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# Bacteria-mediated RNA interference in *Rhynchophorus ferrugineus* (Olivier, 1790)


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O Presidente do Júri,

Porto, _____/_____/_____

# Sworn Statement

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Diogo Miguel Santos da Silva

Porto, November 15th, 2022

# Scientific publications and communications developed during this work year

## Articles in scientific journals

**Silva, D.,** Oliveira, J., Matos, I., Rangel, L., Santos, M. J., Ayra-Pardo, C. (2022). Bacteria-mediated RNA interference in *Rhynchophorus ferrugineus* (Olivier, 1790). Manuscript in preparation for Journal of Invertebrate Pathology.

## Oral communications

Matos, I., **Silva, D**., Oliveira, J., Pereira, N., Gonçalves, C., Alves, R., Rangel, L., Santos, M. J., Ayra-Pardo, C. (2022). Phoretic and parasitic organisms associated with *Rhynchophorus ferrugineus* Olivier, 1790 in Northern Portugal. IJUP 2022- 15th Meeting of Young Researchers of the University of Porto.

## Poster communications

**Silva, D.**, Matos, I., Santos, M. J., Ayra-Pardo, C. World distribution of mites associated with the Red Palm Weevil (*Rhynchophorus ferrugineus* Olivier, 1790) (2022). IJUP 2022- 15th Meeting of Young Researchers of the University of Porto.

**Silva, D.**, Matos, I., Santos, M. J., Ayra-Pardo, C. World distribution of mites associated with the Red Palm Weevil (*Rhynchophorus ferrugineus* Olivier, 1790) (2022). 15th International Congress of Parasitology Copenhagen, Denmark.

Matos, I., **Silva, D**., Oliveira, J., Pereira, N., Gonçalves, C., Alves, R., Rangel, L., Santos, M. J., Ayra-Pardo, C. (2022). Site distribution analysis of mites from *Rhynchophorus ferrugineus* Olivier, 1790 (Coleoptera: Curculionoidea): first report in Portugal. 15th International Congress of Parasitology Copenhagen, Denmark.

Matos, I., **Silva, D**., Rangel, L., Santos, M. J., Ayra-Pardo, C. (2022). Identification of pathogenic fungi associated with the red palm weevil-mite complex in Portugal. 15th International Congress of Parasitology Copenhagen, Denmark.

Oliveira, J., Matos, I., **Silva, D**., Santos., M. J., Ayra-Pardo, C., Rangel, L. (2022). Distribution of mites on *Rhynchophorus ferrugineus* Olivier, 1790 (Coleoptera: Curculionidae) in Northern Portugal and its consequences on public health. 17th YES Meeting – European Young Scientist – Porto, Portugal.

Oliveira, J., **Silva, D**., Matos, I., Santos., M. J., Rangel, L., Ayra-Pardo, C., (2022). RNA Interference in Red Palm Weevil by feeding larvae with insect dsRNA-expressing bacteria. 1ª Edição do Congresso de Ciências. Concurso de Posters. Faculdade de Ciências da Universidade do Porto.

## DNA sequences published in GenBank

### Fungi

*Scopulariopsis* sp. isolate Av1 ON854670
*Scopulariopsis* sp. isolate Av2 ON854671
*Scopulariopsis* sp. isolate Av3 ON854672
*Alternaria* sp. isolate Vi2 ON931226
*Fusarium* sp. isolate Pt1 ON931476
*Fusarium* sp. isolate Pt2 ON931477
*Fusarium* sp. isolate Pt3 ON931478
*Fusarium* sp. isolate Av4 ON931479
*Fusarium* sp. isolate Br5 ON931480
*Penicillium* sp. isolate Av3 ON931626
*Penicillium* sp. isolate Br6 ON931627
*Penicillium* sp. isolate Av7 ON931628
*Penicillium* sp. isolate Av8 ON931629
*Penicillium* sp. isolate Vi9 ON931630
*Penicillium* sp. isolate Vi10 ON931631

### Mites

*Centrouropoda* sp. isolate 7A ON951975
*Nenteria extremica* isolate 13A ON951976
*Acarus* sp. isolate 16A ON951977
*Dendrolaelaps* sp. isolate 12A ON951978
*Dendrolaelaps* sp. isolate 12B ON951979

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Hope I have made everyone proud.

# ABSTRACT

*Rhynchophorus ferrugineus,* commonly known as the Red Palm Weevil (RPW), is currently one of the most important pests of palm trees around the world. This species is responsible for killing the palm trees used mainly for food and ornamental purposes, causing serious economic damages. There is an urgent need to control this insect population and that starts with laboratory trials with the aim of implementing the work in the field.

In the first part of this work, the laboratory rearing of RPW was optimized by testing different diets, different substrates for egg hatching, and different conditions to keep the insects healthy. Of the two diets tested, it was found that a diet consisting of components such as maize flour, wheat germ, vitamins, brewer's yeast, mold inhibitors and apple allowed the insect to complete its life cycle under laboratory conditions. One of the major advantages of this diet is that all components are readily available not only in Portugal but also in most other European countries where this pest is a problem. As for the egg hatchability, eggs laid in cotton pads with a 20% honey solution hatched faster and at a higher rate than the eggs laid in apple slices. As for the rearing conditions, the insects were able to grow and molt between larval stages when fed the diet, but they were not able to pupate. The latter only happened when the larvae were incubated with sugarcane stalks in the last stage and the photoperiod was set to complete darkness, which could be explained by the boring nature of RPW larvae.

In the second part of this work, the larvae were fed with bacteria expressing double-stranded RNA (dsRNA) of different fragments of the RPW genes *α-actin* and *β-tubulin* at different cell concentrations to induce RNA interference (RNAi) in the insect. The bacterium used as a dsRNA carrier was the *Escherichia coli* strain HT115 (DE3), which carries a mutation that switches off the expression of the dsRNA nuclease RNAse III. The results showed that both target genes were successfully downregulated in the treated larvae, with suppression of *α-actin* better affecting larval weight gain and mortality. In general, RNAi-induced insect mortality was dependent on dsRNA size for *β-tubulin* and dose-dependent for both genes. The *β-tubulin* was just not as effective as the *α-actin*, but it also suppresses the expression of *α-tubulin* from the tubulin gene superfamily, which means that it is probably not a safe target in an RNAi-based strategy to control RPW.

