 3.115	19	3.92	0.0009
Control	-9.031 ± 4.897	15	1.84	0.0850

Table 2.4. Mean \pm SEM of rates of burst impulses 22-23 d after treatment in 30d-age-group compared to same palm 15 d previously

Treatment	Change in No. impulses s ⁻¹	df	<i>t</i>	<i>P</i>
10 ⁶	- 13.622 \pm 4.156	9	3.28	0.0096
10 ⁴	- 9.331 \pm 2.375	19	3.93	0.0009
Control	- 2.364 \pm 2.716	15	0.87	0.3977

2.4.3 Effects of *B. bassiana* Treatments on Larval Survival.

When the palms were dissected 25 d after treatment, the decreases in the numbers of surviving larvae generally reflected the decreases observed in burst rates in the different treatments. There was at least one surviving larva in each initially infested palm at day 25 and tunneling damage was observed in association with all surviving larvae (e.g., Figure 2.4D–F). Five of the 10 larvae in palms with the 10⁶ treatment had survived. The five that died exhibited hyphal growth and other visual signs of *B. bassiana* infection (e.g., Figure 2.4A–C). Seventeen of the 20 larvae in palms with the 10⁴ treatment survived to day 25. The three that died also exhibited hyphal growth. In addition, 2 of the 16 larvae in the untreated control died of unknown causes and 1 larva had pupated.



Figure 2.4. Examples of mortality and hyphal growth observed for larvae exposed to 10^6 treatment (A–C), and tunneling damage observed with untreated control larvae (D–F).

2.5 Discussion

The results suggest that the effects of different *B. bassiana* treatments against *R. ferrugineus* can be assessed successfully by three methods that compare different aspects of larval movement and feeding activity: the rate of bursts, the count of impulses per burst, and the rate of burst impulses, as seen in Figure 2.3 and Tables 2.2–2.4. The mean burst rates, counts of impulses per burst, and rates of burst impulses for the 10^6 and 10^4 treatments were significantly lower on the final two days of testing, days 22 and 23, than on days before any trends in burst rates had emerged, e.g., days 7 and 8 after treatment. In correlation with the reduction to 50% survival observed when the palms with 10^6 treatment larvae were dissected on day 25, the mean rate of bursts on days 22–23 decreased to 26% of the 0.363 bursts s^{-1} mean on days 7 and 8. Similarly, the 10^6 treatment mean count of impulses per burst decreased to 23% of the mean of 39 impulses per burst, and the mean rate of burst impulses decreased to 10% of the mean of 15.2 impulses s^{-1} . In correlation with the reduction to 85% survival observed when the palms with 10^4 treatment larvae were dissected, the mean rate of bursts on days 22 and 23 decreased to 47% of the days 7 and 8 mean of 0.428 bursts s^{-1} . Similarly, the 10^4 treatment mean count of impulses per burst decreased to 62% of the days 7 and 8 mean of the 32 impulses per burst, and the mean rate of burst impulses decreased to 41% of the days 7 and 8 mean of 15.8 impulses s^{-1} . Of the three different activity level measures, the rate of burst impulses on days 22 and 23 had the greatest fractional change from its mean value on days 7 and 8 because it factored in both effects of a decrease in burst rate and a decrease in the rate of impulses per burst.

The effects of *B. bassiana* treatments on activity and survival of hidden larvae correlate well with previous studies conducted outside of hidden environments (Fargues *et al.*, 1994, Gindin *et al.*, 2006; Nussenbaum and Lecuona, 2012). In such studies, live spores or conidia germinated when they contacted the insect cuticle. After germination, the fungus penetrated into the insect cuticle and grew within the body of its host which, once infected, reduced its feeding and movement activities. High doses of fungal treatments such as the 6.3×10^7 to 3.0×10^9 conidia ml^{-1} treatments in Dembilio *et al.*, (2010a) have been shown to cause 100% larval mortality in *R. ferrugineus* within 6–7 d. Malarvannan *et al.*, (2010) found the least pupation (43.33%) in *Spodoptera litura* (F.) larvae treated with their highest dosages (2.4×10^7 conidia ml^{-1}) of *B. bassiana*.

Correlation was observed in this experiment between mean activity levels and subsequently observed fungal treatment effects even though the rate of sound production is variable and often decreases during periods of molting or pupation (Mankin *et al.*, 2011). The ages of the larvae played a role here because the larvae were near their final stages of development but only one larva (an untreated control) had pupated by the end of the experiment.

Thus, there is considerable potential for use of acoustic methods as tools for nondestructive assessment of effects of biological control treatments against hidden insect pests under field conditions. The use of such methods could assist in optimization of doses and delivery methods for development of entomopathogenic fungal treatments as alternative pest control methods.



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Chapter 3

Monitoring Red Palm Weevil Field Management using Acoustics and GIS



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Chapter 3

Monitoring Red Palm Weevil Field Management using Acoustics and GIS

3.1 Summary

The red palm weevil (RPW) *Rhynchophorus ferrugineus* (Olivier) is an important pest of palms. RPW damage to palms and effects of management treatments have been assessed on individual palms using symptom scales. RPW adults have been monitored in the field with pheromone traps but these are not permitted in Spanish historical patrimony palm groves. We therefore, developed methods to apply acoustic sensors and geographic information system (GIS) techniques to monitor the spatial spread of RPW larval activity in 12 palm plots with RPW management treatments near a historical palm grove in SE Spain. Presence of RPW was mapped using GIS techniques to determine effects of biocontrol *Beauveria bassiana*, Chemical control (Imidacloprid), and their combination against RPW compared with untreated control plots. Trains of impulses (bursts) were identified using improved spectral and temporal acoustic patterns of RPW larvae recorded in field infested palms. RPW palm damage was assessed using a visual scale. These enabled detection and mapping of RPW hot spots using GIS methods. *B. bassiana* and Imidacloprid treatments were found to reduce RPW infestation in the field. Activity of RPW in field infested palms can be monitored successfully with a combination of acoustic, visual, and GIS methods. Our results may help to make treatment decisions for control of RPW infestations.

3.2 Introduction

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Oliver, 1790) (Coleoptera: Dryophthoridae), is the main economically important palm pest in Asia, the Middle East (El-Sabea *et al.*, 2009), and the Mediterranean Region (Ferry *et al.*, 2002, Mizzi *et al.*, 2009; Manachini *et al.*, 2013). In South Eastern Spain (Elche-Alicante), historical date palm groves (*Phoenix dactylifera* L. Arecales: Arecaceae) are kept for ornamental purposes, date production, craft and other agricultural uses (Ferry *et al.*, 2002; Rivera *et al.*, 2015). Locally grown date palms are also an important cultural asset in the tourism industry in Alicante Province (SE Spain).

In Elche (SE Spain), when historic palms become old, they are replaced by new young palm using the seed of the historic palms continuously grown for this purpose. When palms in public parks are damaged by RPW, the landscape managers of the municipality of Elche usually replace them with fully grown palms at cost of several thousand euros each (Ferry *et al.*, 2002; Rivera *et al.*, 2015)

RPW infested palms are difficult to manage and often die because of the cryptic habits of this insect pest (Giblin-Davis *et al.*, 2013). Integrated pest management (IPM) is a preferred method for combining different pest control tactics into one programme to manage RPW under field conditions (Giblin-Davis *et al.*, 2013; Mukhtar *et al.*, 2011; El-Mergaway and Al-Ajlan, 2011; Justin *et al.*, 2008). Many IPM programs against RPW include chemical insecticides (Dembilio *et al.*, 2015; Abo-El-Saad *et al.*, 2012; Llàcer *et al.*, 2012; Sacco *et al.*, 2011; Hernandez-Marante *et al.*, 2003). However, reliance on insecticide treatments alone can harm non-target organisms and subsequently accumulate in the food chain (Al-Samarrie and Akela, 2011; Pillay *et al.*, 2003). Repeated use of these agrochemicals frequently results in development of resistance in insects. Furthermore, pests may survive in situations where the chemical does not reach them, is washed off or is applied at an improper rate (William and Pillay, 2011)

Entomopathogenic fungi such, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Clavicipitaceae) have been proposed as biological control agents in IPM (Lo *et al.*, 2015; Ricaño *et al.*, 2013; Güerri-Agulló *et al.*, 2011; Dembilio *et al.*, 2010a; Sewify

et al., 2009). Entomopathogenic fungi are important regulators of insect populations under natural conditions (Augustyniuk-Kram and Kram, 2012; Shahid *et al.*, 2012). Although slow-acting against RPW, fungi such as *B. bassiana* have been used for the biological control of insect pests, coleopterans in particular (Erler and Ates, 2015; Gkounti *et al.*, 2015). *Beauveria bassiana* in a solid formulation remains effective at least 3 months after application under semifield conditions (Ricaño *et al.*, 2013). This *B. bassiana* solid formulation when applied in the field, induced great RPW adult mortality (Güerri-Agulló *et al.*, 2011) and persisted for at least one year. *B. bassiana* can be applied for RPW management either as a preventive or a curative (Güerri-Agulló *et al.*, 2011)

Combinations of chemical pesticides and entomopathogenic fungi have been used to control insects. The insecticide Imidacloprid increases virulence of *Metarhizium anisopliae* against the dengue vector *Aedes aegypti* (L.) (Diptera: Culicidae) (Paula *et al.*, 2011). Imidacloprid and the entomopathogenic nematode *Steinernema carpocpsae* showed positive effects in controlling RPW infestation in the field (Dembilio *et al.*, 2010b). In this work, we apply a combination of the chemical insecticide, Imidacloprid and the entomopathogenic fungus, *B. bassiana*. Both agents are effective separately against RPW to protect young date palms from RPW infestation in the field.

An effective integrated pest management program typically contains several interacting components, including i) identifying and monitoring pest problems, ii) selecting the best management tactics to reduce the detected infestation and iii) recordkeeping and evaluating the program (El-Mergaway and Al-Ajlan, 2011). Early detection of RPW is crucial in IPM programmes. Unfortunately, clear visual symptoms of RPW infestation (RPW feeding indicators and reduced plant growth) appear only after advanced stages of attack (Giblin-Davis *et al.*, 2013).

Monitoring RPW activity over a wide area is essential for keeping a close watch on the establishment and subsequent build-up of pest infestation because RPW can fly long distances. The flight mill technique in the laboratory show that RPW adults have a large potential for dispersal of more than 5000 m with an average speed of approximately 0.5 km h⁻¹ (Àvalos *et al.*, 2014). Consequently, after initial reports of infestation, it is imperative to monitor the area-wide activity of adult weevils as well the activity of young larvae inside the individual dates palm in the infested field.

RPW adults can be efficiently monitored using pheromone traps (Fiaboe *et al.*, 2011) but this technique is prohibited in protected areas such as in the Historical Palm Grove of Elche (SE Spain). Other monitoring techniques are a visual pest symptoms index (Güerri-Agulló *et al.*, 2011) use of dogs for detecting RPWs infestation (Nakash *et al.*, 2000) and X-ray computed tomography (CT) scanning (Ma *et al.*, 2012). X-ray CT scanning is expensive. Using dogs for RPW detection is also costly because they have considerable maintenance costs and must be frequently retrained to detect infested palms.

Acoustics is being increasingly used to study animal species and environments for their monitoring and conservation (Depraetere *et al.*, 2012). Thus, applications of acoustics have been integrated in insect pest management (Mankin *et al.*, 2011). Detection of RPW sounds allows early detection and monitoring of RPW infestation on coconut tree (Siriwardena *et al.*, 2010; Martin *et al.*, 2015), canary palm (Herrick and Mankin, 2012; Jalinás *et al.*, 2015) and date palm (Soroker *et al.*, 2004; Mankin *et al.*, 2011; Hetzroni *et al.*, 2016). Portable and user friendly acoustic devices have been used for detecting RPW infestation in the field (Siriwardena *et al.*, 2010). Although commercial sensors for detecting acoustic signals from insects activities are still expensive, an affordable prototype sensor has been developed for detecting RPW activity (Rach *et al.*, 2013).

A mathematical method to automatically detect acoustic activity of RPW in palm offshoots (Potamitis *et al.*, 2009) and computer based programming (Hetzroni *et al.*, 2016, Potamitis *et al.*, 2009; Pinhas *et al.*, 2008) are required for detecting hidden activities of RPW. New instrumentation and signal analyses for detecting RPW have also been conducted (Mankin *et al.*, 2011). Manual and automated acoustic monitoring is now feasible for monitoring young palm trees (Hetzroni *et al.*, 2016).

However, capturing RPW acoustic signals in the field can be challenging whereby the background sounds from different origins and directions may affect the quality of the recordings (Fiaboe *et al.*, 2011). In a previous greenhouse experiment, RPW larvae artificially inoculated inside palms were successfully detected and the effect of *B. bassiana* treatment could be monitored using acoustics (Jalinás *et al.*, 2015). However, wind-induced tapping sounds in the field are difficult to distinguish from larval sounds, partly because the larval sounds and palm leaf-tapping sounds can have similar resonance frequencies (Mankin, 2011). To accommodate the greater noise levels in field compared to laboratory studies, we made use of a previously developed method of analysing temporal

patterns of detected signals to identify bursts of larval sounds (Jalinas *et al.*, 2015). In addition, we identified and made use of additional distinctive spectral features of sounds produced in palms by larvae to better discriminate the larval sounds from background noise.

Geographic information system (GIS) technology has recently been used for assessing the activity of the red palm weevil in infested host plants in Saudi Arabia (Massoud *et al.*, 2012), India (Faleiro *et al.*, 2002) China (Rui and Ajlan, 2011). GIS can be a useful tool for implementing area-wide RPW–IPM programmes in large areas, enabling the logging of data pertaining to insect activity assessment in addition to the tracking of infestation locations (Pontikakos *et al.*, 2015; Fiaboe *et al.*, 2012; Oehlschlager *et al.*, 1995). GIS has also been used for predicting the potential worldwide distribution of the red palm weevil *Rhynchophorus ferrugineus* (Fiaboe *et al.*, 2012). We therefore incorporated the acoustic assessment of infestation activity levels into a GIS system to monitor the spatial activity of RPW and the effects of the treatments based on acoustic activity, visual pest symptom and RPW signs.

The aim of this chapter was to improve acoustic analysis for RPW field detection under noise background conditions. Secondly, assessing RPW infestation and invasion by spatially mapping measures of acoustic activity and visual symptoms of RPW infestation using a GIS system. This step aims to evaluate the effects of *B. bassiana* and Imidacloprid treatments to protect young date palms from RPW field infestation.

3.3 Materials and Methods

3.3.1 Experimental plots and palm information referencing

The Hort de Bernia site (Elche, SE Spain) (38°16'36.06''N 0°40'57.30''W) (Figure 3.1A-B) was selected for our study. It is a new date palm plantation (15-year-old palms) in the vicinity of the Elche's Historical Palm Grove (UNESCO Patrimony) (Ferry *et al.*, 2002) with no signs of RPW infestation. The 1.47-Ha study site contained twelve *P. dactylifera* palm plots (1,027.215±10.15 m² per plot) from which 355 palms (0.4±0.02 m height) were selected for experiments from October 2013- November 2014 (Figure 3.2). An additional plot (1,094±0.50 m²) containing 58 palms (0.697±0.175 meter height) at the edge of the experimental plots, designated as “external plot,” was added to the study because it

included four palms observed to be infested when the experiment began (Figure 3.2). The infested palms were confirmed by assessment of visual symptoms for RPW infestation (Güerri-Agulló *et al.*, 2011). Palms were selected and used with the approval of Municipality of Elche. All palms were georeferenced using GPS (Series GeoXM, Trimble GeoExplorer). The GIS software used in this investigation was ArcGIS® (Release 10, 2011) ESRI 2011. ArcGIS Desktop: Release 10. Redlands, CA: Environmental Systems Research Institute

3.3.2 RPW Management treatments

Replicated control design for RPW management treatments were assigned. Entomopathogenic fungi, *B. bassiana* isolate (203) used in this project was isolated from naturally infected *R. ferrugineus* adults in SE Spain (Daimès, Elche; CBS 121097; Güerri-Agulló *et al.*, 2010). The fungus is maintained in our fungal collection on corn meal agar (CMA; BBL Sparks, MD) at 4°C in the dark. A solid formulation of *B. bassiana* with 3.10^9 conidia per gram was prepared according to Güerri-Agulló *et al.*, 2010). Ninety-three palms from plots 4, 8 and 12 were dusted (stem and crown) with the dry formulation (1 kg per palm) of the entomopathogenic fungus *B. bassiana* (*Bb 203*), 89 palms from plots 2, 6, and 10 sprayed with 0.015% Imidacloprid in water (10L /palm) only, 87 palms from plots 3, 7 and 11 with *Bb 203* formulation + Imidacloprid and 86 palms from plots 1, 5 and 9 were left untreated as controls. Treatments were applied four times (October 2013, April, June and September 2014) (Figure 3.2). The four palms just outside the experimental plots that had been acoustically identified as RPW infested were also treated with Imidacloprid by the Elche Council Pest Management staff.

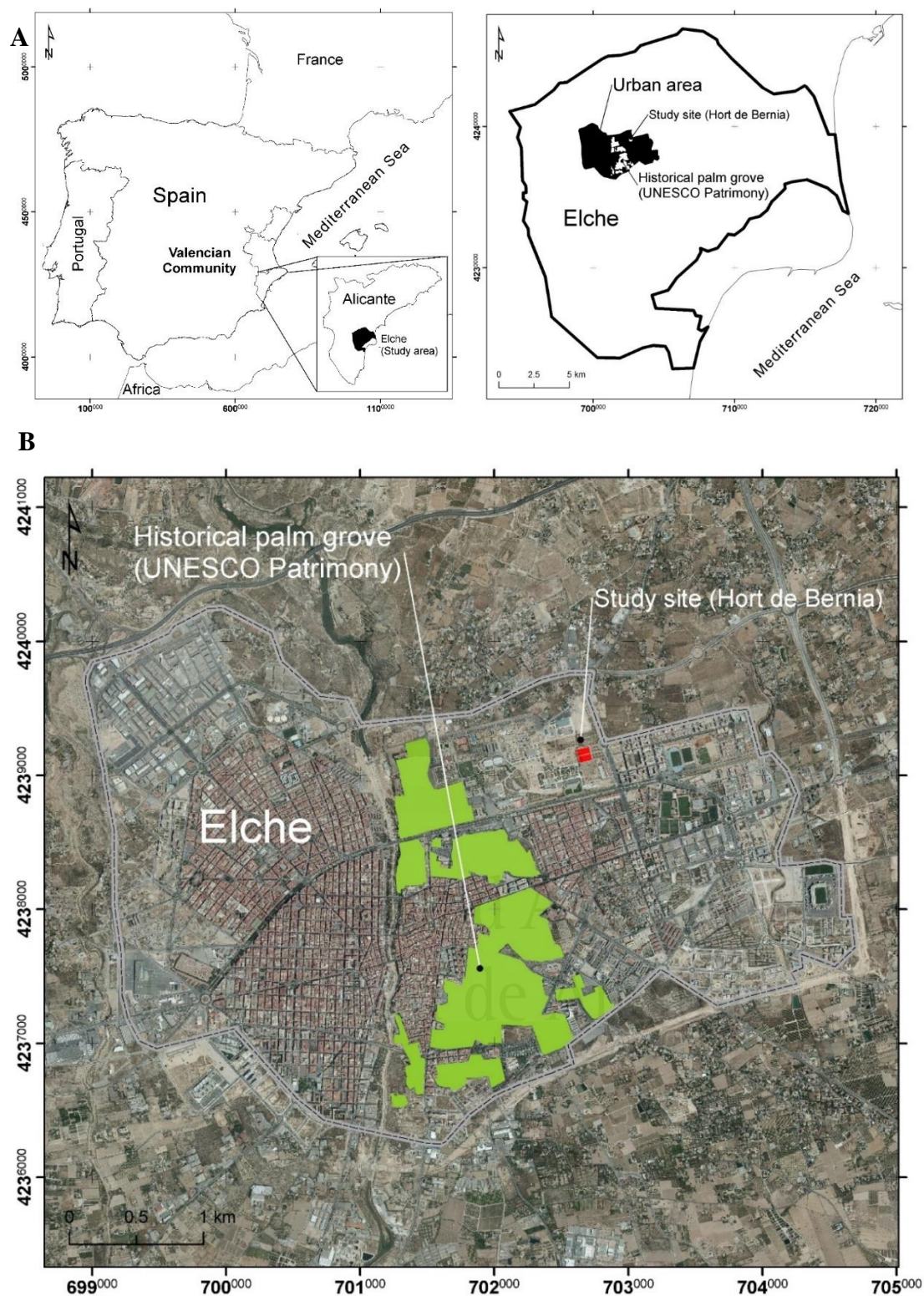


Figure 3.1. Location of experimental site. A) Elche (SE, Spain). B) Experimental plot (1.466 Ha) within the Hort de Bernia in Elche close to the Historical Palm Grove.