RNAi technology is very promising and has been shown to be an effective and safer alternative to chemical pesticides in controlling recalcitrant insect pests. As there is a

great need for a fast-acting, environmentally friendly control method for RPW, taking into account our results, this technology should be further explored in this pest species.

Keywords: RNA Interference; *Rhynchophorus ferrugineus*; Red Palm Weevil; Bacteria-mediated; Pest Control; Insect Rearing

# RESUMO

*Rhynchophorus ferrugineus*, vulgarmente conhecido como Escaravelho-vermelho da Palmeira (RPW), é atualmente uma das pragas de palmeira mais devastadoras do mundo. Esta espécie é responsável pela morte de palmeiras que são usadas principalmente como alimentação e também para fins ornamentais, causando graves danos económicos. É urgente controlar a população deste inseto e isso começa com testes laboratoriais com o objetivo de implementar os trabalhos no terreno.

Para testar novas tecnologias e estratégias em laboratório, é necessário manter uma colónia de RPW para que os insetos necessários para os testes estejam sempre disponíveis. Neste trabalho, foi realizada uma otimização da cultura de RPW em laboratório, testando diferentes dietas, diferentes substratos para a eclosão de ovos, e diferentes condições para manter os insetos saudáveis. Das duas dietas testadas, observou-se que uma dieta composta por ingredientes como farinha de milho, gérmen de trigo, vitaminas, levedura de cerveja, inibidores de crescimento de fungos, e maçã permite ao inseto completar o seu ciclo de vida em condições laboratoriais. Uma das principais vantagens desta dieta é que todos os componentes são facilmente acessíveis não só em Portugal mas também noutros países. Para a incubação de ovos, os ovos colocados em almofadas de algodão com uma solução de mel de 20% mostraram uma taxa de eclosão mais rápida e mais elevada quando comparados com os ovos colocados em fatias de maçã. No que diz respeito às condições de criação de insetos, estes alimentaram-se e cresceram quando expostos à dieta, mas não iniciaram o processo de pupação A pupação só começou a ocorrer quando o fotoperíodo foi definido para obscuridade total, o que pode ser explicado devido à natureza das larvas de RPW que passam as fases larvais dentro do tronco das palmeiras.

A segunda parte deste trabalho envolveu alimentar as larvas com bactérias recombinantes, neste trabalho foi usado o sistema L4440-*E. coli* HT115(DE3), que expressam moléculas de RNA de dupla cadeia de fragmentos de dois genes de RPW, *α-actina e β-tubulina*, em diferentes concentrações a fim de induzir uma resposta de RNA de interferência. Os resultados mostraram que ambos os genes-alvo foram suprimidos com sucesso em larvas tratadas, mas com melhores resultados na supressão da *α-actina* afetando o ganho de peso e induzindo uma mortalidade mais acentuada. Em geral, a indução da mortalidade por RNAi era mais dependente do tamanho do fragmento no gene da β-tubulina, e era dependente da concentração em ambos os genes. O RNA de dupla cadeia (dsRNA) do gene da *β-tubulina* também causou mortalidade e afetou o ganho de peso das larvas, mas além de não ser tão eficaz

como o dsRNA da *α-actina*, também suprimiu a expressão da *α-tubulina*, que pertence à superfamília do gene da tubulina*,* o que significa que provavelmente não é um alvo seguro para usar esta tecnologia no controlo da RPW.

A tecnologia de RNA de interferência é muito promissora e demonstrou ser uma alternativa eficaz e segura aos pesticidas químicos no controlo de pragas de insetos recalcitrantes. Como existe uma grande necessidade de um método de controlo de insetos rápido e seguro para o ambiente, esta tecnologia deve ser estudada mais aprofundadamente.

Palavras-chave: Rna Interferência; *Rhynchophorus ferrugineus;* Controlo de pragas; Criação de insetos; Bactérias como vetor para fornecimento de dsRNA

# TABLE OF CONTENTS

Sworn Statement ..... v
Scientific publications and communications developed during this work year ..... vi
Articles in scientific journals ..... vi
Oral communications ..... vi
Poster communications ..... vi
DNA sequences published in GenBank ..... vii
Fungi ..... vii
Mites ..... vii
ACKNOWLEDGMENTS ..... viii
ABSTRACT ..... ix
RESUMO ..... x
LIST OF TABLES ..... xiv
LIST OF FIGURES ..... xv
LIST OF ABBREVIATIONS ..... xviii
INTRODUCTION ..... 1
The Red Palm Weevil (RPW) ..... 1
Economic Impact ..... 2
Control Methods ..... 3
Sterile insect technique ..... 3
Trapping and semiochemicals ..... 3
Chemical control and insecticides ..... 3
Associations and Biological control ..... 4
Viruses ..... 4
Fungi ..... 4
Nematodes ..... 5
Bacteria ..... 5
RNA Interference ..... 6
RNA Interference in insects ..... 8
OBJECTIVES ..... 10
MATERIALS AND METHODS ..... 11
Diet preparation ..... 11
Insect collection and rearing ..... 12
RNA extraction, cDNA synthesis and PCR amplification of target genes ..... 13

Bacterial strain and construction of dsRNA expression vectors ..... 14
Transformed bacteria preparation and dsRNA expression ..... 15
Delivery of recombinant bacteria expressing dsRNA to RPW larvae ..... 15
Bioassay of the bacteria expressing dsRNA ..... 16
Gene expression analysis with quantitative real-time PCR (qPCR) ..... 16
Statistical analysis ..... 16
RESULTS ..... 17
Insect rearing and diet optimization ..... 17
Bacterial transformation and dsRNA expression ..... 18
RNAi-induced insect mortality ..... 20
RPW larval weight variation after fed diet containing recombinant bacteria expressing dsRNA of the target genes ..... 22
DISCUSSION ..... 24
CONCLUSION AND FUTURE PERSPECTIVES ..... 29
REFERENCES ..... 31
ANNEXES ..... 49

# LIST OF TABLES

**Table 1 -** List of ingredients and quantities used to make the diets for the RPW rearing in laboratory conditions.

**Table 2** – Primers used to amplify the target genes.

# LIST OF FIGURES

**Figure 1** - *Rhynchophorus ferrugineus* life cycle. The cycle starts with the egg, then a larva emerges and goes through 10-15 instars. When the signal to start the metamorphosis is received, a cocoon is made and the state of pupa is reached. Lastly an adult emerges from the cocoon.

**Figure 2** – RNA interference pathways: (A) siRNA pathway. In this pathway, long double-stranded RNA (dsRNA) is cleaved by Dicer 2 enzyme. Then, a duplex siRNA is loaded into Argonaute 2 (AGO2), and a RNA-induced silencing complex (RISC) is assembled. The sense strand of the siRNA suffers degradation while in the RISC and this allows the antisense strand to cleave the complementary target RNA by AGO2 and RISC; (B) miRNA pathway. In the miRNA pathway, the endonucleases Drosha and Pasha cur the primary miRNA (pri-miRNA) into hairpins to form precursor miRNA (pre-miRNA). This precursor miRNA is then cleaved by Dicer 1 to create a miRNA duplex. This miRNA matures and is loaded into AGO1, a RISC is assembled, and complementary target mRNA are cleaved.; (C) PIWI- interacting RNA pathway. De novo piRNA production begins when the transcription of long single-stranded antisense RNAs by RNA polymerase II from piRNA clusters that contain transposable elements. The antisense piRNA precursor is sliced into primary piRNA by Zucchini (Zuc) endonuclease. The antisense piRNA that results from this step is loaded into PIWI and Aubergine (Aub). The PIWI/Aub complex cuts a sense transposon transcript degrading the target, and crating new sense piRNA. AGO3 uses the new sense piRNA to induce the cleavage of complementary antisense transposon transcripts to generate more antisense piRNA, that can be loaded into the PIWI/Aub complex to cleave more complementary sense transposon transcripts. Created with BioRender.com.

**Figure 3** - Picusan® trap used to capture weevils (A); Aggregation pheromone used (B). The pheromone is placed inside the trap in a dedicated compartment. Traps were inspected weekly, and the insects collected and transported to laboratory in plastic containers.

**Figure 4** – Lanes 1 and 2: cDNA synthetized from the RNA extracted from RPW larval gut using First-strand cDNA synthesis Kit (Nzytech). M, NZYDNA ladder III.

**Figure 5 -** Diagram of the dsRNA production system in bacteria. The RNAi target gene fragment is inserted in the multiple-cloning site which is flanked by two T7 promoters in opposing directions. Then the transformation is performed in *Escherichia coli* strain

HT115 (DE3) cells. IPTG is used to induce RNA transcription by the T7 promoter. dsRNA is then extracted and purified.

**Figure 6** – (A) Larva in the first stages dead after feeding on the Diet A. Length: 1.8cm, Width: 0.4cm.; (B) Larva in a more advanced stage feeding on the Diet B. Length: 4cm, Width: 1.5cm.

**Figure 7**– Mean number of eggs hatched per day while on different substrates. It is possible to observe a greater daily hatching rate in eggs placed in cotton pads embedded in a solution of 20% honey.

**Figure 8** – Results of the bacterial transformation (positive colonies marked with the orange arrows): (A) positive colony containing the *β-tubulin* long fragment (TubL) (613 bp); (B) positive colonies containing the *α-actin* short (ActS) (300 bp) and long (ActL) (640 bp) fragment, and colonies containing the Kanamycin resistance gene (KanR) (540 bp); (C) positive colonies containing the *β-tubulin* short fragment (TubS) (334bp). M, NZYDNA Ladder III.

**Figure 9** – Detection of dsRNA produced by recombinant bacteria. Arrows indicate dsRNA fragments. M: Ladder; ActL: *α-actin* long fragment; ActS: *α-actin* short fragment; TubL: *β-tubulin* long fragment; TubS: *β-tubulin* short fragment; EV: empty vector; KanR: Kanamycin resistance gene fragment.

**Figure 10** – Reverse transcription quantitative PCR (RT-qPCR) analysis of the relative gene expression between treatments and control. (A) target: $\alpha$*-actin*; (B) target: *β-tubulin*. EV: Empty vector (negative control). KanR: Kanamycin resistance gene. Gene knockdown was observed in the target genes actin and *β-tubulin*. An off-target effect in the *α-tubulin* was observed when targeting the *β-tubulin* gene. Neither target genes caused a downregulation of the Kanamycin resistance gene. Asterisks represent a statistical difference between the treatments and the negative control. * ($p<0.05$); **($p<0.05$); ***($p<0.001$).

**Figure 11 -** Survival curve of larvae exposed to treatment and controls. Larvae exposed to the higher concentration of dsRNA of the $\alpha$*-actin* fragments showed 100% mortality by the 7th day. The *β-tubulin* short fragment in the highest concentration induced 100% mortality by the 12th day. The other treatments induced some degree of mortality by the last day, but not quite as high. Asterisks indicate significant difference when compared to the negative control: * ($p<0.05$); ** ($p<0.005$); *** ($p<0.001$).

**Figure 12** – RPW larvae subjected to diet containing recombinant bacteria. (A) 12 well plate with RPW larvae exposed to one of the treatments. (B) Dead larvae that resulted from the treatment with the target genes. (C) Alive larvae from the control group. Larvae exposed to the treatment started to show signs of melanization, which lead to death.

**Figure 13** - Weight increment of RPW larvae exposed to diet containing recombinant bacteria expressing dsRNA of the actin gene. (A) *α-actin* long fragment; (B) *α-actin* short fragment. Asterisks represents significant difference in means when compared to the control group: *** ($p<0.001$); **** ($p<0.0001$). Lack of asterisk means no significative difference.

**Figure 14** - Weight increment of RPW larvae exposed to diet containing recombinant bacteria expressing dsRNA of the *β-tubulin* gene. (A) *β-tubulin* long fragment; (B) *β-tubulin* short fragment. Asterisks represents significant difference in means when compared to the control group ($p<0.005$). Lack of asterisk means no significative difference.

**Figure 15** - Weight increment of larvae exposed to diet containing bacteria expressing dsRNA of the Kanamycin resistance gene (off-target control). Lack of asterisk means no significative difference.

**Figure 16** -RPW larvae going through pupation. (A) Cocoon made inside of sugarcane, (B) Pupation occurring without cocoon, (C) Adult emerging from sugarcane.