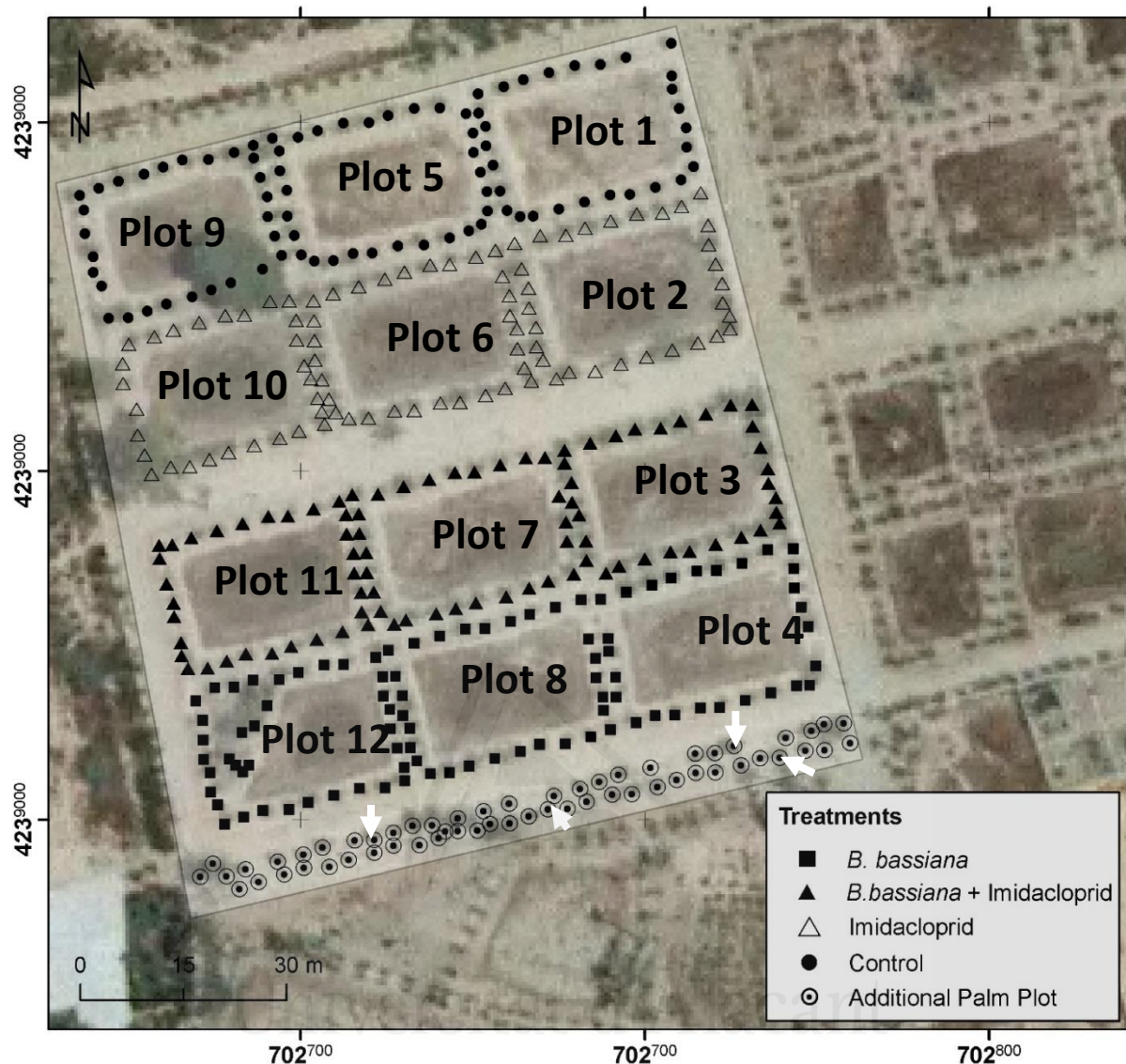


Figure 3.2. Red palm weevil Integrated Pest Management treatments. Plots 4, 8 and 12 were treated with a dry formulation of *B. bassiana* (*Bb 203*) (black squares), plots 3, 7 and 11 with (*Bb 203*) + Imidacloprid (black triangles), plots 2, 6 and 10 with Imidacloprid (white triangles). Plots 1, 5 and 9 were left untreated as control (black circle). A palm plot containing 58 palms (circles with a central dot) adjacent to the experimental included four RPW naturally infested palms (white arrows) which were detected at the start of our experiments also is shown.

3.3.3 Acoustic recording procedures

Before RPW management treatments were carried out, seven palms from each plot were randomly selected for acoustic recording. The four RPW naturally infested palms initially found at the external plot were also recorded (Figure 3.2). Other palms in this plot later identified as infested by the visual RPW symptom assessment scale were also monitored acoustically. A 6 x 60-mm screw (PZ3, Standers, Leroy Merlin, Lezennes, France) was inserted near the base of the palm leaf petiole for signal waveguide. Recordings of 180 s

each were made approximately every two weeks between 10:00 and 17:00h using a sensor–preamplifier module (model SP-1 L, Acoustic Emission Consulting [AEC] Inc., Sacramento, CA) connected by a magnetic attachment to the signal waveguide screw (Jalinas *et al.*, 2015; Dosunmu *et al.*, 2014). The signals were fed from the sensor module through an amplifier (AED-2010, AEC Inc. Sacramento, CA) to a digital audio recorder (model HD-P2, Tascam, Montebello, CA) at a 44.1-kHz digitization rate. In addition, signals were also monitored with headphones as they were being collected to avoid faulty readings or periods of loud background noise. Precaution was taken during recording to minimize contact by wires and sensors with the palm leaves (leaflets).

3.3.4 Signal processing and discrimination of RPW sounds from non-target background noise

Because the palm orchard was in an urban area periods of high environmental noise (e.g. people talking, walking, trains, dogs, or building construction), wind, and bird calls were detected on different occasions. To reduce interference from background noise, signal processing was performed to discriminate RPW signals from non-target background noise.

In the first stage of signal processing, recordings were screened using the Raven 1.3 software sound analysis program (Charif *et al.*, 2008) and converted to 16-bit. wav format. Individual sounds were then classified by the custom-written, DAVIS insect signal analysis program (Mankin *et al.*, 2008a and 2008b). Like most insects sounds and unlike typical background noise, RPW larval sounds are produced as trains (groups) of 1-10-ms impulses with similar, distinctive spectral and temporal patterns. The types of spectral and temporal patterns in any individual train depend on the type of feeding or movement activity in progress at the time of recording.

The first set of signals analyzed in this process came from the field recordings of four palms originally outside the experimental plots (Figure 3.2) that had been identified as infested during the beginning of the experiments. In preliminary analyses, four larval RPW sound profiles (Jalinas *et al.*, 2015) from previous semifield experiments were matched against each impulse (Jalinas *et al.*, 2015; Mankin, 2011a). Impulses that matched closely with a larval profile were classified as larval impulses, and trains containing >6 and <200 larval impulses were classified as larval bursts, used previously as indicators of the

likelihood of insect presence (Jalinas *et al.*, 2015; Mankin, 2011a; Mankin *et al.*, 2008a). The time and type of each burst and the counts of impulses in each burst were scored in a spreadsheet (Jalinas *et al.*, 2015).

Because multiple types of profiles were present in almost every recording, a total rate of bursts, r_{4p} , was calculated as the total number of bursts of all four types divided by the recording interval. In addition, a quantitative assessment based on total rate of bursts was assigned for ratings of RPW infestation likelihood (Mankin *et al.*, 2008a)

Low	$r_{4p} < 0.02 \text{ bursts s}^{-1}$,	
Medium	$0.02 \text{ bursts s}^{-1} \leq r_{4p} < 0.06 \text{ bursts s}^{-1}$, and	Eq. 1
High	$r_{4p} \geq 0.06 \text{ bursts s}^{-1}$.	

Larval RPW sounds detected by DAVIS from the positive control palms were rescreened in Raven 1.3 software to enable listeners to check the assessments, and to observe the amplitudes, spectral features, and temporal patterns. Positively identified impulses inside larval bursts were stored in a library database of known RPW sounds for scouts surveying RPW infestations in the field. Different types of background sounds which had been recorded during this study were identified and stored in the database. The library of sounds was used to discriminate RPW sounds and non-target background signals.

Early in the collection and analysis of recordings, it became apparent that significant percentages of RPW sounds which were being detected failed to match the spectral profiles obtained in our original semifield study (Jalinas *et al.*, 2015) that had used 30-d-old RPW larvae. For example, repetitive squeal sounds (Rach *et al.*, 2013) were identified in RPW infested palms in the field. To accommodate the additional RPW signals, new profiles from squeal activities were constructed and integrated into analysis for improving the detection system (See results section 3.4.2). In addition, sounds of young, 15-d-old RPW larvae were added to the sound library database by visual confirmation of chewing activities by lab-reared larvae inside an artificial tunnel in a palm petiole. Video recording (Carl Zeiss Tessar HD 1080p-Logitech) was conducted in a plastic box (40 cm x 32 cm x 22cm). The sounds were recorded as in section 3.3.3 above. These additions to the sound library enabled identification of squeals of older larvae, and sounds of young larvae in infested palms under field conditions. All palm recordings thereafter were analyzed with and without the use of squeal profiles to test their effect on the outcome of RPW infestation assessments.

3.3.5 Inverse distance-weighted interpolation spatial models for distribution of RPW infestation based on acoustic activity

Inverse distance weighting (IDW) was used as an interpolation method (Philip and Watson, 1982; Mitchell, 2005) in GIS to make density maps and to estimate the spread of RPW based on the acoustic activity levels (rates of larval bursts). The interpolator operates on the assumption that entities in close proximity to one another are more alike than those farther away. The effects of the treatments on the spatial distribution of RPW larval acoustic activity were evaluated based on the assessments in Eq. 1.

3.3.6 Visual symptom scale for RPW palm damage

The effect of the treatments for RPW management was also assessed scoring visual symptoms (Güerri-Agulló *et al.*, 2011) in January 2014, April 2014 and September 2014 using a 5-level scale. Level 1 included palms without visual symptoms of RPW damage. Level 2 included palms with notches in leaves and larval feeding damage in expanded leaves. Improved techniques on visual symptoms for level 2 was also carried with the finding of the drying of emerging leaves (unopen immature leaves) at the central of palm crown, drying of young offshoots and appearance of chewed palm tissues. Level 3 included palms with large numbers of leaves asymmetrically placed in the crown. Level 4 included palms in an advanced stage of RPW infestation, mostly with flat or bent down leaves. Level 5 included dead palms with no living leaves. All palms in plots were georeferenced and visually scored.

3.3.7 *B. bassiana* presence in RPW

RPW infested material in the plots was carefully searched for insects (larvae, pupae and adults) and their numbers scored. Each insect was scored as alive or dead. Dead RPW were taken to the laboratory and placed in moist chambers (Güerri-Agulló *et al.*, 2011; Marti *et al.*, 2005) to determine the presence of *B. bassiana*. Moist chambers were observed daily for 15 days for fungal sporulation. Suspected signs of *B. bassiana* infection were confirmed by plating on corn meal agar (Becton Dickinson). Cultures were incubated at 20-28 °C and examined microscopically daily.

3.3.8 Insect sound burst statistical analyses

The rates of bursts with and without inclusion of squeals, r_{4p+s} and r_{4p} , respectively, were tested as potential indicators of RPW larval activity. Two-sided, paired t-test were conducted to compare mean rates of bursts and mean rates of combining larval bursts and squeals using R studio software version 3.1.3.

3.4 Results

3.4.1 Detection of RPW sounds in field experimental plots

Examples of RPW larval sound impulses detected from infested palms in the experimental plot are shown in Figure 3.3. A train of at least 7 impulses defines a RPW sound burst (Figure 3.3A). Figure 3B shows longer RPW bursts. RPW larvae sounds recorded in the laboratory had similar temporal pattern and characteristics as those in the experimental field plots illustrated in a supplementary figure 1.

3.4.2 Improvement of RPW signal discrimination in field sound recordings

To improve the discrimination of RPW sounds from non-target background noise commonly encountered in the experimental plots, we incorporated a set of new spectral profiles into the digital signal analysis process. They included distinctive squeals that listeners readily identified as RPW sounds in field recordings. Supplementary Figure 2 displays as an example, the new profiles incorporated into DAVIS sound analysis program. They facilitated detection of the numerous squeals sounds in recordings from RPW infested palms (Table 3.1).

These profiles helped signal analysis because the palm plots were < 100 m from construction sites with sirens, horns, and machinery sounds. The RPW squeals are loud can be detected even with background noise. We identified three sources of background noise which affect acoustic recording and the detection of RPW signals: (i) wind gust and associated leaf tapping sounds (Supplementary figure. 3A-3B) (ii) hammering, horns, and sirens at construction sites (Supplementary figure. 3C-3E) (iii) non-target animal sounds

(Supplementary figure 3F-3H). Bird calls and chirping have low (1.862-3.626 kHz), medium (5.880-7.252 kHz) and high-frequencies (11.074-14.210 kHz, 16.660-19.500 kHz) ranges. These background sounds match with RPW larval impulse profiles. However their temporal patterns impulses usually exceeded the maximum train length of 200 impulses for RPW bursts. This discards them as background noise instead of true RPW larval sounds.

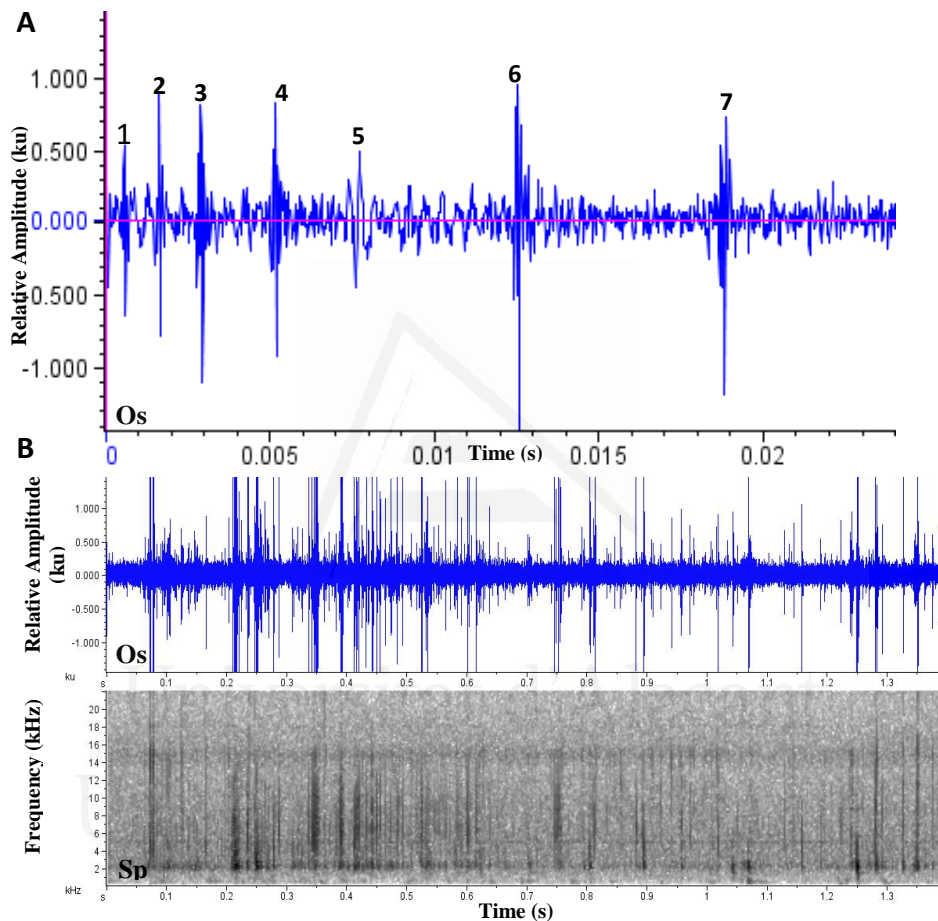


Figure 3.3. *Rhynchophorus ferrugineus* larval sound impulses. **A)** Oscillogram (Os) of a 0.021 s period of seven impulses the minimum number of impulses in a larval burst, recorded from a palm with the scale 2 assessment of visual pest symptom. These seven impulses matched with one of published RPW profiles for larval feeding and moving activities. **B)** 115 impulses in period of 1.40 s.

Table 3.1. Improvement of RPW signal detection

Palm	Listener Rating	Rate of Bursts (No. per s)	Rate of Bursts (No. per s) + Rate of Squeals (No. per s)	df	<i>P</i>	Visual Pest Symptom
P.17	High	0.124±0.027 (a)	0.168±0.053 (a)	4	0.05248	Level 2
P.28	High	0.346±0.035 (a)	0.508±0.084 (b)	4	0.006156	Level 2
P.50	High	0.373±0.083 (a)	0.597±0.078 (b)	4	0.004757	Level 2
P.36	High	0.470±0.107 (a)	0.632±0.087 (a)	4	0.3329	Level 2

Different letters within rows indicate are non-significant difference at $P < 0.05$. (\pm SEM; N=5 sounds in 180 s recording for each palms).

See Figure 4 for location of P.17,P.28, P50 and P.36 RPW infested palm in the heat maps

3.4.3 Assessment of RPW Palm Infestation Likelihood

Quantitative RPW infestation assessments, we performed in the external plot at the beginning of our experiment. We scored palms with a high likelihood of infestation (Table 3.1) in early and mid-October 2013 recordings before treatment with Imidacloprid. The mean rate of bursts was $0.33 \pm 0.04 \text{ s}^{-1}$ ($n=20$ observations from all infested palms). RPW detection rate increased to $0.48 \pm 0.06 \text{ s}^{-1}$ when the new RPW profiles were included in the sound analyses. However, when r_{4p} and r_{4p+s} (see section 3.2.4 and equation 1) were compared for individual palms, the difference was significant ($P < 0.05$) for only two of the initial 4 palms tested (Table 3.1). In early October 2013, there were no RPW visual symptoms in the experimental plots surveys. In addition, the mean rate of bursts from the seven palms acoustically sampled in each experimental plot remained below 0.02 s^{-1} , the threshold for low likelihood of infestation.

3.4.4 Spatial distribution of RPW larvae in the field

Figure 3.4 shows the spatial distribution of RPW larvae in the field (early October 2013) using the GIS-Heat map technique. We used the acoustic activity of RPW larvae to estimate its occurrence. The presence of RPW at the beginning of the experiments at the external palm plots correlated with high values in the heat map for RPW activity inside the palms.

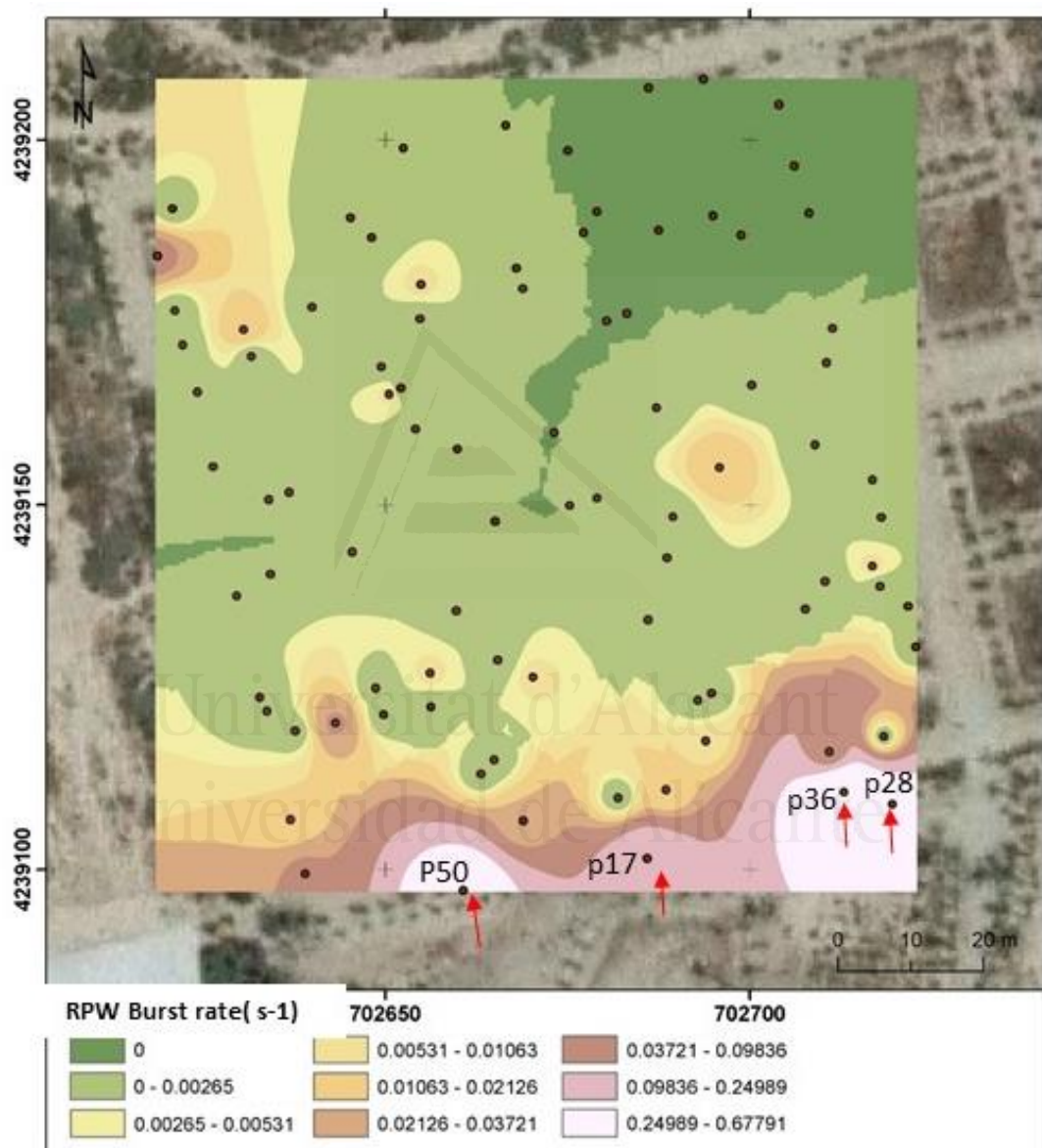


Figure 3.4. Spatial distribution of RPW larvae in the experimental field (October 2013). Red arrows show RPW infested palms detected based on visual symptoms.

3.4.5 Effect of RPW management treatments on RPW larval spatial distribution

Figure 3.5 shows the effect of management treatments on the spatial distribution of RPW. Our results (Fig. 3.5A-C) show that during the deployment of the treatments RPW remained mostly confined to the external plot. In the external plot, only palms with symptoms were sprayed with Imidacloprid

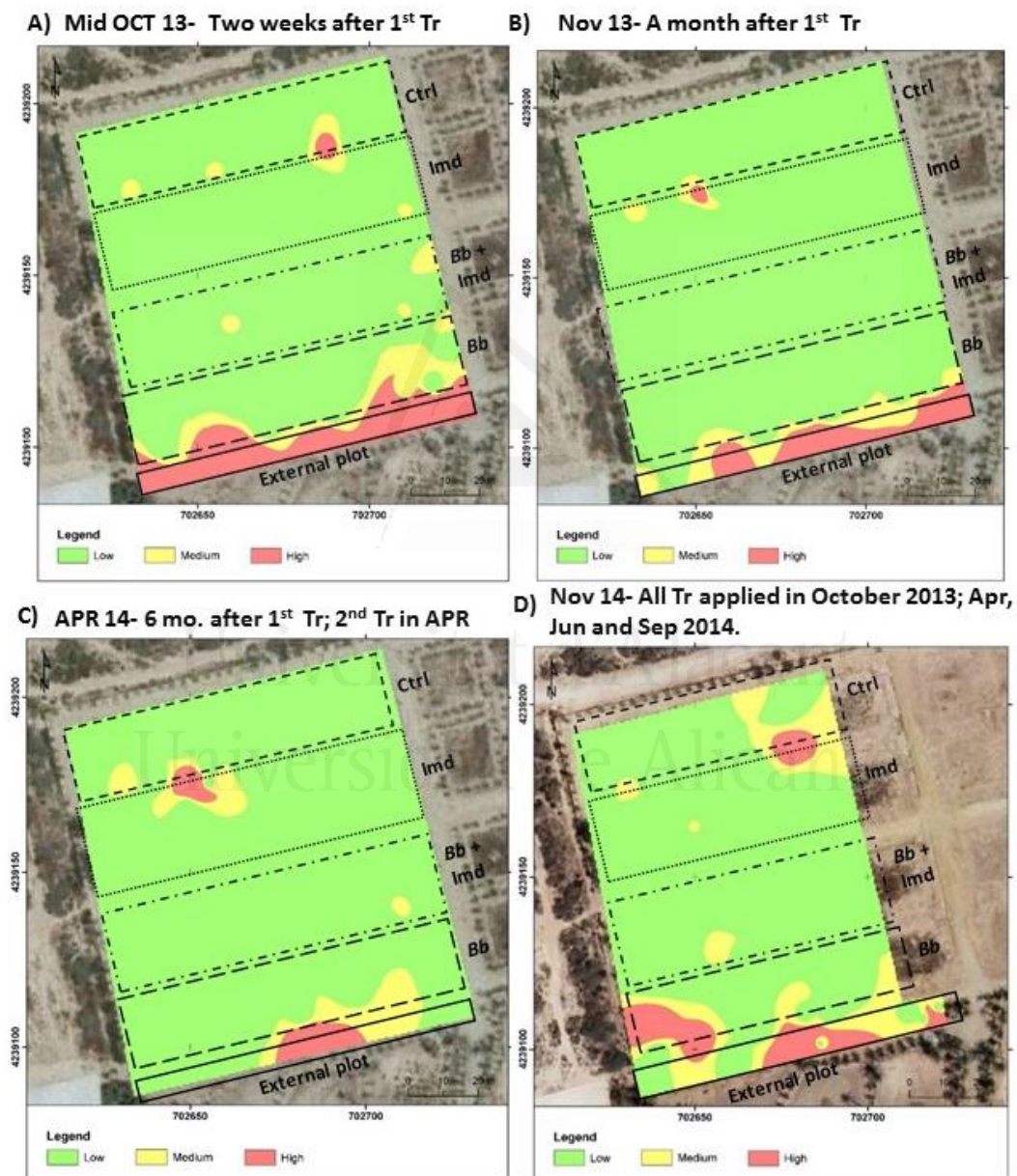


Figure 3.5. Evolution of RPW distribution in experimental field A) mid-October 2013 B) November 2013 C) April 2014 D) November 2014. Boxes indicate RPW Management treatments. Abbreviations: mo (Months), Tr (Treatment), Ctrl (Control), Imd (Imidacloprid), Bb + Imd (*B. bassiana* and Imidacloprid), Bb (*B. bassiana*).

In Mid-April 2014, several palms in plots 1, 2, 3 and 4 (Figure 3.5D and Supplementary figure 4E-F) were removed for school construction. In November 2014 (1 year after initial treatments) (Figure 3.5D), RPW infestation spreaded from the external plot to the experimental plots. This matched with the results of visual estimation of RPW damage in palms (Supplementary figure 4).

The percentage of RPW infested palms in treated experimental plots (Table 3.2) was < 1.5%, lower than the > 2.0% infestation in untreated plot (control). The fraction of infested palms in the outer, external plot increased from 6.90% (October 2013) to 13.79% (January, April and September 2014). These visual survey results suggest that the treatments applied in the experimental plot provided protection from RPW-infested palms in the nearby external plot.

Table 3.2. Visual symptom assessment on palms in the experimental palms

	January 2014			April 2014			September 2014		
	n= No. palms assessed	RPW infested palms		n= No. palms assessed	RPW infested palms		n= No. palms assessed	RPW infested palms	
		no.	%		no.	%		No.	%
Control	86	2	2.33	71	2	2.82	71	2	2.82
Imd	89	1	1.12	72	1	1.39	72	1	1.39
Bb +Imd	87	1	1.15	71	1	1.41	71	1	1.41
Bb	93	1	1.08	78	1	1.28	78	0	0.00
Outside	58	8	13.79	58	8	13.79	71	8	13.79
Total	413	13	3.15	350	13	3.71	350	12	3.43

No. = total palms assessed with visual RPW palm damage symptom scales; no. = RPW infested palms
Abbreviations: Imd (Imidacloprid), Bb + Imd (*B. bassiana* and Imidacloprid), Bb (*B. bassiana*).

Acoustic assessment of the four infested palms (October 2013) after Imidacloprid treatments showed reduction in the mean rates of bursts (Figure 3.6). After the chemical treatments, dead RPW were found around the palms with signs of *B. bassiana* infection (Figure 3.7). These palms were only treated with Imidacloprid, but they were located near palms in the plots of *B. bassiana* treatments. Possibly, *B. bassiana* inoculum spread to new locations non- initially treated. Imidacloprid treatments initially reduced RPW acoustic activity. However, GIS heat map showed (Figure 3.5) that the chemical did not killed RPW since acoustic activity remained in several infested palms after treatments.

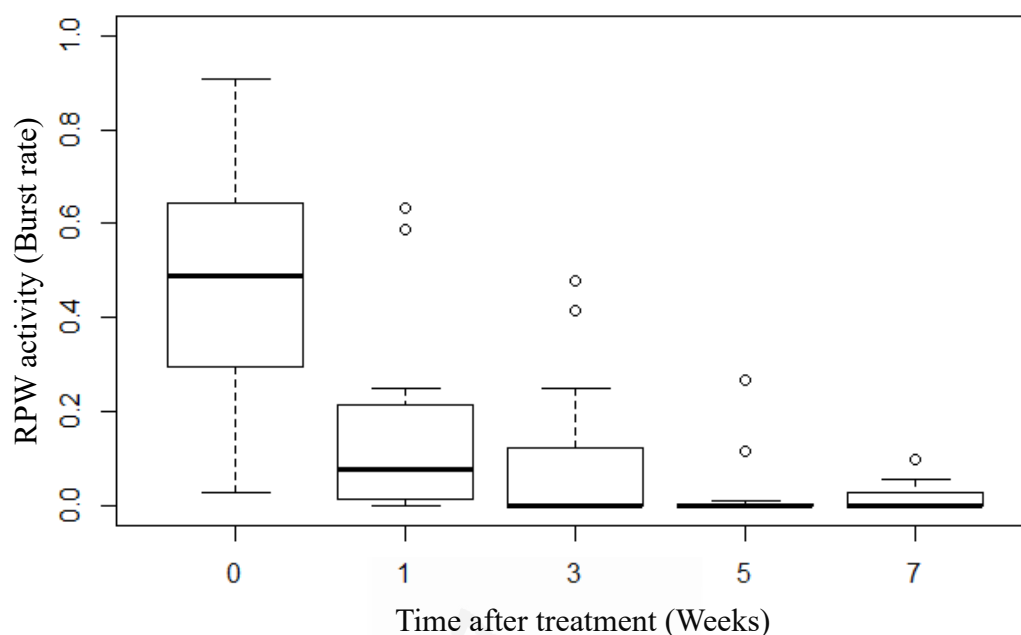


Figure 3.6. Reduction of RPW activity (acoustic estimation) in RPW infested palms after Imidacloprid Treatment. Values are the averages of infested palms ($n = 4$) in the external plot.

Five newly infested palms (16, 27, 35 and 58) (Supplementary Fig. 4D) and a palm no. 43 (Supplementary Fig. 4F) were discovered in the external plot from November 2013 to September 2014. A series of Imidacloprid treatments were applied upon discovery. The visual pest symptoms for two palms thereafter decreased from level 2 to 1. However, palm no. 43 soon exhibited an increase of visual symptoms to level 4, associated with a high rate of bursts in five acoustic monitoring tests, $0.40 \pm 0.05 \text{ s}^{-1}$. This palm was consequently removed to reduce risk of RPW spread.

3.4.6 *B. bassiana* epizootics in experimental field

B. bassiana epizootics in experimental field appeared in RPW adults and pupae, (Table 3.3). We found signs of *B. bassiana* infection from RPW collected in the field (Supplementary Fig. 5). A month after application of *B. bassiana* formulation in the field (November-December 2014), 17.4 % of RPW samples was dead with signs of *B. bassiana* (Figure 3.8A). Interestingly, 15% of RPW samples were dead with *B. bassiana* infection from infested palms treated with Imidacloprid only (Table 3.3). The number of dead RPW samples with signs of *B. bassiana* in January and February 2014 increased to 34.78 %

(Figure 3.8A) (Supplementary Figure 6). During the second set of treatments (March to April 2014) the percentage of dead RPW with signs of *B. bassiana* increased to 80% (Figure 3.8A) (Supplementary Figure 7). In total, 76 individuals collected (Nov-April 2014) were found dead, per stage 28.57% of the pupae, and 33.3% of the RPW adults collected had signs of *B. bassiana* infection (Figure 3.8B).

RPW mortality in Nov-Dec 2013



Figure 3.7. Spatial distribution of RPW dead adults in the experimental field (November-December 2013). Arrows show RPW with *B. bassiana* infection. (N= number of RPW infected with *Bb*) in the field. First treatment at experimental plots was October 2013 and first Imidacloprid treatment on infested palms was November 2013.

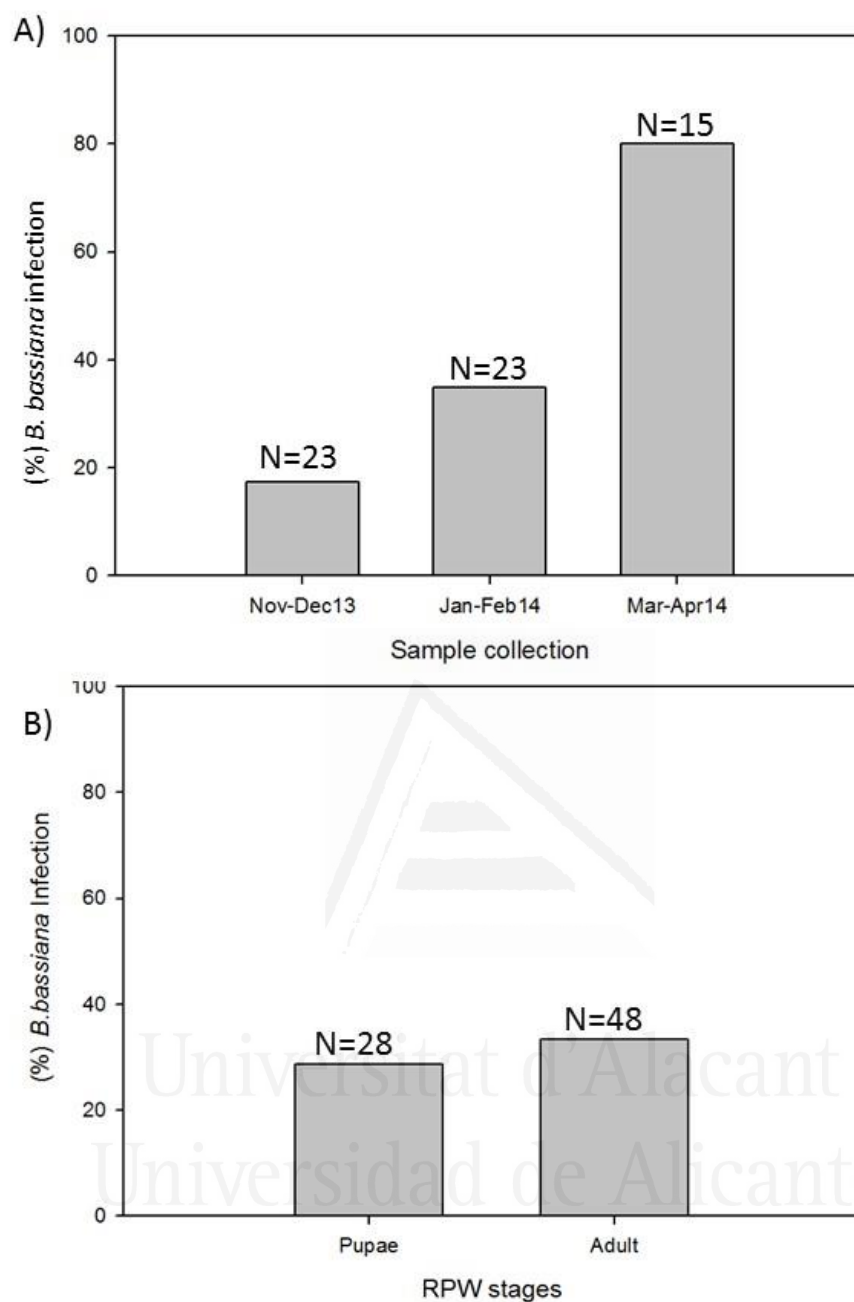


Figure 3.8. *Beauveria bassiana* presence in RPW. (A) Dead RPW with signs of *B. bassiana* infection per sample collection (B) Total presence of *B. bassiana* per RPW stage. N= total number of insects scored.