**Figure 17** – RPW adults exposed to apple slices soaked in a dsRNA-expressing recombinant solution. (A) Adults exposed to *α-actin* long fragment (ActL) dsRNA, (B) Adults exposed bacteria containing an empty vector, (C) Adults exposed to apple cubes soaked in a PBS solution. 100% mortality was observed in adults exposed to recombinant bacteria expressing actin dsRNA, while adults exposed to both control showed no signs of phenotypic alterations or mortality.

# LIST OF ABBREVIATIONS

RPW – Red Palm Weevil

IPM – Integrated Pest Management

DNA – Deoxyribonucleic Acid

RNA – Ribonucleic Acid

CPV – Cytoplasmatic polyhedrosis virus

ssRNA – Single-stranded RNA

dsRNA – Double-stranded RNA

RNAi – RNA interference

siRNA – Short interfering RNA

miRNA – Micro RNA

piRNA – PIWI-interacting RNA

TE – Transposable elements

RISC – RNA-induced silencing complex

AGO – Argonaute

TdT – Terminal deoxynucleotidyl transferase

HCC – Higher concentration of cells

LCC – Lower concentration of cells

ActL – *α-actin* gene long fragment

ActS – *α-actin* gene short fragment

TubL – *β-tubulin* gene long fragment

TubS – *β-tubulin* gene short fragment

KanR – Kanamycin resistance gene

RPS6 – Ribosomal protein S6

MoA – Mode of Action

# INTRODUCTION

## The Red Palm Weevil (RPW)

*Rhynchophorus ferrugineus* (Olivier, 1790) (Coleoptera: Curculionidae), commonly known as Red Palm Weevil (RPW), is one of the most damaging pests of palm trees in the world (Milosavljević et al., 2019). It is originally from South Asia but has been spreading across the Middle East, North Africa, and the Mediterranean area, mostly due to the trade of infected palms.

The RPW completes its life cycle inside the trunk of the palm tree. It has four stages of development – egg (2-5 days), larva (60-105 days), pupa (13-17 days), and adult (100-120 days) (Al-Dosary et al., 2016) (**Fig. 1**). RPW larvae are the main responsible for the harm done to the palm trees. These larvae are creamy white, pyriform, legless, and with a brown russet-red head. Female weevils lay their eggs individually in separate holes at the base of the trunks and leaves, and these eggs may hatch in three days, depending on the temperature (Dembilio & Jaques, 2015; Ferry & S, 2002). The eggs have an oblong shape and are creamy-white in colour. Right after hatching, the larvae begin to feed on soft tissues and terminal bud tissues. Until the larvae fully develop and just before pupation, the larvae move to the interior of the palm creating large cavities and making tunnels. However, the pupation process occurs on the periphery of the palm, where the larvae forms a cocoon using palm fibres (Ferry & S, 2002). Adults then emerge from the cocoon, and usually don't leave the palm tree while they can feed on it. RPW adults are rusty red, have a distinctively curved rostrum, and have dark spots on the dorsal side of the thorax. Males and females in this species have characters that allow their distinction. Males have short hairs on the dorsal half of the rostrum, while females lack these hairs and have a longer, narrower rostrum (Dembilio & Jaques, 2015). Adults have well-developed wings, and although most of them are considered short distance flyers (<100 m), some individuals have been observed to fly long distances (>5000 m) (Ávalos et al., 2014), which explains the RPW high dispersal ability.

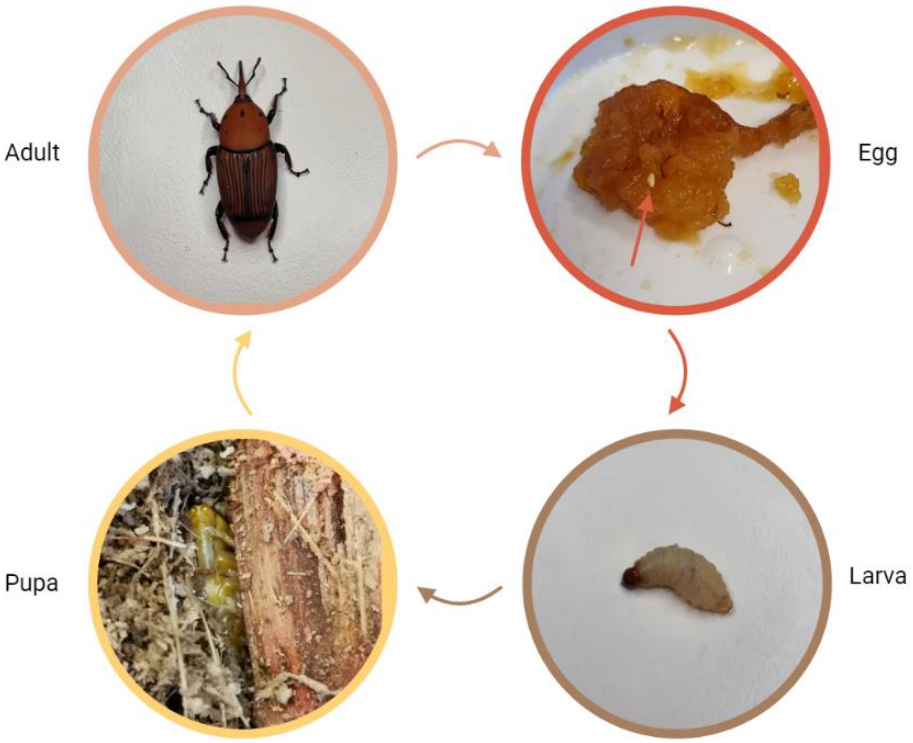



**Figure 1 -** *Rhynchophorus ferrugineus* life cycle. The cycle starts with the egg, then a larva emerges and goes through 10-15 instars. When the signal to start the metamorphosis is received, a cocoon is made and the state of pupa is reached. Lastly an adult emerges from the cocoon.

## Economic Impact

This species has been described to attack over 20 different species of palm trees. It was reported for the first time on the Coconut palm tree (*Cocos nucifera*) in Southeast Asia. Later on, it was recorded attacking the Date palm tree (*Phoenix dactilyfera*) in the Middle East, and in Europe it is showing a clear preference for the Canary Island palm tree (*Phoenix canariensis*) (Abdel-Hamed, 2018; Dembilio & Jaques, 2015). The *Phoenix* genus is composed of 14 species, some used for decoration and construction purposes, and some used for food and religious purposes (Grosbalthazard, 2013).

The *Phoenix* genus is very popular because of its adaptability to a great array of climates, meaning that these palms are one of the most frequently grown in the world (Dembilio & Jaques, 2015). Usually, the harm caused by this pest is not visible in the very beginning of the infection, and when the first symptoms appear, usually it is already too late for treatment, resulting in the death of the palm. As a result, the damage done to these palms by the RPW have a serious economic toll all around the world but is especially devastating in the arid regions, where palm trees are very useful because they are able to withstand unpropitious weather conditions, and provide a micro-climate for farmers to grow different crops (Al-Dosary et al., 2016). In the Mediterranean area, palm

trees worth up to € 483 million have reportedly been destroyed by this insect (Macleod & Hussein, 2016).

## Control Methods

Several methods have been used to try to control the RPW dispersion and its damage to the palm trees. Early detection of infections is a critical step to avoid palm death and it is a crucial element of an Integrated Pest Management (IPM) program to control RPW, which encompasses public awareness, efficiency of control methods, and precautionary measures (Dembilio & Jaques, 2015; Mazza et al., 2014).

### Sterile insect technique

The objective of this method is to expose target species to radiation in order to induce sexual sterility and release the individuals back in the wild (Hussain et al., 2013; Klassen & Curtis, 2005; Robinson, 2005). This technique has shown that it is possible to induce 90% sterility among exposed male populations of RPW (Al-Ayedh & Rasool, 2010), however when the males were allowed to mate with unexposed females, it was observed the production of fertilized eggs, which the authors suggested that it was due to the resistance of sperm to radiations. These findings were obtained in the laboratory and there is no report of a successful control in the field, mainly due to the concealed nature of the RPW, and the choice of females to mate with normal males (Hussain et al., 2013).

### Trapping and semiochemicals

Heavy trapping of adults with the help of pheromone traps is a widely used procedure for the management of the RPW (Faleiro, 2006). The main aggregation pheromone used is Ferrugineol (4-methyl-5-nonanol), which is reported to preferentially attract females over males (2:1), meaning that females are captured before they initiate oviposition (Dembilio & Jaques, 2015). This result could be explained by greater dispersion of females to find oviposition sites, food and mates (Firdaus et al., 2020).
Although this method offers some advantages, it can also attract this pest to un-infested areas (Hussain et al., 2013).

### Chemical control and insecticides

Insecticides are agents of chemical or biological origin and have been used for a long time to control insects. These compounds have several advantages as well as disadvantages, the main disadvantage being the exposal of non-target animals and even people to the chemicals (Askary, 2010; Gupta et al., 2019; Ware, 2004).

The use of insecticide treatments of date palms is practiced to limit the spread of the RPW. These chemicals are used in preventive treatments, such as the covering of exposed wounds, and periodic showering with insecticides, and in curative treatments, such as the injection of insecticides in cavities performed by the weevils in the trunk. These cavities can also be man-made (Faleiro, 2006).

Although the chemical treatment is rather effective, several concerns about the pesticides side-effects on the environment and the potential rapid evolution of resistance in RPW populations has led to a restriction in the use of said products. So, biological control has been gaining popularity as the preferential method of management of the RPW (Mazza et al., 2014; Murphy & Briscoe, 1999).

## Associations and Biological control

The RPW is known to be associated with more than 50 natural organisms. Some of these interactions may be beneficial, some may be prejudicial to the RPW, and other may be considered neutral (Dilipkumar et al., 2015). Viruses, fungi, nematodes, mites, and bacteria have been reported to interact with *R. ferrugineus* (Al-Dosary et al., 2016; Dilipkumar et al., 2015).

### Viruses

Entomopathogenic viruses are intracellular parasites composed of either DNA or RNA encapsulated by a capsid. There are 11 families that have been isolated from a large number of species, being reported to be very effective in controlling populations of Hymenoptera and Lepidoptera (Kalha et al., 2014). The cytoplasmatic polyhedrosis virus (CPV) is the only one that has been isolated from RPW, infecting all stages of the insect, and causing phenotypical alterations and reducing this pest population when the infection occurs in the last larval stage (Gopinadhan et al., 1990; Mahmoud et al., 2018; Mazza et al., 2014).

### Fungi

Entomopathogenic fungi are usually Zygomycota, Ascomycota and Deuteromycota. Most species attacking insects belong to Entomophtorales in the Zygomycota division, and Hyphomycetes in the Deuteromycota division (Shah & Pell, 2003). *Beauveria bassiana* is one of the most studied species of entomopathogenic fungi and has been found to cause death in RPW, infecting all life stages (Dembilio et al., 2010; Mazza et al., 2014).

Several other species of fungi have also been isolated from RPW such as *Metarizhium anisopliae*, *Aspergillus* sp., *Fusarium* sp., *Trichotecium* sp., and *Penicillium*

sp. that could also have some effect on the control of this pest (Francardi et al., 2012; Tarasco et al., 2008).

### Nematodes

The use of nematodes as biological control is currently one of the most popular strategies to control the RPW .(Hamadttu Abdel Farag & Jose Romeno, 2020). The interest in this method has been increasing over the last few decades, and it can be used to control not only insects but also mollusks and other plant nematodes (Askary, 2010; Grewal et al., 2005; Mazza et al., 2014). Other advantages associated with the use of nematodes are their broad host spectrum, easy application, ease of mass culturing and storage, and safeness of non-target organisms (Hussain et al., 2013).

The main worms used to control the RPW belong to Steinernematidae and Heterorhabditidae. These nematodes carry specific pathogenic bacteria in a modified portion of the intestine, which are released in the hemocoel of the insect. The proliferation of these bacteria in the host hemocoel prevents the growth of other microbes that may be harmful to the nematode, allowing them to pass several generations in the host (Hussain et al., 2013).

This method of biocontrol has proven to be very efficient. In both laboratory and field-like conditions, bioassays showed a very high success rate as a curative measure and as a preventive measure against RPW infection (Atwa, 2004; Llácer et al., 2008; Saleh et al., 2011).