Table 3.3. RPW mortality and *B. bassiana* incidence in the Experimental Site

Treatment plot	Stage	November-December 2013			January-February 2014			March- April 2014			May-June 2014			October-November 2014		
		Dead RPW	No. RPW with <i>Bb</i>	RPW with <i>Bb</i> %	Dead RPW	No. RPW with <i>Bb</i>	RPW with <i>Bb</i> %	Dead RPW	No. RPW with <i>Bb</i>	RPW with <i>Bb</i> %	Dead RPW	No. RPW with <i>Bb</i>	RPW with <i>Bb</i> %	Dead RPW	No. RPW with <i>Bb</i>	RPW with <i>Bb</i> %
Ctrl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Imd	Pupae	-	-	-	6	2	33.33%	-	-	-	-	-	-	-	-	-
	Adult	1	-	-	5	-	0%	-	-	-	-	-	-	-	-	-
<i>Bb</i> + Imd	Pupae	-	-	-	1	-	0%	2	2	100%	-	-	-	-	-	-
	Adult	1	-	0%	1	-	0%	-	-	-	1	0	0	-	-	-
<i>Bb</i>	Larvae	-	-	-	-	-	-	-	-	-	-	-	-	1	-	0%
	Pupae	-	-	-	1	-	0%	-	-	-	-	-	-	1	-	0%
	Adult	1	1	100%	-	-	-	2	2	100%	1	0	0	1	-	0%
Outside	Pupae	-	-	-	5	3	60%	3	1	33.33%	-	-	-	9	-	0%
	Adult	20	3	15%	4	3	75%	8	7	87.5%	1	0	0	1	-	0%
	Total RPW No.	23	4	17.40%	23	8	34.78%	15	12	80%	3	0	0	13	0	0%

Abbreviations: Ctrl (Control untreated plot), Imd (Imidacloprid treatment plot), *Bb* + Imd (*B. bassiana* and Imidacloprid), *Bb* (*B. bassiana* treatment plot), Outside (Additional Palm Plot)

3.5 DISCUSSION

Our results demonstrate that acoustics can detect RPW infestation in palms under field conditions. The combination of temporal and spectral analysis of background sounds reduce the rate of misclassified background sounds which have spectra with peaks similar to those of RPW (Mankin *et al.*, 2008a). In this study, we show that the efficacy of RPW management treatments can be monitored using the RPW acoustic records (burst rate) supported by visual pest symptom assessment. In our previous study (Jalinas *et al.*, 2015), we demonstrated that burst rate sequential recording can monitor infection of RPW larvae with *B. bassiana* under semifield experiments. Furthermore, the reduction of burst rates in RPW infested palms (external plot) after Imidacloprid treatments indicates that the acoustics can also to assess the effect of chemical treatments for RPW management.

Newly characterized “squeal” sounds improved the process of acoustic detection of RPWs field activity in the infested palms. Kočárek, (2009) analyzed sound production and chorusing behaviour of *Icosium tomentosum* (Coleoptera: Cerambycidae) during larvae feeding in the host plant. Visual confirmation indicated that sound production occurred by larvae knocking their heads against the wall of their burrows. Squeal sounds are different from those caused by RPW mandibles cutting the palm fibers as well as head knocking the wall of larval tunnel. The mechanisms of the “click” or chorusing sounds are not well understood. Another study by Corcoran *et al.*, (2010) reported that the click sounds produced during flexion and relaxation by tymbal organs. Insects including order of Coleoptera produce vibratory signals by body vibration such as tymbal mechanisms and stridulation (Čokl and Virant-Doberlet, 2009). Thus, our finding of squeal sounds in the field study suggest RPW is able to produce vibratory signals similar as tymbal mechanisms or stridulating inside the infested palms. Under these conditions, RPW larvae producing vibration sounds are not only from palm fiber cutting sounds, but also click sounds.

Thus, the early insect detection afforded by the use of acoustic methods enables managers to undertake suitable monitoring and control measures at an early stage of infestation. This prevents further build-up of the pest population. Our results show that integration of RPW larvae acoustic activity in a GIS system could estimate the spatial RPW

activity the experimental plots. The effect of RPW management treatment could be observed through the evolution of spatial activity of RPW in the experimental plots. Massoud *et al.*, (2012) used annual RPW captures in pheromone traps to be integrated in GIS based techniques. This allowed monitoring of the spatial spread of RPW in the field and correlate with the number of palms infested with RPW. The GIS based techniques help the decision making for the critical points of infestation.

RPW have ability to migrate to nearby palms. The mean value of dispersion for *Rhynchophorus ferrugineus* obtained from the computer simulations is 0.5 km h^{-1} (Ávalos *et al.*, 2014). These authors reported that 54% of the insect population in their laboratory study were classified as short-distance flyers (covering <100m). In our study, there was an increase over time in the number of infested palms outside our experimental plot. This result suggest that RPW in the external plot migrated to nearby palms within same row which were not protected with treatments.

Our results show the risk of RPW infestations calculated by interpolating RPW larvae acoustic activity using the Inverse Distance Weighting (IDW). This technique was used in an areawide pest management of corn rootworms (CRW) (Beckler *et al.*, 2005). This technique showed the predictions of emergence of CRW (Coleoptera: Chrysomelidae) based on number of CRW adults trapped (Beckler *et al.*, 2005). RPW adults in the experimental plot showed that RPW begun to migrate nearby palms from just outside to new territories inside the experimental plot. However, the experimental plot with RPW treatments showed less RPW activity than the untreated control and external plots. Therefore, the results indicate that the RPW management treatments reduced the risk of RPW attacks on palms in the experimental treatments plots.

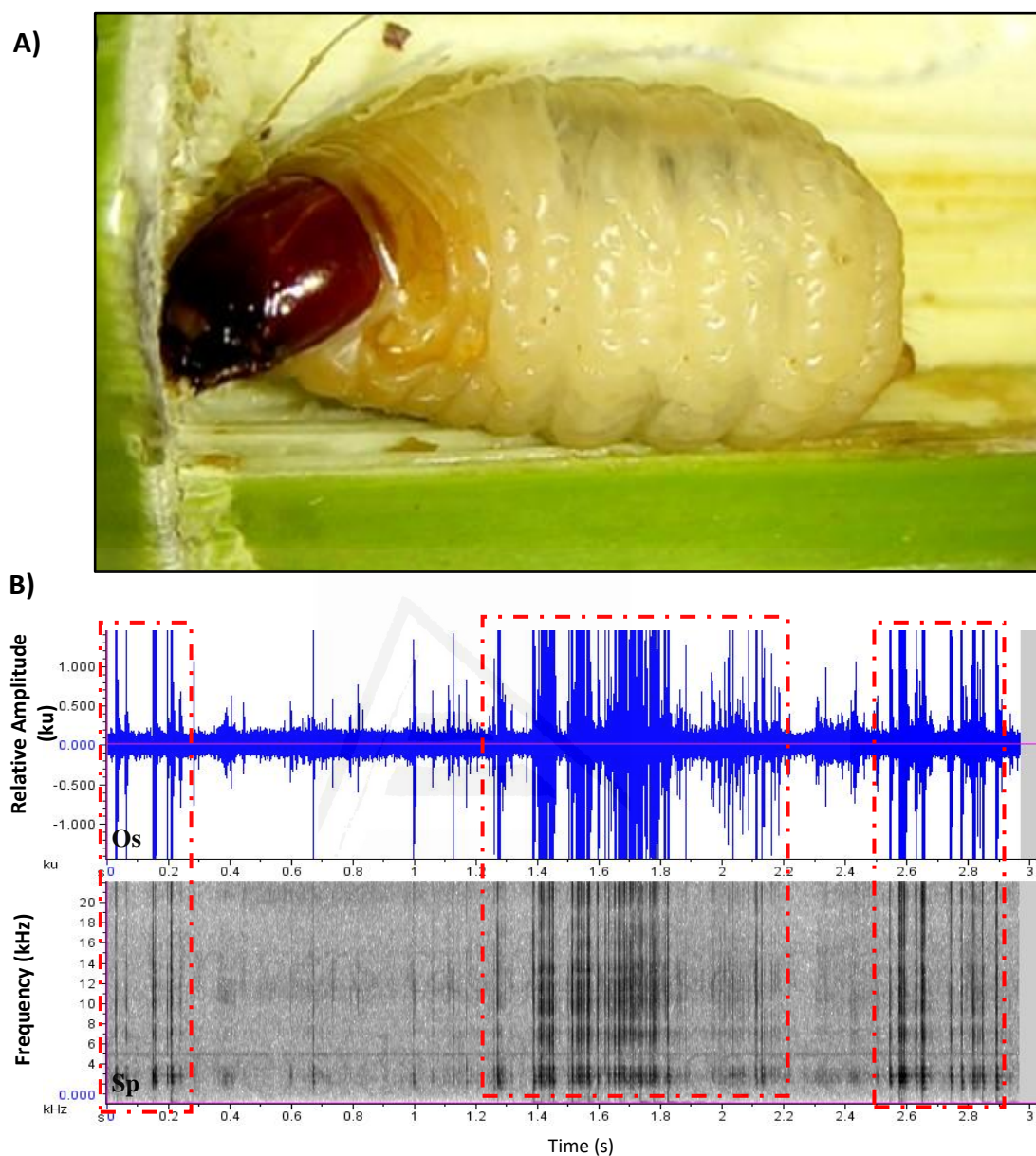
The spatial distribution of RPW activity aggregated at the infested palms outside the experimental plots suggested that RPW management treatment might be able to avoid RPW attack and infestation. RPW has special antennae which detect host plant volatile compounds which known as kairomones. The experiments suggest that the treatments might have influence on the migratory movement towards the selection of new host plants. A previous study showed that fungi produced volatile organic compounds with insecticidal and repellent activities (Strobel *et al.*, 2010). There are also reports of avoidance of fungi, *B. bassiana* by *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) (Ormond *et al.*, 2011).

In this study, our fungal treatments created an artificial epizootics on the RPW population in mostly outside the experimental plot. More than twice RPW insects were dead with signs of *B. bassiana* outside the experimental plot where the fungus was applied. RPW adults can survive a few days after *B. bassiana* infection, and may therefore act as vectors of the fungus (Ricaño *et al.*, 2013). Moreover, untreated palms in this experiment were close to palms treated with *B. bassiana*. Conidia are easily transmitted through air by wind (Ropera *et al.*, 2010) or water (Dobbs, 2006) during storms and irrigation. A former field study also showed that *B. bassiana* application in the field induced artificial epizootics (Güerri-Agulló *et al.*, 2011).

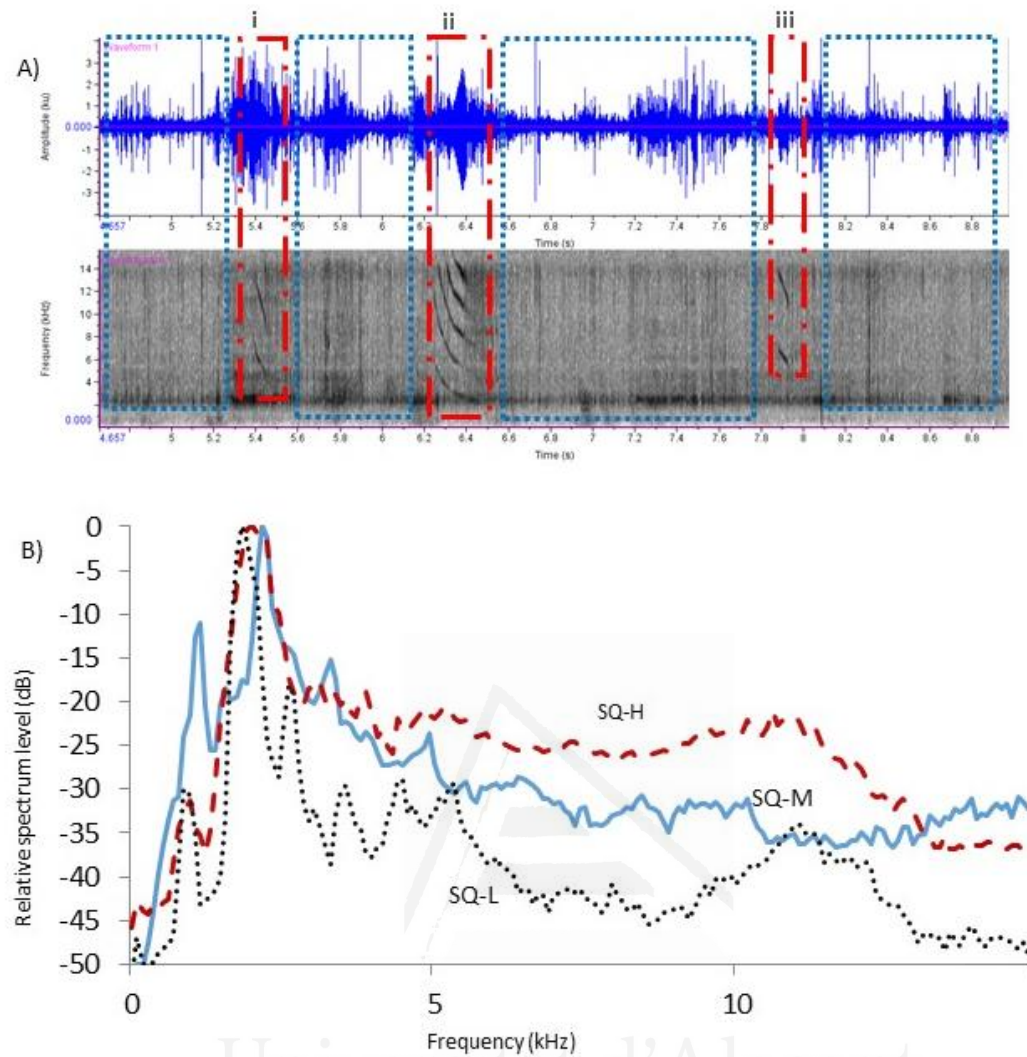
The results showed the combination of Imidacloprid and *B. bassiana* showed less palm damage than palms in the control untreated plots. This combination worked synergistically reducing RPW spatial activity in the experimental plots. The combination of Imidacloprid (Confidor®) and *B. bassiana* caused higher mortality when used together (Malik *et al.*, 2016) for second and fourth instar of RPW larva under laboratory conditions. Furthermore chemical treatments only resulted in low rates of bursts detected and visual pest symptoms which did not progress indicating that the efficacy of treatments. However, this study suggested that RPW infested palms may not be completely protected in the long-term with chemical treatment alone.

Thus, the development of acoustic detection information integrated into a GIS mapping system could facilitate surveillance of the distribution patterns of RPW in a palm grove and could estimate the invasive activity of RPW and the effect of the treatments for RPW management. As a result, there is potential to reduce the costs of labor and for monitoring the RPW infestation in the field, especially in historically protected areas.

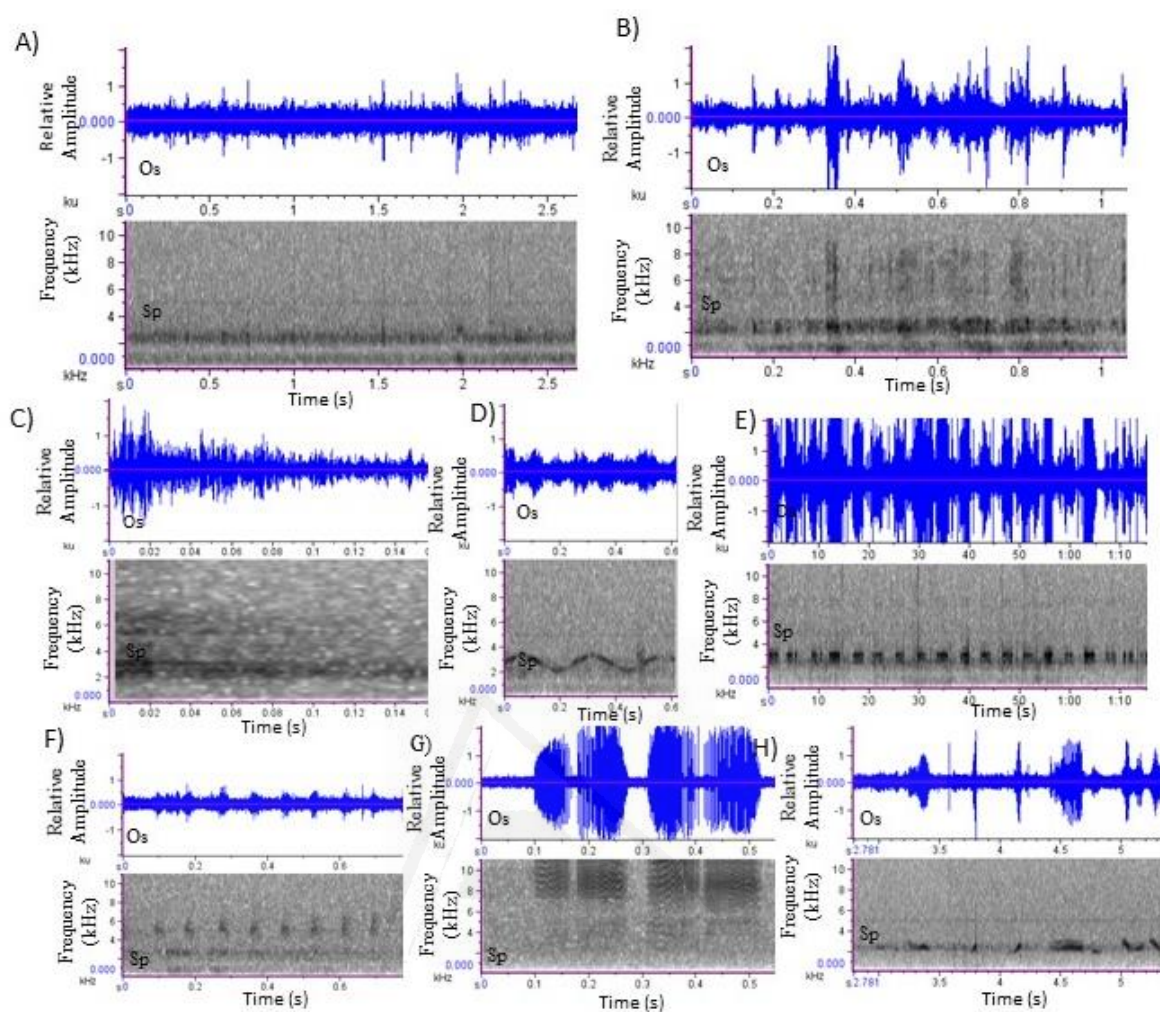
Supplementary figures



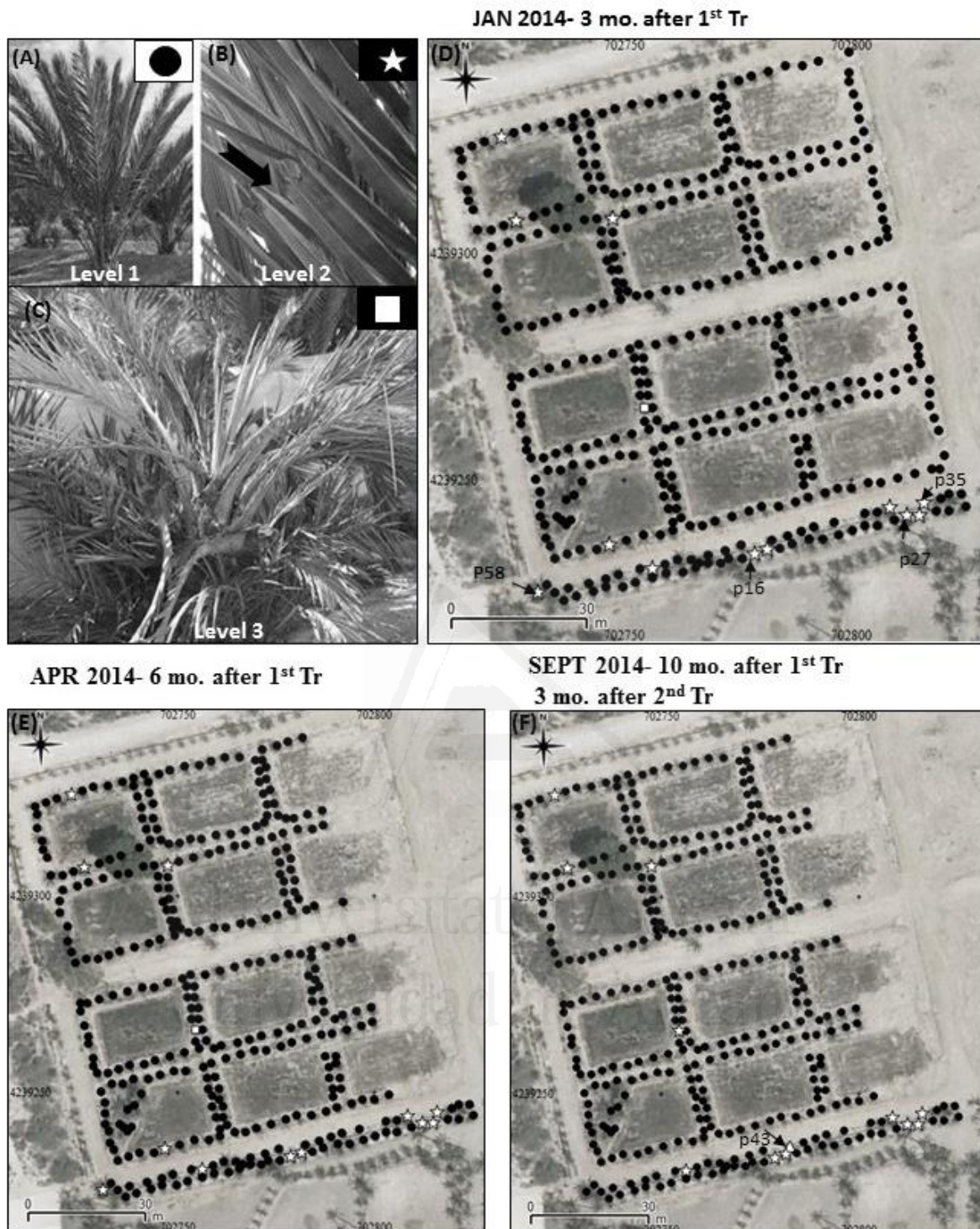
Supplementary figure 1. Visual confirmation of RPW larvae chewing activities. A) The chewing activities of RPW larvae inside an artificial tunnel in a palm petiole. B) Oscillogram (Os) and Spectrogram (Sp) show the chewing activities of RPW larvae (red box) during 3 seconds of recording.