Although the use of nematodes to control the RPW has proven to be quite useful, there are some drawbacks: 1) nematodes are very susceptible to hot weather conditions, meaning that the success rate of this method decreases as the temperature increases (Faleiro, 2006), 2) ethanol, ethyl acetate and acetic acid generated by RPW-infested palm tissue, inhibits the activity of some nematode species, 3) mites associated with the RPW can be predators of nematodes eliminating their effect (Mazza et al., 2011).

### Bacteria

Entomopathogenic bacteria based microbial pesticides are one of the most used biocontrol options nowadays, due to their specificity, safety, and cost-effective production (Charles et al., 2013; Glare et al., 2017).

Bacteria in the order Bacillales are the most successful entomopathogens used in commercial products (Hussain et al., 2013; Mazza et al., 2014). Some authors have reported a successful control of RPW under laboratory conditions using *Bacillus thurigiensis*. This species produces insecticidal proteins comprised of Crystal (Cry) and Cytolitic (Cyt) toxins. These toxins are specific to the target insects, are biodegradable,

and are innocuous to vertebrates, plants, and humans, which makes them a viable alternative for the control of insect pests (Bravo et al., 2007).

Another method of biocontrol involving bacteria is using them to deliver double-stranded RNA (dsRNA) of essential genes to insects, in order to suppress the expression of corresponding genes via a mechanism known as RNA interference (RNAi). This method has some benefits, the main ones being that the production of dsRNA-expressing recombinant bacteria is inexpensive, an efficient delivery option, and is specific to the target organism (Laudani et al., 2017; Ramya Shanivarsanthe Leelesh & Lynne K. Rieske, 2020; Papić et al., 2018; Zhang et al., 2019).

## RNA Interference

RNA interference (RNAi) is a post-transcriptional system that silences specific genes in eukaryotes and is triggered by double-stranded RNA (dsRNA) molecules (Laudani et al., 2017; Zhang et al., 2019). This mechanism starts when small RNA molecules of approximately 21 nucleotides in length, induce sequence-specific gene silencing. This response acts at the post-transcriptional level by cleaving or blocking long RNAs containing matching sequences (Vogel et al., 2019). These small RNAs are mainly separated in three different classes generated by independent biogenesis pathways, short interfering RNAs (siRNAs), microRNAs (miRNAs), and Piwi-interacting RNAs (piRNAs) (Nandety et al., 2015). siRNAs and miRNAs are mainly involved in RNA degradation/cleavage or translation repression, while the primary function of piRNAs is to silence transposable elements (TEs) (Bourque et al., 2018; Siomi et al., 2011).

siRNAs are Dicer dependent. Exogenous or endogenous long double-stranded RNA (dsRNA) molecules are processed by this enzyme generating double-stranded siRNAs (dssiRNAs) in the cytoplasm (Nandety et al., 2015). Mature siRNA is loaded in the RNA-induced silencing complex (RISC), being associated to Argonaute (AGO) protein (Agrawal et al., 2003; Hammond, 2005; Siomi & Siomi, 2009). Exogenous dsRNAs include experimentally introduced dsRNAs and viral RNAs, while endogenous dsRNAs come from overlapping transcripts, structured loci, and transposable elements (Ghildiyal et al., 2008; Zhu & Palli, 2020) (**fig. 2A**).

The miRNAs are single-stranded RNAs (ssRNAs) that originate from endogenous primary transcripts (pri-miRNAs) due to the action of two Rnase III type nucleases, Drosha and Dicer. Drosha acts in the nucleus, excising the stem-loop of the pri-miRNA which results in the generation of a precursor microRNA (pre-miRNA). After being transported to the cytoplasm, the pre-miRNAs are processed by Dicer enzymes into double-stranded miRNAs (dsmiRNAs). One of the strands of dsRNA is selected and

loaded into the RISC, which is composed by the ssmiRNA and the AGO protein (Kim et al., 2009; Okamura & Lai, 2008) (**fig. 2B**).

The piRNAs are germ-cell-specific small RNAs, and their main function is to silence transposable elements (TEs). While miRNAs and siRNAs are generated by the Dicer endonuclease, piRNAs originate through Dicer-independent mechanisms, and associate

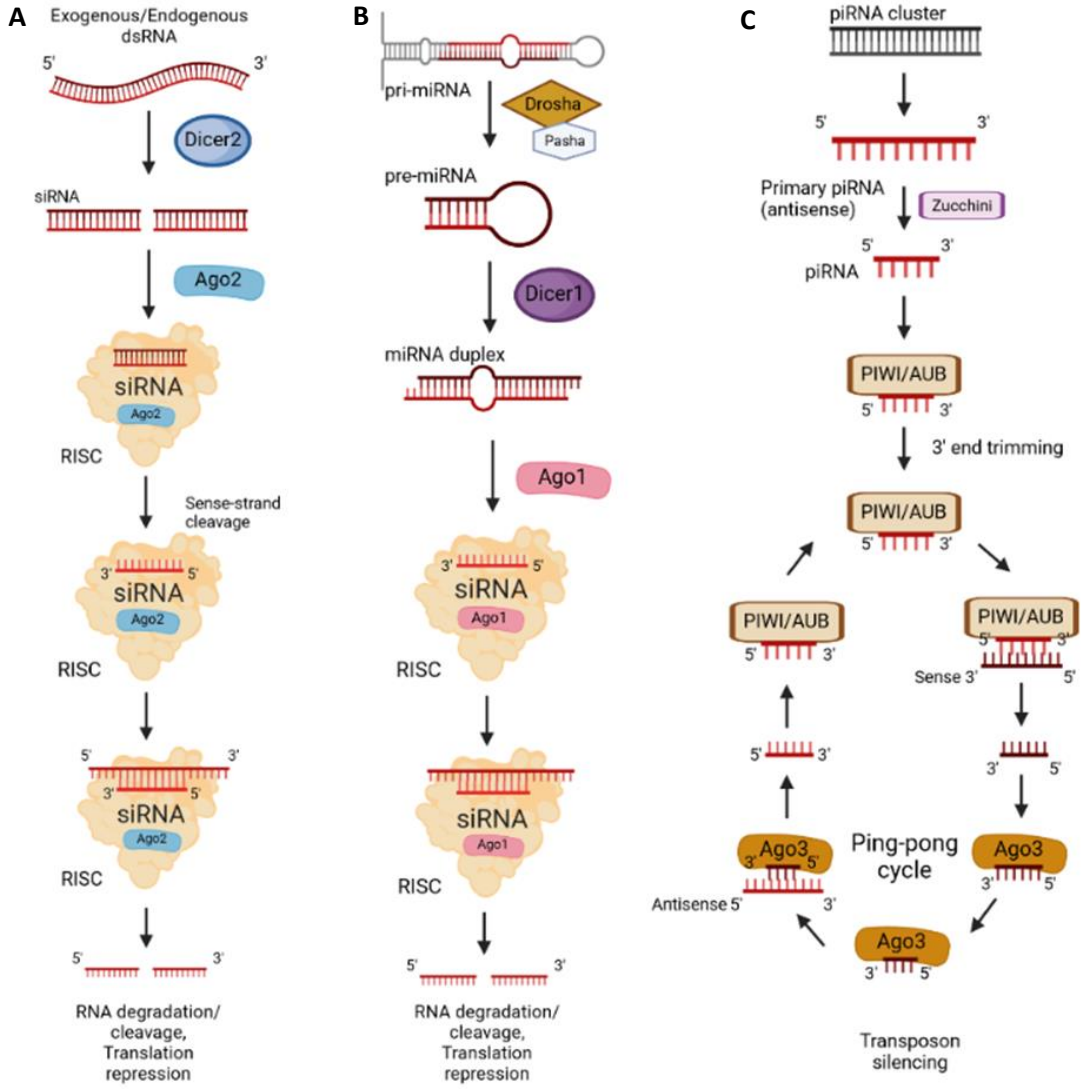



**Figure 2 -** RNA interference pathways: (A) siRNA pathway. In this pathway, long double-stranded RNA (dsRNA) is cleaved by Dicer 2 enzyme. Then, a duplex siRNA is loaded into Argonaute 2 (AGO2), and a RNA-induced silencing complex (RISC) is assembled. The sense strand of the siRNA suffers degradation while in the RISC and this allows the antisense strand to cleave the complementary target RNA by AGO2 and RISC; (B) miRNA pathway. In the miRNA pathway, the endonucleases Drosha and Pasha cur the primary miRNA (pri-miRNA) into hairpins to form precursor miRNA (pre-miRNA). This precursor miRNA is then cleaved by Dicer 1 to create a miRNA duplex. This miRNA matures and is loaded into AGO1, a RISC is assembled, and complementary target mRNA are cleaved.; (C) PIWI- interacting RNA pathway. De novo piRNA production begins when the transcription of long single-stranded antisense RNAs by RNA polymerase II from piRNA clusters that contain transposable elements. The antisense piRNA precursor is sliced into primary piRNA by Zucchini (Zuc) endonuclease. The antisense piRNA that results from this step is loaded into PIWI and Aubergine (Aub). The PIWI/Aub complex cuts a sense transposon transcript degrading the target, and crating new sense piRNA. AGO3 uses the new sense piRNA to induce the cleavage of complementary antisense transposon transcripts to generate more antisense piRNA, that can be loaded into the PIWI/Aub complex to cleave more complementary sense transposon transcripts.

with the PIWI group proteins, which are germline-specific members of the AGO family (Nandety et al., 2015) (**fig. 2C**).

The RNA interference mechanism was discovered in the late 1980s and early 1990s in plants (Agrawal et al., 2003; Hammond, 2005; Lindbo, 2012) when plant biotechnology researchers were attempting to increase the pigment content in petunia flowers by inserting selected petunia genes paired with very strong promoters into the plant genome. While the researcher were expecting an increment in floral color, some plants exhibited a co-suppression of both the inserted gene and the homologous endogenous gene, often showing the opposite of the expected results (Lindbo, 2012). Meanwhile, this mechanism was also being discovered in *Caenorhabditis elegans.* Investigators injected dsRNA corresponding to a segment of *unc22* gene, and the decrease of this gene activity is supposed to produce a twitching phenotype. They found that the injected individuals manifested weak twitching, while other individuals showed strong twitching. The investigators also found that this loss-of-function could also be achieved with dsRNA of four other nematode genes (Fire et al., 1998). After these discoveries, this phenomenon of RNAi has been demonstrated to occur in many vertebrates and invertebrates.

## RNA Interference in insects

In insects, RNAi activity can be performed by all three classes of small RNAs, each one showing different specific activities, and some being very important for natural gene regulation and insect development. Some of these activities include defense against viral infections via siRNA-pathway, regulation of gene expression via miRNA-pathway, and suppression of germline transposon expression via piRNA-pathway (Zhu & Palli, 2020). Since RNAi can be artificially induced via delivery of gene-specific dsRNA molecules, which triggers the siRNA-pathway resulting in functional knockdown effects, this technique is being studied and implemented in agriculture with the aim of controlling specific target insect pests (Kim et al., 2015; Vogel et al., 2018; Wynant et al., 2014).

There are several dsRNA delivery methods, such as injection, and ingestion. The most common one is microinjection, in which dsRNA of a target gene is directly injected into the test organism (Yu et al., 2013). This method has several advantages but also disadvantages compared to other methods. The main two advantages are that it is possible to get the dsRNA directly into the target tissue or into the hemolymph, and it is possible to know exactly the amount of dsRNA introduced into the organism. However, this method is strenuous and challenging, and surviving insects often show upregulated immune responses and trauma, leading to possible misinterpretations of external alterations (Whitten et al., 2016). Also, some factors such as needle choice and place of injection are very important and vary between organisms.

The delivery of dsRNA through ingestion can be performed by two main strategies: dsRNA can be expressed in genetically modified bacteria, often *Escherichia coli*, or it can be synthesized *in vitro*, mixed with food, and supplied to the organisms. This method has been proven to be successful in a wide variety of insects and has some advantages over the delivery by injection. It is cheaper, easier to perform, labor-saving, and eco-friendly (Pu et al., 2017; Tian et al., 2009). At the same time it is a less invasive process, and is highly applicable for high-throughput gene screening (Kamath et al., 2000). On the other hand, the efficiency of RNAi by ingestion of dsRNA differs among species, possibly due to the distinct gut environment, and also it is not possible to determine the amount of dsRNA that has been ingested (Yu et al., 2013).