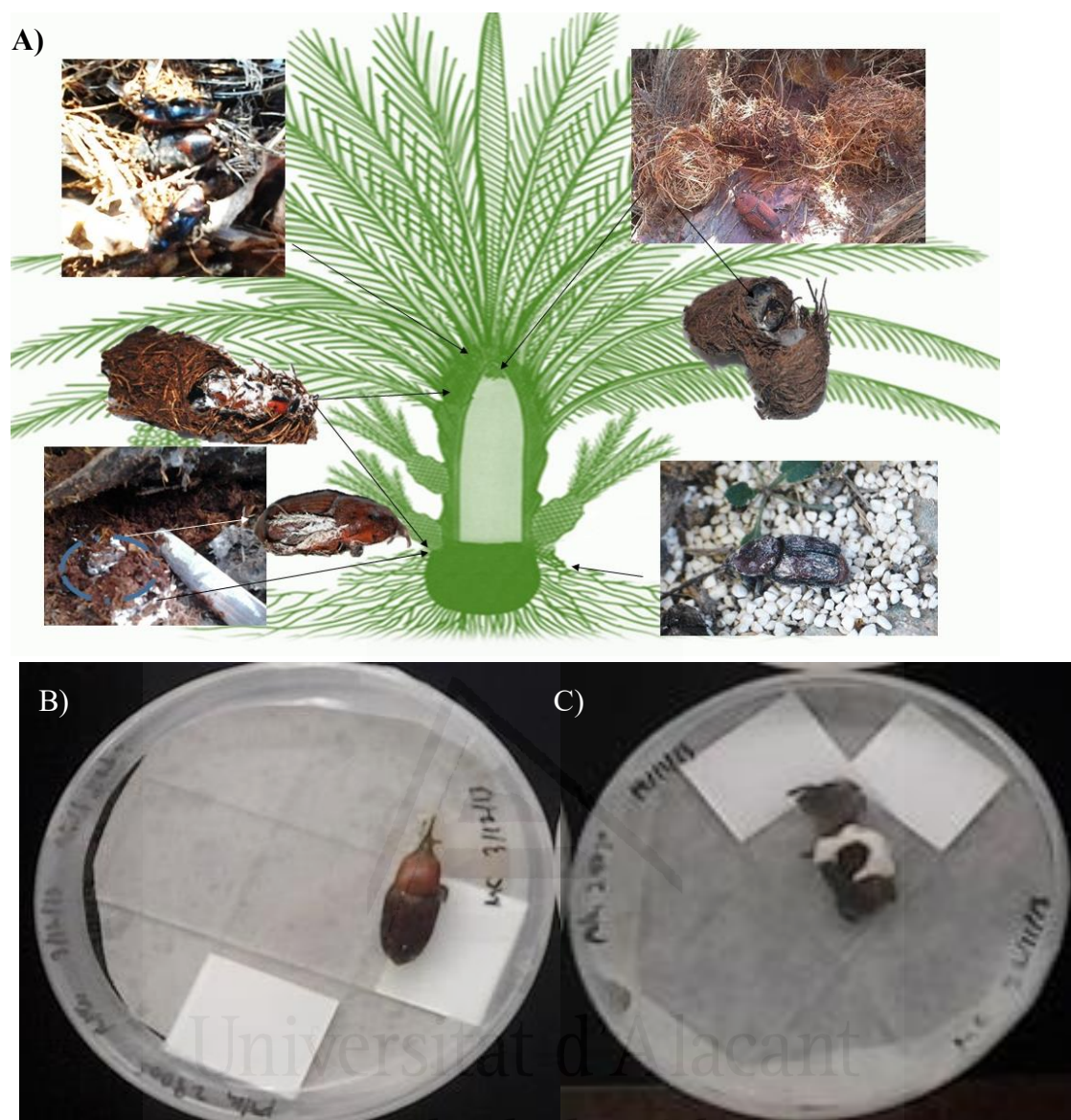
Supplementary figure 2. RPW squeal sounds. **A)** RPW squeal sounds (red box) which audibly differed from the feeding activities (blue box) were identified in an infested palm. The sound impulses audible as distinctive squeals when played back on headphones were identified in the field recordings **B)** The three RPW squeal profile spectra differ from our four original published profiles (Jalinas *et al.*, 2015) by the prominence of high-energy peaks between 1.8 and 2.1 kHz. The SQ-H profile had energy over a broad range of frequencies up to 10.9 kHz.



Supplementary figure 3. Background (“Noise”) sounds recorded in the experimental field. **A)** wind gust, **B)** wind gust mixed with leaftapping , **C)** hammer sounds **D)** siren sounds, **E)** horn sounds **F)** unknown animal, **G)** unknown insect , **H)** bird chipping. Abbreviations: OS (Oscillogram), SP (Spectrogram)

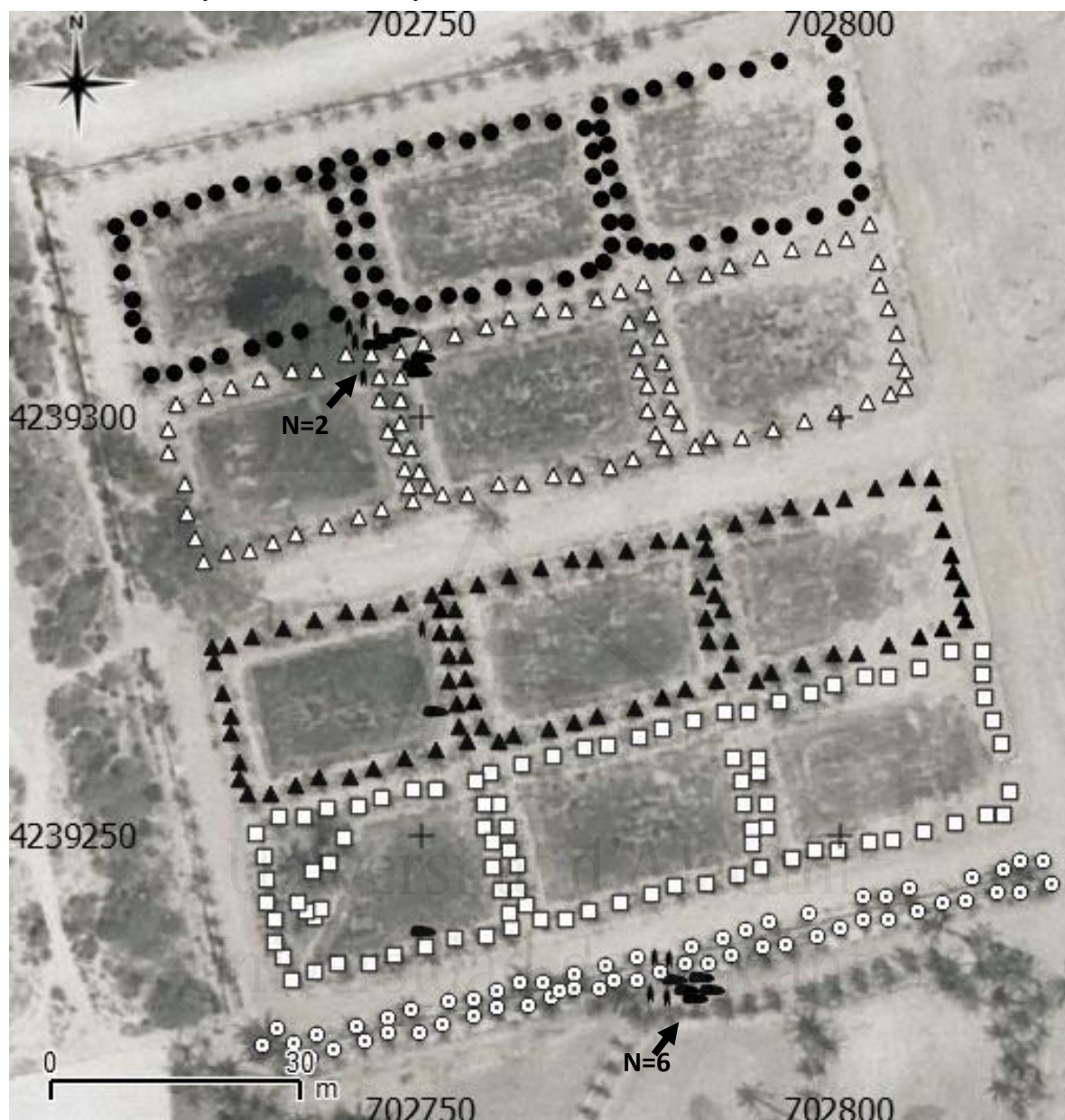


Supplementary figure 4. Visual estimation of RPW damage in palms **A)** Level 1: no RPW damage **B)** Level 2: Palm with notches in leaves (Arrow) **C)** Level 3: Leaves asymmetrically placed in the crown in **D)** January, **E)** April and **F)** September 2014. From April 2014 onwards plots had missing palms due to construction works near the Experimental Site. Abbreviations: mo. (Months), Tr (Treatment). Level 4 (dead palm) (triangle-p43).



Supplementary figure 5. RPW mortality and *B. bassiana* infection in the field
A) Sites of RPW recovery **B-C)** Mycoses for RPW collected in the field

RPW mortality in Jan-February 2014

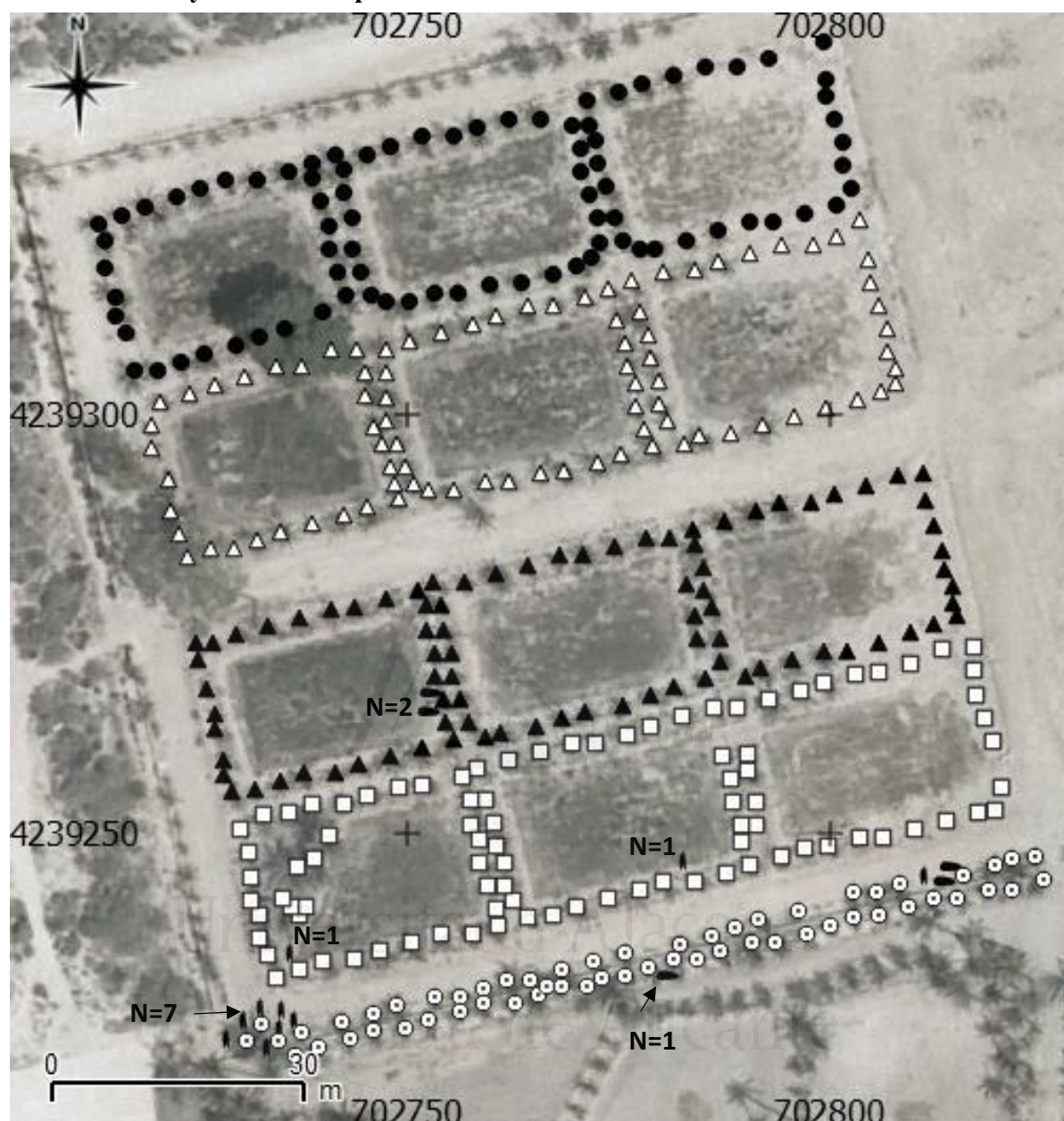


Supplementary figure 6. Spatial distribution of RPW adults in the experimental field (January-February 2014). Arrows show RPW with *B. bassiana* infection (N= number of RPW infected with *Bb*) in the field. First treatment at experimental treatment plots was October 2013 and first Imidacloprid treatment on infested palms which located outside plot was November 2013.

Treatments

- *B. bassiana*
- ▲ *B. bassiana* + Imidacloprid
- △ Imidacloprid
- control
- Outside
- † RPW adult
- RPW pupae

RPW mortality in March-April 2014

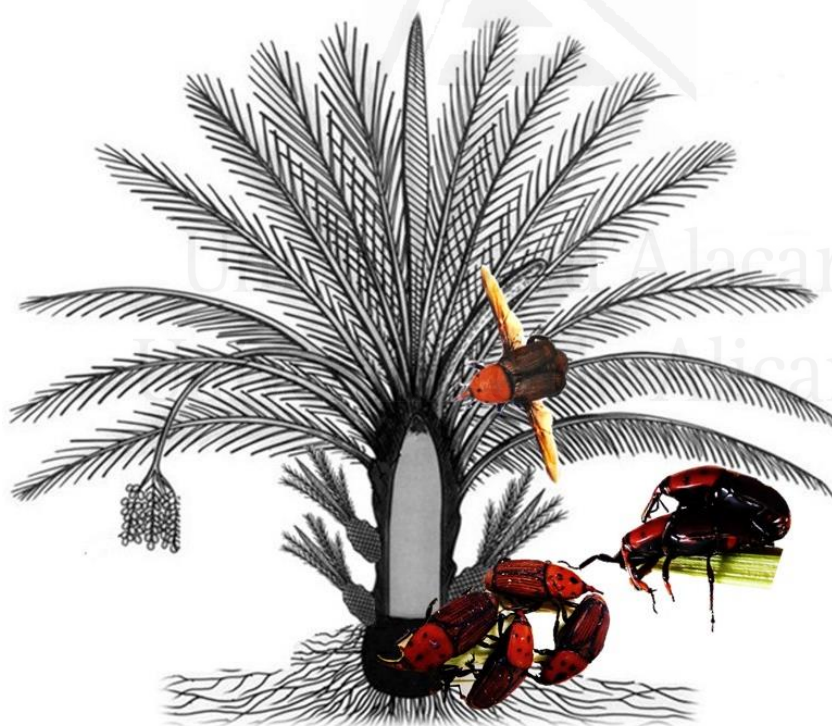


Supplementary figure 7. Spatial distribution of RPW adults in the experimental field (March-April 2014). Arrows show RPW with *B. bassiana* infection (N= number of RPW infected with *Bb*) in the field. First treatment at experimental treatment plots was in October 2013 and second treatment was in April 2014.

- Treatments
- *B. bassiana*
 - △ *B. bassiana* + Imidacloprid
 - Imidacloprid
 - control
 - ⊙ Outside
 - ┆ RPW adult
 - RPW pupae

Chapter 4

Beauveria bassiana (Hypocreales: Clavicipitaceae) Volatile Organic Compounds (VOCs) Repel *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) Females.



Chapter 4: Johari Jalinas, Frutos C. Marhuenda-Egea, and Luis Vicente López-Llorca. *Beauveria bassiana* (Hypocreales: Clavicipitaceae) volatile organic compounds (VOCs) repel *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) females. Manuscript in preparation.

Chapter 4

Beauveria bassiana (Hypocreales: Clavicipitaceae)
Volatile Organic Compounds (VOCs) Repel
Rhynchophorus ferrugineus (Coleoptera:
Dryophthoridae) Females.

4.1 Summary

Fungi produce a large number of volatile organic compounds (VOCs). Some fungal VOCs can repel insect. Entomopathogenic fungus, *Beauveria bassiana* is used to control the red palm weevil (RPW) *Rhynchophorus ferrugineus* (Oliver) and can reduce RPW infestations in the field. We demonstrate that *B. bassiana* that significantly repels RPW females in Y-tube bioassays. VOCs from *B. bassiana* were collected and analyzed by gas chromatography – mass spectrometry (GC–MS). VOCs from *B. bassiana* solid formulation differed from uninoculated rice controls. Two specific VOCs from *B. bassiana* repelled RPW females. Our findings suggest that VOCs and *B. bassiana* could act complementary to avoid RPW infestation to palms.

4.2 Introduction

The red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera:Dryophthoridae) is the most serious insect pest for date palms (Ferry *et al.*, 2002; El-Mergaway and Al-Ajlan, 2011). The invasion of RPW in Alicante Province (SE, Spain) is getting worst and it endangers the UNESCO heritage palms in cities of Elche and Alicante (South Eastern Spain). In Spain, the most common method to control RPW is using pesticides. Excessive use of insecticides causes development of resistances in insect targets and environmental pollution (Williams and Pillar, 2011). To minimize the problems caused by current treatments, research interest has been focused on the search for new and environmentally friendly strategies that can provide effective pest control. Thus, the understanding of chemical communication by RPW is essential to find better solutions to control and prevent the RPW spread to palms.

RPW invasion and spread is due its searching behaviour to acquire food (Giblin-Davis *et al.*, 1996, Poorjavad *et al.*, 2009), mates (Rochat *et al.*, 1991), oviposition and breeding sites (Saïd *et al.*, 2003). Furthermore, RPW are strong flyers (Ávalos *et al.*, 2014) which can climb very tall palms to find resources. These efficient searching mechanisms of RPW are based on antennae that function primary as chemo and mechanoreceptors (Saïd *et al.*, 2003; Antony *et al.*, 2016). These receptors provide crucial information for increasing RPW chances of survival and reproduction in the environment. Environmental signals are detected with chemosensory and mechanosensory organs which are arrayed on the antennae. Chemical signals known as semiochemicals are detected by the antennae, and these events are transformed into electrical signals that are further processed by the central nervous system (Antony *et al.*, 2016). Chemical signals such pheromones and allelochemicals intercepted by antennae may alert the insect to the presence of prospective mates, food, suitable places to lay eggs or avoiding any chemical dangers (Antony *et al.*, 2016). Thus, any chemicals that could interrupt and modify behaviour of RPW searching ability will provide a new strategy to control RPW.

Volatile organic compounds (VOCs) are carbon based solids and liquids that enter the gas phase by vaporizing at 20°C and 0.01 kPa (Pagans *et al.*, 2006). VOCs appear as intermediate and end products of various metabolic pathways and belong to numerous structure classes such as mono- and sesquiterpenes, alcohols, ketones, lactones, esters or

C8 compounds (Korpi *et al.*, 2009). Fungi produce various mixtures of gas-phases (Crespo *et al.*, 2006, Müller *et al.*, 2013) which have been shown to be involved in different biological processes such as biocontrol or communication between microorganisms and their living environment. It has been proposed that the biological interactions among insects and fungi, are mediated by volatile organic compounds (VOCs) (Yanagawa *et al.*, 2009). Fungal volatile organic compounds were repellents to a maize grain insect pest (Herrera *et al.*, 2015). The grooming behaviour of the termite was found to remove conidia of entomopathogenic fungi from the insect cuticle (Yanagawa *et al.*, 2009). These studies have revealed the importance for an organism to be able to recognize the chemical signals from the environment that surrounds it, because an incorrect identification could result in poor nutrition, in its intoxication, or it being the target of a predator.

Fungal VOCS are also being investigated for their insecticidal and repellent activities. For example, the VOCs produced by *Muscodor spp.*, including nitrosoamide, have been shown to kill insects (Strobel *et al.*, 2010). *Muscodor vitigenus* produces naphthalene, formerly used in “mothballs”, and functions as an effective insect repellent (Daisy *et al.*, 2002). In addition, VOC profiles have been correlated with varying levels of pathogenicity of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, studied for their potential as biocontrol agents to reduce termite populations (Hussain *et al.*, 2010a). The volatile profile of the virulent inocula contained n-tetradecane and alkenes, while the non-virulent strains contained many branched alkanes (Hussain *et al.*, 2010b). The ability of insects to detect and respond to entomopathogenic fungi within the order Hypocreales has been widely assessed, with reports of avoidance of fungi by species within the Coleoptera (Ormond *et al.*, 2011), Isoptera (Yanagawa *et al.*, 2009), Hemiptera (Meyling and Pell, 2006) and Orthoptera (Thompson *et al.*, 2007). In addition, VOCs from fungi showed neurotoxicity effects in *Drosophilla melanogaster* (Inamdar *et al.*, 2010).

A potential function of fungal VOCs in the interactions between fungus and insect is discussed in **this Chapter 4**. The principal objective this study was to determine the effect of a *Beauveria bassiana* solid formulate on *Rhynchophorus ferrugineus* activity. Another objective of this investigation was to investigate VOCs of *B. bassiana* to determine the potential insecticidal or repellent activities of these VOCs against red palm weevil females. This would establish a basic knowledge for developing a new RPW repellent.

4.3 Materials and methods

4.3.1 Insects and Palm Petioles Used in the Bioassays

A population of RPW adults were collected in Dolores, Alicante Province, SE Spain using pheromone traps baited with 4-methyl-5-nonanol and 4-methyl-5 nonanone (Kaakeh *et al.*, 2001). Insects were maintained in the laboratory in an incubator at $25 \pm 0.5^{\circ}\text{C}$ in darkness. Plastic boxes (40 by 30 by 21 cm) were set with a folded piece of moistened filter paper containing thin green apple slices that were replaced three times per week. Adults from the stock were sexed by visual inspection of their snouts (Prabhu and Patil, 2009). Healthy female RPW were randomly selected from the stock population of RPW for Y-tube bioassay experiments.

Palm petioles of *Phoenix dactylifera* were randomly collected in the University of Alicante, (SE Spain). Spines and leaves were removed and the stem was cut into 3 cm x 2 cm in length. These cuttings were used for the Y-tube bioassay experiments within 3 hours.

4.3.2 Entomopathogenic fungus, *Beauveria bassiana*

Entomopathogenic fungus, *B. bassiana* strain used in the experiment, *Bb* 203, was isolated from naturally infected RPW adults in southeast Spain (Daimès, Elche; CBS 121097; Güerri-Agulló *et al.*, 2010) and is maintained in the fungal collections of Glen Biotech and Department of Plant Pathology, University of Alicante. The fungus was kept in darkness at 4°C on corn meal agar (CMA; BBL Sparks, MD). A solid formulation of *B. bassiana* using rice substrate (*Oryza sativa*) was prepared according to Güerri-Agulló *et al.*, (2010).

4.3.3 Description of Y-tube olfactometer

Y olfactometers were constructed (Rhodes *et al.*, 2012) for laboratory bioassays to test the response of red palm weevil females. The olfactometer was constructed entirely from detachable heat-resistant glass tube sections each connected by airtight cone (5mm). The glass tubes had an internal diameter of 30mm, and stem 200 mm and the arms of the Y junction were all 150 mm in length. The angle between each arm and the main body was

75°. The end of each arm was joined to a glass cage (60 mm in length, 25 mm in diameter) in which odour sources were placed. The airspeed inside each arm of the olfactometer was kept constant for all experiments by natural airflow. The outlet tubes were partially covered to prevent the exit of insects (Supplementary Figure 1). When a tested substance was changed, the jars were washed with sterile distilled water (Onagbola and Fadamiro, 2011) and rinsed with n-hexane to remove all remnants of the previous odour (Doddala *et al.*, 2015).

4.3.4 Behavioral (Y-tube olfactometer) bioassays

Initial experiments for Y tube bioassays were performed to observe the ability of an RPW female to detect and to respond on attractant (palm petiole) or fungus in two-choice experiments. Three series of experiments were performed with 20 individuals each were tested in a Y-tube olfactometer and recorded for maximum 10 min when a given a choice between control arm (nothing) and stimulus arm. Stimuli were (i) fresh palm petiole of *Phoenix dactylifera*, ii) solid formulation of the entomopathogenic fungus *B. bassiana* iii) autoclaved rice. Rice (*Oryza sativa*) was used for the two choice experiments because it was the substrate of the solid of formulation of *B. bassiana*. A video camera (Logitech Carl Zeiss Tessar HD 1080P) was used for video recording of the RPW movement in Y-tube olfactometer in the Recording box 30 L (Curve plastic Iberia. S.A).

For each experiment, data obtained on the percentage of responses of red palm weevil females to each stimulus versus control arm were subjected to Pearson's chi-squared (χ^2) analyses to test for significant deviation at $p < 0.01$ from an expected 1:1 (stimulus: control) response.

4.3.5 Effect of the presence of *B. bassiana* solid formulation on the behavioral (Y-tube olfactometer) bioassays

To ascertain if *B. bassiana* was responsible for the repulsion behaviour of RPW females, another series of y-tube olfactometer test were done for RPW females (n= 10) to compare between rice inoculated with *B. bassiana* and rice only with similar methods (10 minutes). Further trials for 60 min (n= 3) of recording were also performed with a given a choice between rice and fungi. Mean time spent for RPW in the both arms was scored.

4.3.6 Analysis of volatile organic compounds (VOCs) from *B. bassiana*

The analysis of VOCs from entomopathogenic fungi requires to use an adequate adsorbent resin to capture the VOCs from fungi. In this experiment, Tenax in Glass TD Tubes 7" TD glass Gerstel tube (Supelco, Bellefonte, PA USA) was used as adsorbent resin for trapping Volatile Organic Chemicals (VOCs) from the entomopathogenic fungus *B. bassiana* grown on a rice substrate (1 month after inoculation, with 3.10^9 conidia per gram). The blank profile for VOCs analysis started with Tenax which was thermally cleaned with Gerstel ThermoDesorption System (TDS) (300 ° C, 1 h in Nitrogen stream). The peak for mass spectra of the tenax were used as control and standard blank for normal Tenax profile.

To have a rapid collection and trapping of VOCs from the entomopathogenic fungus, *B. bassiana* a 10 g sample of solid formulation of *B. bassiana* on rice substrate in a glass tube was air flowed for 10 min using an air pump. We also collected and trapped VOCs from a 20 g sample of solid formulation of *B. bassiana* on rice substrate. The end of the glass tube was connected with a Tenax tube for collecting and trapping VOCs from the sample. VOCs from a same amount of 10 g of uninoculated rice (autoclaved) were collected and trapped as explained. All processes were repeated three times.

The samples of Tenax tubes containing VOCs were subjected to thermal Desorption (TDS) as before in split into the analytic device, gas chromatography (GC) (Agilent Technologies, 6890 Network GC System) using Column 1: HP5-MS 30m×0.25mm×0.25 µm (Agilent, Waldbronn, Germany). The vaporised VOCs from the sample passes into a ionisation chamber of a mass spectrometer. The compounds separated are subsequently analyzed by mass spectrometry (Agilent, 5973 Network Mass Selective Detector). Compounds were then identified using a library database of mass spectra, or by comparison of retention times and spectra with those of known standards.

4.3.7 Behavioral (Y-tube olfactometer) bioassays on candidate chemicals

In this experiment, high volatile compound 1, C1 and low volatile compound 2, C2 were identified as *B. bassiana* VOCs and chosen as the candidate chemicals for Y-tube bioassay to test their repulsion activity towards RPW females. Fast chemical release was obtained by injecting 0.5 ml of chemicals at chemical stimuli arm. RPW female (one at time) was

than released into the opening of the central arm of the Y-tube through and exposed to two arms i) chemical stimuli and ii) control arm. A total number of 40 RPW healthy females were used for these experiments and 20 RPW randomly selected for each chemical stimulus.

Meanwhile, a modified-method for slow chemical release technique was applied (Zada et al. 2009) by using silica gel to trap and slowly release the chemical. This was done by placing 0.5 ml of chemical in 2 g of white silica gel (70-230 mesh, 60 angstrom, Aldrich). Chemical loaded silica-gel was wrapped with cotton wool and placed in the chemical glass cage (60 mm in length, 25 mm in diameter). An individual RPW female was released into the opening of the central arm of the Y-tube when control arm cage and chemical glass cage inserted into chemical stimuli arm.

RPW females were tested as for fast chemical tests (see above). All RPW were given a maximum of 10 min in the Y-tube to make a choice. A negative response or repulsion behaviour was scored when an insect either moved away from chemical stimuli arm or only stayed in control arm during the test recording. After each test, the RPW was removed, the Y-tube was rinsed with soap, n-hexane (Sigma-aldrich), and water and finally dried with tissue and filter paper.

4.3.8 Data assessment

For each experiment, data obtained on the percentage responses of RPW adult females to each stimulus versus control arm were subjected to chi-square (χ^2) analyses. R studio version 3.1.3 to test was used for significant deviation (at $p < 0.01$ from an expected 1:1 (stimulus: control) response. A positive (+) response indicates avoidance and a negative response (-) indicates attraction to the chemical tested at a given concentration.

4.4 Results

4.4.1 Passive airborne volatiles from entomopathogenic fungus, *B. bassiana* repel Red Palm Weevil Females.

The results of olfactometer Y tube indicated that RPW females ($n=20$) showed attraction to *P. dactylifera* petiole compared to control arm ($\chi^2 = 14.2383$, $df = 1$, $P = 0.0001611$).

Meanwhile the behaviour of RPW females (n=20) vs. rice and control arm showed no significant differences ($\chi^2 = 4.0599$, df =1, P =0.04391). RPW females showed repellent activity towards airborne volatiles from the entomopathogenic fungus *B. bassiana*. In a two choice experiment between Entomopathogenic fungus, *B. bassiana* and control arm, RPW females were significantly attracted to control arms ($\chi^2 = 12.8$, df =1, P =0.0003466) (Figure 4. 1).

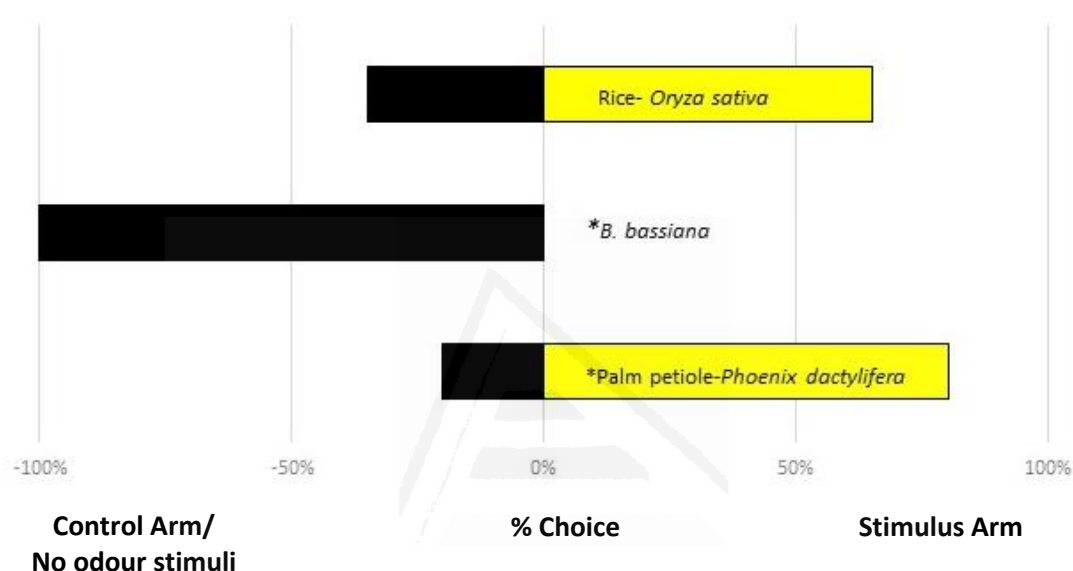


Figure 4.1 Response of *Rhynchophorus ferrugineus* female (%) in a Y-tube olfactometer (10 min) when given a choice between environmental air (control) and odour stimulus (host palms and Solid formulation of entomopathogenic fungus, *B. bassiana*). N=20 individuals per choice test. *Indicates significant difference within a choice test (P<0.01, chi-square)

We observed the choice of RPW females (n=10) on uninoculated rice vs. *B. bassiana* formulate. Eighty percent RPW female avoided *B. bassiana* arm and chose the rice arm instead during a 10 min recording. RPW female recorded for 60 min in a Y-tube avoided *B. bassiana* (0.083 ± 0.06 min) and chose rice instead (35.333 ± 13.172 min) (Figure 4.2).

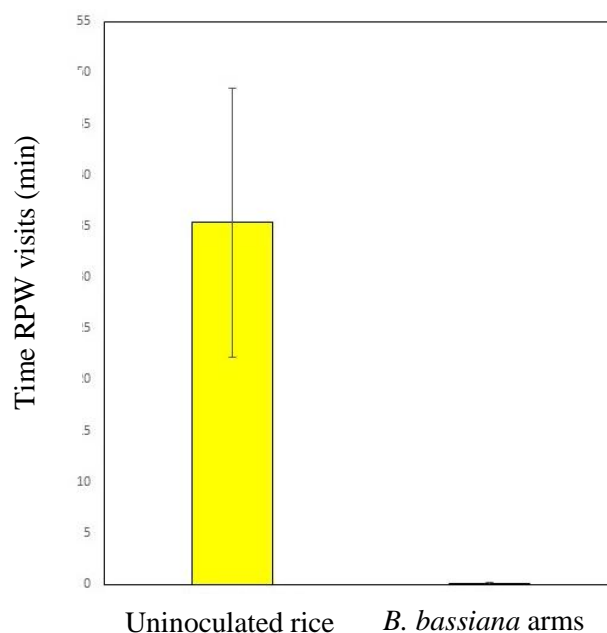


Figure 4.2. Mean time RPW female (n=3) visits in 60 min recording in Y-tube olfactometer between rice arm and *B. bassiana* arm

4.3.2 VOCs production of *B. bassiana* on solid formulation

GCMS analysis for control-blank (Tenax) detected two main peaks only (Figure 4.3A and Table 4.1) and seven peaks were found from GCMS analysis for uninoculated Rice (substrate for solid formulation, *B. bassiana*) (Figure 4.3B and Table 4.2). Eleven peaks were found from GCMS analysis for 10 g of *B. bassiana* (Figure 4.3C and Table 4.3). Finally 22 main peaks were observed from GCMS analysis for 20 g samples of *B. bassiana* formulate (Figure 4.3D and Table 4.4). Peaks 12 and 13 of 20 g *B. bassiana* samples corresponded to the same compound found in tenax sample (Peak 1, Table 4.1).

A Venn diagram analysis was performed to identify the unique VOC compounds only present in *B. bassiana* samples (Figure 4.4). This diagram was very helpful to see the intersection relation between samples. In the Venn diagram we identified 5 unique compounds from samples of 10 g *B. bassiana* (Figure 4.4A) and 15 compounds from 20 g *B. bassiana* sample (Figure 4.4 B) which were not present in tenax and rice samples.

We then identified three unique compounds present at both samples *B. bassiana* samples (Figure 4.4C). We finally chose two candidate chemicals (C1 and C2) low and high volatile *B. bassiana* unique compounds to run Y tube bioassay experiments.

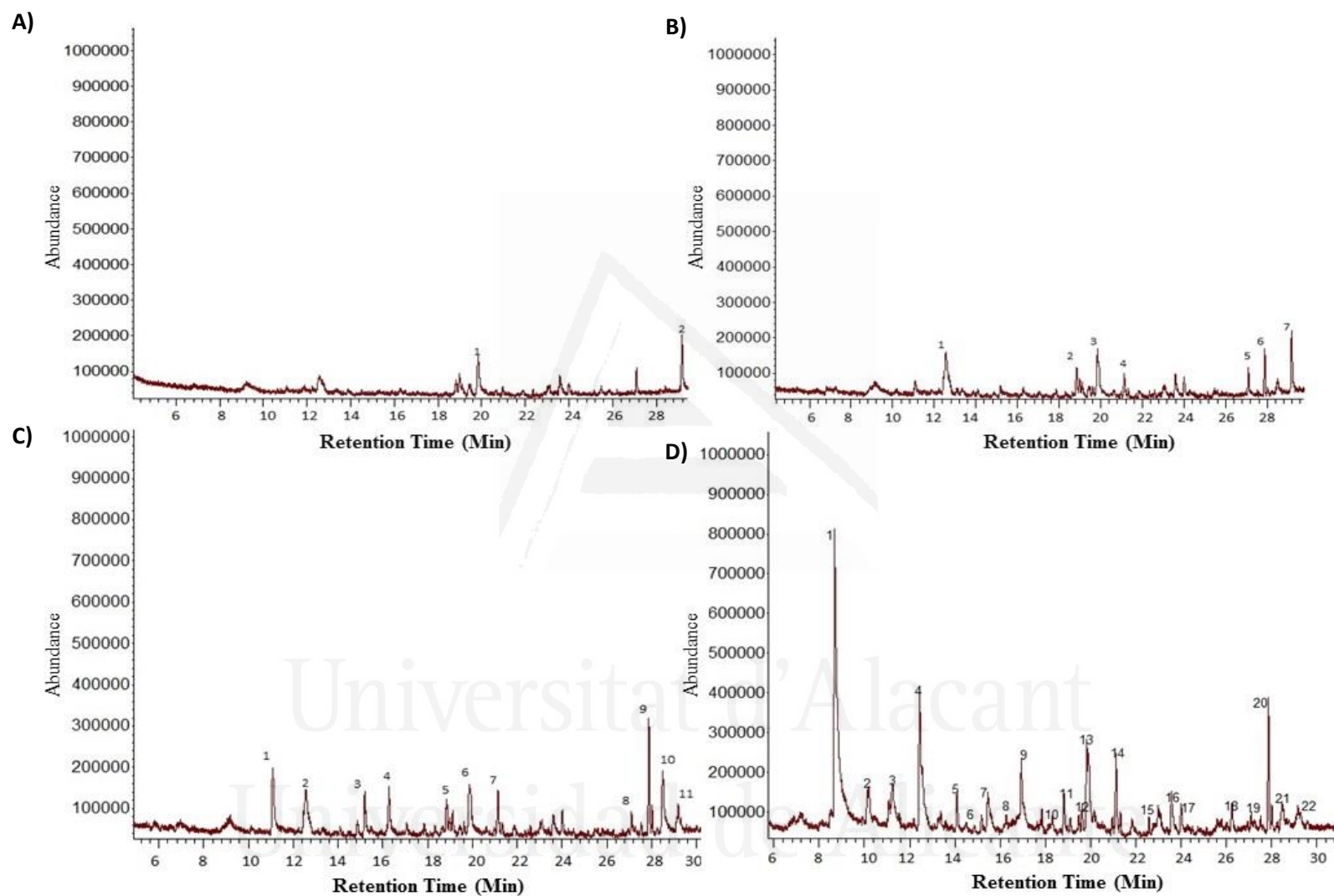


Figure 4.3. GCMS analysis. A) Tenax (control), B)Rice sample , C) 10 grams of *B. bassiana*, D) 20 gram of *B. bassiana*

Table 4.1. Volatile organic compounds (VOCs) detected from sample of Tenax (Control)

Peak No.	R.T	Corr. area	Quality	Compound
1	19.839	6625068	93%	Benzaldehyde
2	29.179	6815159	91%	1-Decanol

Table 4.2. Volatile organic compounds (VOCs) detected from Rice (*Oryza sativa*)

Peak No.	R.T	Corr. area	Quality	Compound
1	12.564	14636750	86%	Cyclotrisiloxane,hexamethyl
2	18.84	3652706	86%	Pentasiloxane, dodecamethyl
3	19.843	10330336	93%	Benzaldehyde
4	21.137	3411857	58%	Undecane, 5-methyl
5	27.099	2619884	76%	Capric Aldehyde
6	27.877	5071929	87%	Benzene, 1,4-bis(1,1-dimethylethyl)-
7	29.179	8110175	91%	1-Decanol

Table 4.3. Volatile organic compounds (VOCs) detected from samples of *B. bassiana* – 10 g

Peak No.	R.T	Quality	Compound
1	11.08	93%	CL 1
2	12.564	86%	CL 2
3	15.186	94%	CL 3
4	16.27	93%	CL 4 (C1)
5	18.84	86%	CL 5
6	19.843	93%	CL 6
7	21.137	58%	CL 7
8	27.099	76%	CL 8
9	27.877	87%	CL 9
10	28.485	93%	CL 10 (C2)
11	29.179	91%	CL 11

C1, C2 are *B. bassiana* unique VOCs used in RPW bioassays

Table 4.4 Volatile organic compounds (VOCs) detected from samples of *B. bassiana* – 20 g

Peak No.	R.T	Quality	Compound
1	8.682	86%	CH 1
2	10.202	62%	CH 2
3	11.251	50%	CH 3
4	12.448	78%	CH 4
5	14.097	81%	CH 5
6	15.186	94%	CH 6
7	15.463	96%	CH 7
8	16.27	93%	CH 8 (C1)
9	16.942	41%	CH 9
10	18.84	86%	CH 10
11	19.63	46%	CH 11
12	19.843	93%	CH 12
13	19.917	86%	CH 12
14	21.137	58%	CH 13
15	22.987	87%	CH 14
16	23.588	95%	CH 15
17	24.01	64%	CH 16
18	26.250	46%	CH 17
19	27.099	76%	CH 18
20	27.877	87%	CH 19
21	28.485	93%	CH 20 (C2)
22	29.179	91%	CH 21

C1, C2 are *B. bassiana* unique VOCs used in RPW bioassays

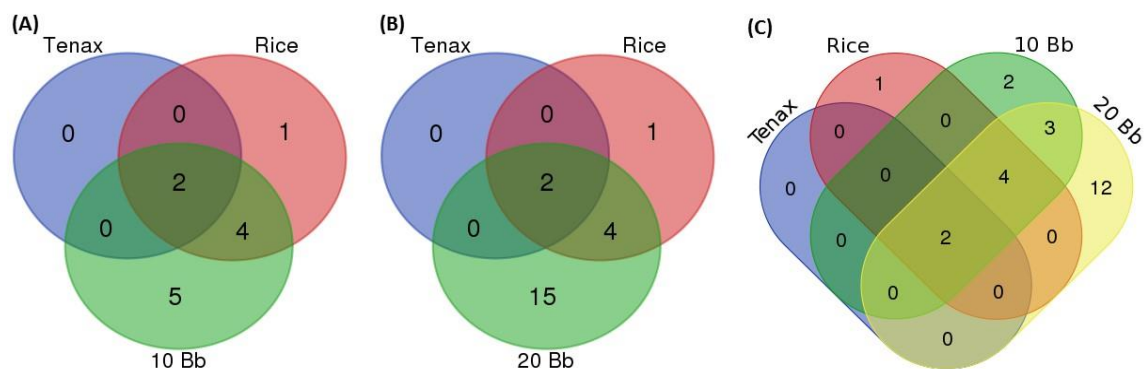


Figure 4.4. Venn diagram depicting the VOCs detected from the samples. **A** i) blank- blue, ii) rice-red, and iii) 10 gram of *B.bassiana*- green **B** i) blank -blue, ii) rice-red iii) 20 gram of *B. bassiana*-green **C** i) blank- blue, ii) rice-red, and iii) 10 gram of *B.bassiana*- green, iv) 20 gram of *B. bassiana*-yellow

4.3.3 Response of red palm weevils females to pure VOCs from *B. bassiana*

Fast VOC release technique. *Rhynchophorus ferrugineus* females showed repulsive behaviour during 10 min when exposed to either C1 ($\chi^2 = 14.2383$, $df=1$, $P=0.0001611$ or C2 ($\chi^2 = 12.9258$, $df=1$, $P=0.0003241$) using a fast VOC release technique (Supplementary Figure 2). It was noticed during the recording that RPW females which chose chemical stimuli arms immediately moved away from both chemical stimuli arms either to control arms or towards the entrance. The fast release technique showed that RPW female much struggled to reach the middle of y-tube for making the decision to which arm go to. In addition, RPW females that failed to move away from the chemical stimuli became less active after the recording

Slow VOC release technique. Results showed that *Rhynchophorus ferrugineus* females showed repulsive behaviour during 10 min exposed to both chemical stimuli for C1 in silica gel ($\chi^2 = 15.0222$, $df=1$, $P=0.0001063$) *Rhynchophorus ferrugineus* females also showed repulsive behaviour for C2 in silica gel ($\chi^2 = 15.5215$, $df=1$, $P=0.00008$) (Figure 4.5). It was noticed that RPW females were more comfortable (slow movement) to reach the middle of y-tube for making decision to which arm go to compared the fast release technique.

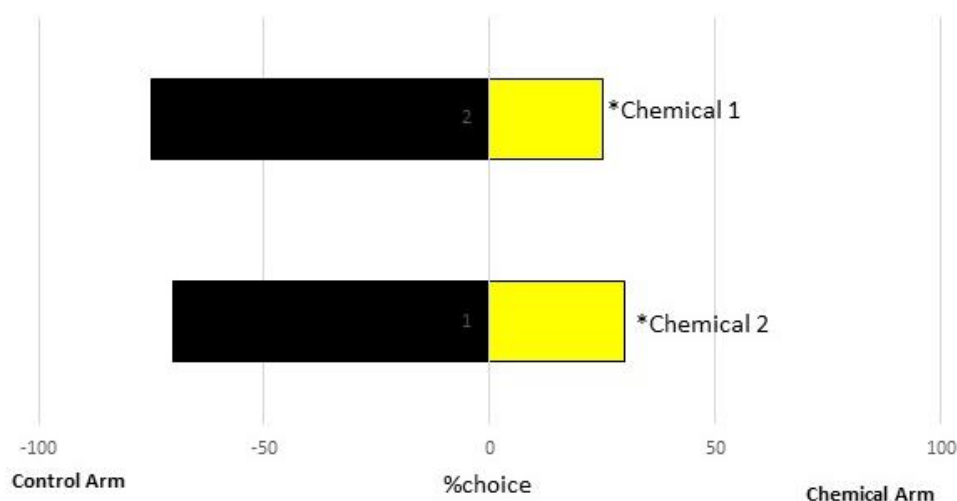


Figure 4.5. Response of *Rhynchophorus ferrugineus* female in a Y-tube olfactometer (10 minutes) when given a choice between environmental air (control) and odor stimulus (Chemical stimuli) with a slow release polymer (silica gel). N=20 individuals per choice test. *Indicates significant difference within a choice test ($P < 0.01$, chi-square).

4.4 Discussion

Our results demonstrate that red palm weevil females are significantly attracted to y-tube arm which contained palm petioles (host palm) compared to control arm. A previous study showed that *Rhynchophorus ferrugineus* are attracted to palm esters (kairomones) emitted by host palms (Guarino *et al.*, 2011). The results show that the Y-tube system is an adequate technique to observe the ecological behaviour of red palm weevil. Using this technique, RPW females showed avoidance to the entomopathogenic fungus, *Beauveria bassiana* and significantly chose control arm. In addition, RPW females showed no significantly effect in choosing between arm control and rice which was used as substrate for the *B. bassiana* solid formulation. The avoidance behaviour observed in our experiments are similar to findings of avoidance of fungi by species within the Coleoptera (Ormond *et al.*, 2011., Seidey *et al.*, 2015), Isoptera (Yanagawa *et al.*, 2009), Hemiptera (Meyling and Pell, 2006) and Orthoptera (Thompson *et al.*, 2007). The avoidance of entomopathogenic fungi (natural enemies) indicate that the red palm weevil females could detect a danger especially in finding suitable places to find food or lay eggs. Entomopathogenic fungi also affect oviposition behaviour in the parasitoid wasp *Trybliographa rapae* (Rännback *et al.*, 2015)

It was noticed that *B. bassiana* growing on solid formulation produced a distinctive odour different from that of autoclaved rice (*Oryza sativa*), the substrate used to grow the

fungus. This distinctive odour is due to fungal VOCs which are produced during both primary and secondary metabolism (Crespo *et al.*, 2006). Different carbon sources used to grow fungi produce different VOCs profiles through metabolic pathways leading to the formation of *B. bassiana* volatiles (Crespo *et al.*, 2006). In this experiment, we detected and identified VOCs emitted by entomopathogenic fungus, *B. bassiana* when growing on rice. Benzene derivatives, benzeneacetaldehyde derivatives, straight even-chain saturated hydrocarbons of 10–12 and 16 carbons, *n*-Decane, ketones and alcohol groups were detected. These groups of compounds were also found by Crespo *et al.*, 2006. In addition, the results showed as the amount of *B. bassiana* formulate increased, the number of VOCs detected. In this experiment, we found three *B. bassiana* unique VOCs that were present from *B. bassiana* samples of 10 g and 20 g. These two candidate chemicals were chosen from high volatile (low retention time) and less volatile (high retention time) compounds.

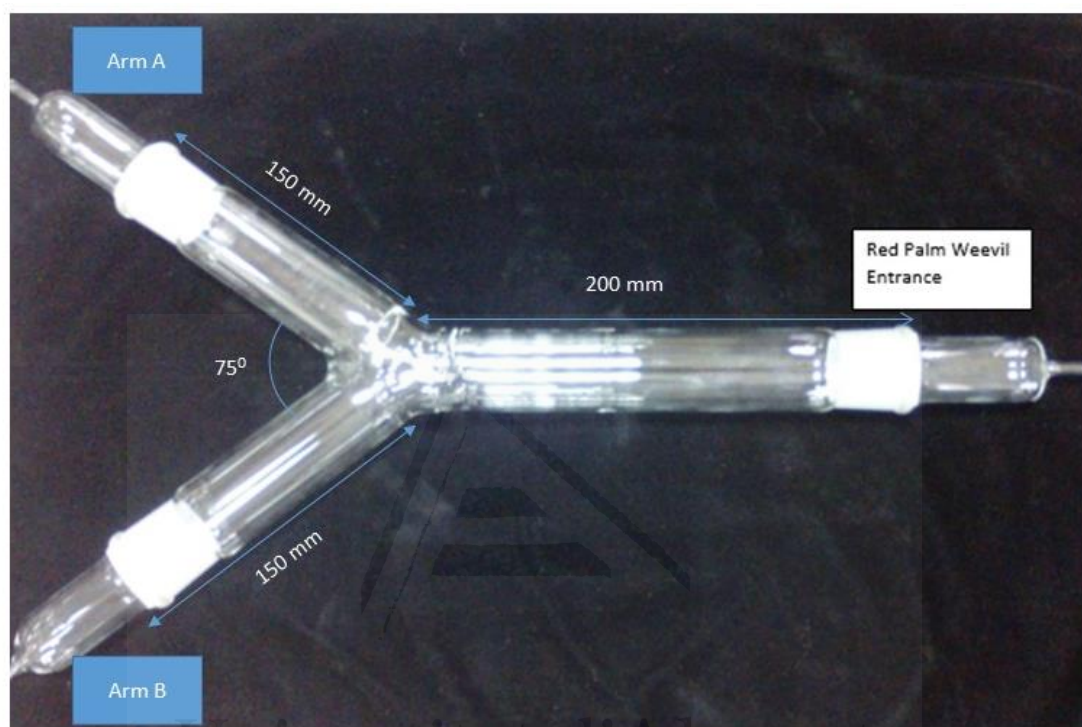
In this study, RPW females showed repulsion effect when exposed to candidate chemicals in y-tube for fast and slow release techniques for both candidate chemicals. In modified slow release technique using silica gel, RPW females smoothly moved smoothly in the y-tube and made the decision in the middle of y-tube to move to y-tube arms. When, female RPW reached to arm control, it remained there. Conversely, female RPW showed repulsion effects when they chose chemical stimuli arm. Gel polymers have been previously used as a technique for slow release of pheromones (Zada *et al.*, 2009). These gels allow a constant rate of chemical release.

These results support our hypothesis that VOCs from the entomopathogenic fungus, *B. bassiana* are responsible to repel RPW females. Previous studies indicate the ability of insects to detect and respond to entomopathogenic fungi within the order Hymenoptera, with reports of avoidance of fungi by species within the Coleoptera (Ormond *et al.*, 2011). In addition, RPW females after exposure to active VOCs from *B. bassiana* become inactive or move slowly. The slowness in movement of RPW suggested that *B. bassiana* active VOCs possess neurotoxicity. A previous study showed fungal produced VOCs which had neurotoxicity effects on mobility of *Drosophila melanogaster* (Inamdar *et al.*, 2010). As RPW repellent agents should allow the use of these bio-chemicals products used in the field.

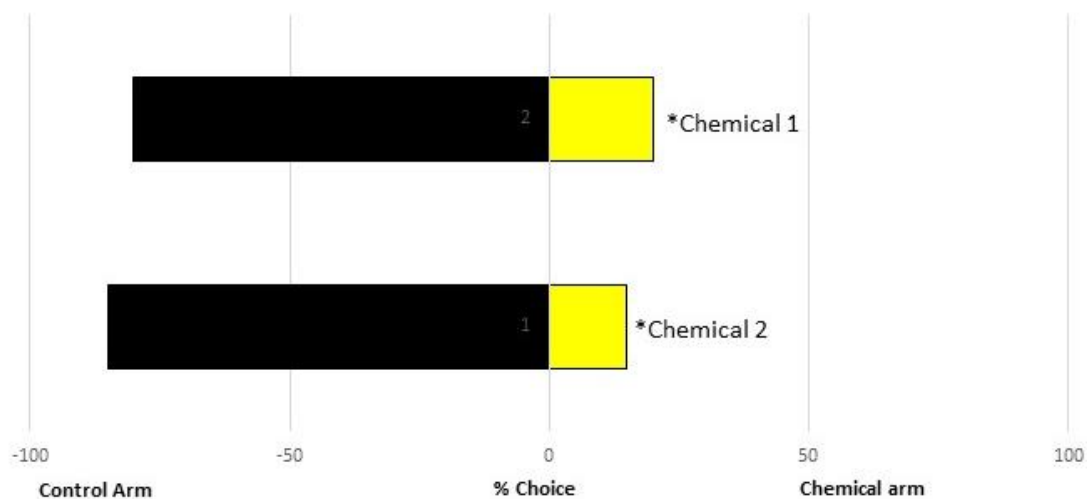
Finally our results show that the use of *B. bassiana* VOCs repellent to RPW is a new approach to manage RPW. Further development of gel dispensers for trapping these

chemicals (RPW repellents) could easily be implemented in IPM programmes for RPW sustainable field management.

Supplementary figures



Supplementary figure 1. Tube Y olfactometer to test the response of adult red palm weevil (female) on different chemical stimuli.



Supplementary figure 2. Response of a *Rhynchophorus ferrugineus* female in a Y-tube olfactometer (10 minutes) when given a choice between environmental air (control) and odour stimulus (Chemical stimuli) individuals per choice test. *Indicates significant difference within a choice test ($P < 0.01$, chi-square)

Chapter 5

General discussion and concluding remarks



Chapter 5

General discussion and concluding remarks

The current red palm weevil (RPW) invasion and infestation is increasing throughout the world. New locations and records for RPW infestation have been identified worldwide. The wide host range (26 palms species in 16 different genera) for RPW has lead this insect to become cosmopolitan (Dembilio and Jaques, 2015). Economic damages by RPW infestations are very common for countries which grow susceptible host plants such as date palm, *Phoenix dactylifera* (Ferry *et al.*, 2002), canary palm, *Phoenix canariensis* and coconut trees (Faleiro *et al.*, 2003). Red palm weevil infestations formerly detected mostly in date and canary palms in Spain, have already reached another palm of economic importance, *Washingtonia filifera* (Dembilio *et al.*, 2015). In South East Asia which is believed to be the origin migration point of red palm weevils, RPW infestations have caused economic damage on coastal coconut trees plantation in Malaysia (Azmi *et al.*, 2014). There is also a potential for RPW destroying oil palm industry in Malaysia based on RPW captures in the pheromone traps in oil palm plantations (Idris *et al.*, 2014). The palm or host plant damage caused by RPW also could potentially lead to other opportunistic pest/pathogen organisms such as nematode ring disease and plant pathogenic fungal diseases such as *Fusarium* wilt (Elliott, 2009). This will reduced even more the survival and the growth of the host plants (Giblin-Davis *et al.*, 2013). Improvement of RPW management in the field is especially required in palm protected areas for reducing economic losses caused by this insect. Earliest RPW detection such as that achieved using acoustics is important to prevent massive RPW infestations extremely difficult to control.

Entomopathogenic fungi such as *B. bassiana* are natural enemies of insects. They have been exploited as biological control agents for managing pest insects (Lacey *et al.*,

2001; Shahid *et al.*, 2012) including RPW (EPPO 2008). Certain entomopathogenic fungi may have special hosts but many entomopathogenic fungi including *B. bassiana* have a wide host range (Toledo *et al.*, 2007). This makes them suitable for controlling many different insect pests in agricultural fields. The use of biological control agents is increasing as alternative treatment strategies to reduce the usage of chemical pesticides in the environment (Butt *et al.*, 2011). Combination of entomopathogenic fungi, chemical insecticides and other natural chemical repellents (semiochemicals) is a way to improve the RPW management treatments as well as to decrease the amount of pesticides used in the agricultural fields.

In Chapter 2 of this PhD Thesis, I have investigated the effects of *B. bassiana* treatments against RPW larval inside the host palms using acoustic detection techniques. The feeding and moving activities of hidden RPW larvae inside the palms produce sound impulses which could be detected by acoustic sensors. The acoustic sensors act as sound pre-amplifiers and sound transducers. They both detect and transmit the emitted sound waves and vibrations emitted by insects which cannot be detected by human ears (Mankin *et al.*, 2011). The emitted sounds are amplified by specific acoustic amplifiers such as AED 2010 which convert the sound waves into electrical waves which could be audio digitized and recorded in the sound recording instruments. The recorded sound can be analysed with the acoustic software for further investigation on temporal and spectral pattern for RPW larvae infected with *B. bassiana*. The decreasing activity of acoustic signals emitted inside the palms which contained RPW larvae treated with the entomopathogenic fungi indicates that the fungus killed the RPW larvae and feeding activity stopped. In our study, a 10^6 conidia ml^{-1} dose of *B. bassiana* reduces feeding activities and effectively kills RPW larvae which otherwise would have caused internal damage on the host palms. The process of detecting RPW larvae activity during the treatments suggest us that this acoustic technique also could be used for managing other species of *Rhynchophorus* such as *Rhynchophorus cruentatus* (Dosunmu *et al.*, 2014) which has similar life history and similar economic impact.

The results of **Chapter 2** of this PhD thesis also suggest us that the effects of biological control agent such as entomopathogenic fungi *B. bassiana* against *R. ferrugineus* can be assessed successfully by acoustic methods. These methods compare different aspects of larval movement and feeding activity: the rate of bursts, the count of

impulses per burst, and the rate of burst impulses. Therefore, the acoustic methods can be used as tools for non-destructive assessment of effects of biological control treatments against RPW under semifield conditions (e.g greenhouses). The use of acoustic methods could assist in optimization of doses and delivery methods for improving entomopathogenic fungal treatments on RPW activities. Furthermore, biological control using entomopathogenic fungi such as *B. bassiana* should be seriously considered as an important component in RPW Integrated Pest Management (IPM) in the field. This was considered in **Chapter 3** of this thesis.

The main challenge of using entomopathogenic fungi under field conditions is the evaluation of their effects against insects which are living in a hidden place. The current technique is based on the evaluation of the external damages to palms caused by RPW larvae through visual observation. This technique relies on external symptoms that appear late when the host is heavily infested. The results from **Chapter 2** provided us a solution to use acoustic detection tools for assessment of RPW field management with *B. bassiana* using acoustic detection tools and automated RPW signal analysis.

We have also explored in this PhD Thesis (Chapter 3) the potential of acoustic monitoring to validate the effects of RPW management treatments under field conditions. The integration of information from RPW infested palms with acoustic detection, visual RPW symptoms and palm damage in the GIS allowed us to study time-space effects of RPW management treatments in the field. Our acoustic method let us monitor, for instance, the dynamics of RPW activity near the Historical Palm Grove of Elche (SE Spain). The acoustic technique also has been used in in other parks such as ‘Pedion Areos’ in Athens (Greece) for RPW management monitoring in Canary palms (Soroker *et al.*, 2013).

The results for **Chapter 3** demonstrated acoustic detection of RPW infestation under field conditions. The combination of temporal and spectral analysis of the background sounds was an important step of the detection process to reduce the rate of misclassified field sounds. This is relevant because some of them had spectra with peaks similar to those of with RPW spectral profiles (Mankin *et al.*, 2008a). In addition, larvae of other similar species such as *Rhynchophorus cruentatus* can be detected acoustically in host trees and fronds in both exposed and enclosed environments (Dosunmu *et al.*, 2014). Newly characterized “squeal” sounds improved the process of acoustic detection of RPW activity in the field. Future studies are needed to understand the mechanism of RPW

“squeal” sounds production. Thus, early detection for RPW infested palms afforded by the use of acoustic enables will enable palm grove managers to undertake suitable monitoring and control measures at an early stage of infestation. This prevents further build-up of RPW population in the field.

Geographic information system is an efficient tool in area-wide RPW–IPM (Integrated Pest Management) programmes in many countries who faced heavy RPW infestation such as Saudi Arabia (Massoud *et al.*, 2011). GIS can log data pertaining to RPW activity assessment, and also track infestation reports (Chefaoui *et al.*, 2005). Our results show that, the integration of information from RPW infested palms with acoustic detection, visual RPW symptoms and palm damage in the GIS allows the study of time-space effect of IPM to RPW in the field.

Geostatistical methods of GIS systems have been used to estimate oviposition and egg hatching periods of RPW in different temperature regions from the Mediterranean basin (Dembilio and Jacas, 2012). The prediction of potential worldwide distribution of the red palm weevil *Rhynchophorus ferrugineus* also has been investigated based on 132 world localities where red palm weevils have been detected (Fiaboe *et al.*, 2012). Our results suggest that, spatial distribution of RPW in the field implemented with acoustic information in GIS systems could be further used in the future to estimate and predict the risk of RPW infestation in whole plantations in Elche and Orihuela (SE, Spain) which are the main locations for palms groves in Spain (Ferry *et al.*, 2002).

In Chapter 3, we also applied a combination of *B. bassiana* and a chemical insecticide (Imidacloprid) under field conditions. Our results in palm plots treated with this combination showed less RPW infested palms compared to control untreated palms. In addition, repeated use of *B. bassiana* treatments in our IPM programme induced artificial epizootics in RPW field populations. This result was found for in Egypt by Sewify *et al.*, (2009), *B. bassiana* is therefore an adequate biological control agent to reduce RPW in integrated pest management programs.

In addition, the cross science fields between research groups of biology and chemistry discovered the toxin properties of entomopathogenic fungi that had caused the dead of insect host (Kucera, 1971) and the enzymes responsible for penetration of entomopathogenic fungi through insect cuticle (Pedrini *et al.*, 2007; Dias *et al.*, 2008; Meng

et al., 2015). Scanning Electron microscopy contributed to understanding the details of the infection process of RPW by entomopathogenic fungus, *B. bassiana* (Güerri-Agulló *et al.*, 2010).

Our results in **Chapter 3** in the field shows spatial distribution of RPW activity only aggregated at the palms outside the experimental treatments plots and neither in *B. bassiana* nor in the combination of *B. bassiana* and Imidacloprid treatments. Entomopathogenic fungi share a co-evolutionary history with arthropods. Secondary metabolism from entomopathogenic fungi produces VOCs (Crespo *et al.*, 2006) that modify insect behaviour (Baverstock *et al.*, 2010; Zimmermann, 2007). Entomopathogenic fungi price is still expensive in the market. The use of natural organic chemicals as repellent agents is a new approach to reduce RPW infestation in field. They could be used together with entomopathogenic fungi in the integrated pest management application. The potential of VOCs from entomopathogenic fungi as repellents of the RPW is investigated in **Chapter 4** of this thesis.

In Chapter 4 of this thesis, our results show that a modified Y-tube system (2-arm bioassay) was an adequate to analyze the behaviour of red palm weevils in response to stimuli. Using this technique, we discovered that RPW females avoided the entomopathogenic fungus *B. bassiana* and significantly chose control arm (y-arm with no stimulus). To this respect we have also observed (Jalinas, 2015; unpublished results) that *B. bassiana* infected RPW individuals repel healthy RPW. However, RPW females showed no significant difference in choosing between control arm and uninoculated rice. The latter was used as substrate for developing *B. bassiana* solid formulation used in our experiments. The avoidance behaviours observed in this experiments using the whole fungus are also similar using only the VOCs detected from *B. bassiana* samples. The avoidance of red palm weevil females to entomopathogenic fungi (natural enemies) indicate that red palm weevils could detect a danger especially in finding suitable places to find food or lay eggs. Entomopathogenic fungi also affect oviposition behaviour in the parasitoid wasp *Trybliographa rapae* (Rännback *et al.*, 2015). RPW females also showed repulsion effect when exposed to pure VOCs identified from *B. bassiana*. These VOCs could be used in the future in combination with *B. bassiana* as a new alternative for sustainable management of RPW. This will help reducing chemical insecticides in the RPW IPM programmes.

To conclude, the results of this PhD thesis suggest that there is a practical potential using acoustic techniques on assessment for RPW infestation. Acoustic techniques could be integrated in GIS systems to monitor RPW infestation and also to evaluate RPW management treatments under real field conditions. The findings of *B. bassiana* repellent effects on RPW movement will open a new treatment strategy for sustainable management of RPW populations.

The main conclusions obtained in this thesis are the following:

1. Acoustic techniques can monitor the progress of infection of RPW with *B. bassiana* under semifield conditions
2. RPW acoustic detection in the field can be improved by noise elimination and adoption of new “Squeal” sounds.
3. Combinations of Imidacloprid and *B. bassiana* improved the protection of palms from the RPW invasion in a young date palm plantation.
4. Application of GIS based method monitored the spatial RPW infestation and invasion and the evolution of RPW management treatments based on acoustic information.
5. VOCs of *B. bassiana* repels RPW females in bioassay laboratory

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Chapter 6

Resumen general



Chapter 6

Resumen general

Esta tesis doctoral contribuye a la comprensión del comportamiento en el campo del picudo rojo (RPW) *Rynchophorus ferrugineus* (Olivier) (Coleoptera: Dryophthoridae). Esta tesis también investiga el uso práctico de los agentes de control biológico (BACs), el hongo entomopatógeno *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Clavicipitaceae) en el manejo integrado de plagas (MIP) para la supervisión, control y evaluación de los efectos de los BACs en RPW en la gestión del terreno mediante detección acústica y Sistemas de Información Geográfica (SIG).

En el **Capítulo uno** de esta tesis se discute sobre palmeras de gran importancia económica y cultural en España. Se ha visto que los problemas de infestación por RPW tienen un efecto negativo sobre el paisaje de algunos lugares turísticos importantes en España. En este capítulo también se ha discutido el comportamiento y el ciclo de vida del RPW con el fin de comprender la etapa perjudicial del RPW en las palmeras huéspedes. El histórico Palmeral de Elche (Alicante, España) está altamente expuesto al riesgo de infestación por RPW. Se ha visto y explorado el actual MIP para RPW. Para proteger las palmeras y prevenir la infestación por RPW se han utilizado insecticidas químicos, el uso elevado de estos productos podría dar lugar a problemas medioambientales. Así, la integración de BACs como hongos entomopatógenos tales como *B. bassiana* podrían reducir el uso de químicos para el tratamiento de RPW.

Se ha observado que *B. bassiana* es patógeno de *R. ferrugineus* tanto en laboratorio como en estudios de campo. Los tratamientos con *B. bassiana* son aplicados en la corona de la palmera (base del peciolo), que es la parte más susceptible a la ovoposición de las hembras de RPW. *B. bassiana* tiene un ciclo de vida multitrófico y puede colonizar endofíticamente los peciolos de las palmeras. La transmisión pasiva mecánica de hongos entre las poblaciones de insectos ha sido detectada en diversos hongos entomopatógenos, entre ellos *B. bassiana*. Los adultos que fueron infectados con esporas de *B. bassiana* transfirieron la infección a las larvas.

La infección de las larvas se mantuvo en el interior de las palmeras hasta emerger en su etapa adulta. No ha sido establecida la realentización en la alimentación y el aumento de la mortalidad de larvas de *R. ferrugineus* tras el tratamiento con *B. bassiana*. Esta tesis investiga el uso de métodos acústicos para evaluar los efectos de larvas de RPW tratadas con *B. bassiana* en la planta huésped, lo que se discute en el **Capítulo dos** de esta tesis.

En el **Capítulo dos**, se hace un estudio utilizando métodos acústicos para evaluar los efectos de los impulsos de sonido producidos por larvas de RPW sin tratar y tratadas con 10^4 y 10^6 conidios de *B. bassiana* en palmeras. Los análisis se realizaron para identificar secuencias de impulsos con patrones característicos (ráfagas) producidos frecuentemente por el movimiento y la alimentación de las larvas, y además raramente tienen interferencias por el ruido de fondo o las vibraciones de los árboles. Las tasas de ráfagas, los recuentos de impulsos de larvas por ráfaga y las tasas de impulsos en ráfagas disminuyeron de forma significativa en el tiempo en ambos tratamientos con *B. bassiana* pero no en el control (sin tratar).

Mientras tanto, en el campo, los daños de RPW a las palmeras y los efectos del MIP fueron evaluados en palmeras individuales utilizando escalas de síntomas. Los adultos de RPW fueron monitorizados en el campo con trampas de feromonas, aunque estas no están permitidas en el patrimonio histórico español del Palmeral. En el **Capítulo tres** de esta tesis, se estudia la aplicación de métodos de evaluación acústica y técnicas GIS para monitorizar la propagación espacial de la actividad de las larvas de RPW en una parcela de un palmeral con tratamientos de manejo de RPW cerca del palmeral histórico de Elche. La presencia de RPW fue detectada mediante técnicas GIS para determinar los efectos del agente de control biológico *Beauveria bassiana*, los efectos del control químico (Imidacloprid) y los efectos de su combinación contra RPW comparada con los controles sin tratamiento.

Los hongos producen un gran número de compuestos orgánicos volátiles (COVs). Algunos COVs pueden repeler insectos. En el **Capítulo cuatro** de esta tesis se demuestra que *B. bassiana* repele significativamente a las hembras de RPW en bioensayos en laboratorio. Los COVs de *B. bassiana* fueron recogidos y analizados por cromatografía de gases – espectrometría de masas (GC-MS). Los COVs de una formulación sólida de *B. bassiana* difieren de aquellos de controles de arroz sin inocular. Se han encontrado dos COVs específicos de *B. bassiana* que repelen a las hembras de RPW.

Esta tesis doctoral concluye en los **Capítulos dos y tres** que hay un potencial considerable para el uso de métodos acústicos como herramientas de evaluación no destructiva de los efectos de los tratamientos de control biológico y de control químico contra plagas de insectos. La detección acústica mejorada en patrones de sonidos de larvas de RPW grabados en palmeras infestadas en el campo ha permitido la detección y los métodos GIS han permitido el mapeo del riesgo de la actividad de RPW dentro de los tratamientos experimentales. Se encontró que los tratamientos con *B. bassiana* e Imidacloprid reducían la infestación por RPW en el campo. La actividad de RPW en palmeras infestadas en el campo puede ser monitorizada satisfactoriamente con una combinación de métodos acústicos, visuales y GIS. Estos resultados pueden ayudar a tomar decisiones de tratamiento para controlar infestaciones de RPW en el campo. Los últimos descubrimientos en el **Capítulo cuatro** en bioensayos de laboratorio sugieren que los COVs y *B. bassiana* podrían actuar de forma complementaria para evitar la infestación de las palmeras por RPW.



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Chapter 7

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Chapter 8

Anexo



Date palm, (*Phoenix dactylifera*) in the University of Alicante

Acoustic Assessment of *Beauveria bassiana* (Hypocreales: Clavicipitaceae) Effects on *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) Larval Activity and Mortality

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ABSTRACT *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Dryophthoridae) is an economically important pest of palm trees in the subtropics. *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Clavicipitaceae), has been shown to be pathogenic against *R. ferrugineus* in laboratory and field studies. However, because they remain inside the trunks until adulthood, the slowing of feeding and increases in mortality of internally feeding *R. ferrugineus* larvae over time after *B. bassiana* treatment has not been established. To explore the potential of acoustic methods to assess treatment effects, sound impulses produced by untreated, 10^4 -, and 10^6 -conidia ml^{-1} *B. bassiana*-treated larvae in palms were recorded for 23 d, after which the palms were dissected and the larvae examined. Analyses were performed to identify trains of impulses with characteristic patterns (bursts) produced frequently by moving and feeding larvae but only rarely (3–8% of the larval rate) by interfering background noise or tree vibrations. The rates of bursts, the counts of larval impulses per burst, and the rates of impulses in bursts decreased significantly over time in both *B. bassiana* treatments but not in the control. This supports a hypothesis that larvae had briefer movement and feeding bouts as they became weaker after infection, which reduced the counts of larval impulses per burst, the rates of bursts, and the rates of impulses in bursts. There is considerable potential for use of acoustic methods as tools for nondestructive assessment of effects of biological control treatments against internally feeding insect pests.

KEY WORDS detection, entomopathogenic fungi, biological control

Rhynchophorus ferrugineus (Olivier) (Coleoptera: Dryophthoridae), the red palm weevil, causes significant damage to a wide range of palm species worldwide. In Spain, *R. ferrugineus* is an important pest of date [*Phoenix dactylifera* L. (Arecaceae: Arecaceae)] and canary palms (*P. canariensis* Chabaud; Ferry et al. 2002, EPPO 2008). Adults can be monitored and trapped with pheromone–food attractant baits, but the larvae feed hidden inside the trunks, making it difficult to detect and control (Fiaboe et al. 2011).

In Spain and other Mediterranean countries where infestation is prevalent in urban areas, there is a strong emphasis on the development of integrated pest

management strategies based on chemical treatments, pheromone traps (Faleiro and Chellapan 1999), and biological control such as entomopathogenic fungi (Shah and Pell 2003, Gindin et al. 2006, Dembilio et al. 2010, Güerri-Agulló et al. 2011). The most commonly used control treatments are insecticides such as Diazinon, Imidacloprid, and Phosmet (Abbas et al. 2010). However, heavy use of chemical treatments causes environmental damage and harms nontarget organisms, and also leads to the development of insecticide resistance. Pheromone traps are excellent monitoring devices but capture only adults, leaving the more harmful larvae to destroy the trunk and emerge later. Moreover, the potential for adults to be attracted to palm trees located near pheromone traps (Roda et al. 2011) and the capability of adults to escape from dry or nearly dry traps (Fiaboe et al. 2011) suggests that pheromone traps may be more useful monitoring or control tools in commercial palm areas than in zones with highly valuable historic palms like Elche-Alicante, Spain. Consequently, there remains interest in development of additional alternatives such as entomopathogenic fungi for *R. ferrugineus* management.

Beauveria bassiana (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) is an entomopathogenic fungus that has been shown to cause *R. ferrugineus* mortality

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Conferences and Seminar

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Use of acoustic technology to monitor the time course of *Rhynchophorus ferrugineus* larval mortality in date palms after treatments with *Beauveria bassiana*. *97th Annual Meeting of the Florida Entomological Society* August 3 - 6, 2014 - Jupiter, FL

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