Although an RNAi effect has been observed in most economically damaging insect orders, such as Diptera, Coleoptera, and Lepidoptera, and this technique has successfully targeted several genes that regulate insect development, the response may vary between species (Bellés, 2010). Some research suggests that Coleoptera is the most sensitive order, with RNAi being greatly efficient and systemic, and gene knockdown being often 90% or higher (Baum et al., 2007; Joga et al., 2016; Laudani et al., 2017; Zhu & Palli, 2020).

Among the RNAi target genes tested in the Coleoptera order are the *actin* and *tubulin* genes. These genes carry out several functions in the organism. Tubulins are a family of eukaryotic structural proteins and their main function is to form microtubules. These microtubules are fundamental components of the cytoskeleton being involved in the mediation of cell division, motility, shape, and intracellular trafficking (Nielsen et al., 2010). The actins are part of a family that differentiated during evolution into genes that encode cytoskeletal actins and muscle actins. These genes can be found performing a variety of functions, mainly being involved in the insects flight muscles, but also being found in the insects sensilla which is a sensory organ that gathers information about the environment that the individual is in (Iwamoto, 2011; Mange & Prudhomme, 1999; Wolfrum, 1990). Both of these genes have been found to induce an RNAi response in coleopteran insect pests (Baum et al., 2007).

# OBJECTIVES

RNAi in RPW has been induced and shown to be effective in downregulating gene expression (Antony et al., 2021; Laudani et al., 2017; Rasool et al., 2021). However, the authors have only reported the use of microinjection or ingestion of naked dsRNA as methods for delivering dsRNA to the insect. In the present work, we aimed to investigate the induction of RNAi in RPW by feeding larvae with recombinant bacteria expressing dsRNA from fragments of actin and tubulin genes.

- First, we tested and optimized a meridic diet for rearing RPW in the laboratory.
- Second, we obtained the recombinant bacteria and performed the bioassays with the insects.

# MATERIALS AND METHODS

## Diet preparation

In our laboratory, conditions were created for mass rearing of RPW, especially by obtaining a new diet for larval growth and development. Two compositions for a meridic diet for RPW larvae were tested. The first diet (Diet A) contained pinto beans, wheat germ, and soybean flour as whole food type ingredients, while the second diet (Diet B) contained wheat germ, maize flour, and apple smoothie. The apple smoothie worked as a token stimulus as the RPW is known to feed on this fruit in the laboratory. This second diet also contained, between other ingredients, methylparaben (Benedict et al., 2009; Funke, 1983) and Fabco-I mould inhibitor (Curkovic et al., 2006). Agar 100 mesh (18.5 g/l) was the gelling agent. Full ingredient list is described in **Table 1**.

**Table 1 -** List of ingredients and quantities used to make the diets for the RPW rearing in laboratory conditions.

| DIET A | | DIET B | |
|---|---|---|---|
| Ingredients | Quantity | Ingredients | Quantity |
| Agar | 15 g | Agar | 18.5 g |
| Distilled water | 900 mL | Distilled water | 900 mL |
| Ascorbic acid | 8 g | Ascorbic acid | 4 g |
| Sorbic acid | 4 g | Sorbic acid | 1.6 g |
| Methylparaben | 6.6 g | Methylparaben | 3.3 g |
| Vitamin + choline | 13.3 g | Vitamin mix | 6 g |
| Pinto beans | 166 g | Fabco-I | 2.5 g |
| Wheat germ | 133 g | Wheat germ | 45 g |
| Soybean flour | 67 g | Maize flour | 45 g |
| Casein | 49 g | Brewer's yeast | 7 g |
| Wesson Salt Mix | 8 g | Peeled apple | 200 g |
| Yeast hydrolysate | 85 g | | |

## Insect collection and rearing

Adults of RPW specimens were collected with the aid of PICUSAN® traps (Koppert), paired with an RPW-specific aggregation pheromone (*Ferrugineol*) (**Fig. 3**) from Ao & Midori Biocontrol S.L. (Spain), placed near infested palm trees in Aveiro, Northern Portugal.




**Figure 3 -** Picusan® trap used to capture weevils (A); Aggregation pheromone used (B). The pheromone is placed inside the trap in a dedicated compartment. Traps were inspected weekly, and the insects collected and transported to laboratory in plastic containers.

Adults of RPW were fed with apple slices. Adults were sexed and paired in plastic containers for mating. Since this species is sexually dimorphic, sexing was done based on body differences between male and female. After mating, females were relocated to a Binder GmbH incubator, model M 240, at 27±1ºC, a photoperiod of 14: 10h (light: dark) and artificial humidification > 80% for oviposition on apple slices. The apple slices were carefully and thoroughly inspected for eggs every two days and replaced with new slices. When detected RPW eggs were then collected and transferred to fresh apple slices or cotton pads soaked with a 20% honey solution. Hatched larvae were transferred to clean 12-well plates containing artificial diet, with one larva per well in order to avoid cannibalism (Elshafie et al., 2013). Larvae were maintained in the same incubator under the same settings. After reaching the final stage, RPW larvae were incubated with sugarcane stalks and in complete darkness to allow them to form the cocoon. When adults emerged, they were sexed and mated again to produce a new generation.

## RNA extraction, cDNA synthesis and PCR amplification of target genes

RPW's housekeeping α-actin *(α-act*) (Acc. Num. KT748776) and β-tubulin (*β-tub*) (Acc. Num. KT748814) genes were selected to assess the insecticidal activity of bacterially-expressed dsRNA since these genes are essential in several stages of the insect life cycle (Yang et al., 2015). Insect total RNA was extracted from the larval digestive system (gut) using NZY Total RNA Isolation Kit (Nzytech), and cDNA was synthesized from the extracted RNA (5 µg per reaction) using NZY First-Strand cDNA Synthesis Kit (Nzytech) (**Fig. 4**). Two fragments of different sizes (~300bp and ~600bp) of each RPW's *β-tubulin* and *α-actin* genes were PCR amplified from the synthesized cDNA using specific primers (**Table 2**) and confirmed by 1% agarose gel electrophoresis. PCR reactions were conducted in a final volume of 50 µL containing 5x Phusion HF Buffer, 1 µL of dNTPs (200 µM), 2.5 µL of each primer (0.5 µM), 0.5 µL of Phusion DNA Polymerase, and 2 µL of cDNA. The two *β-tubulin* fragments (334bp and 613bp) were amplified using the following PCR program: an initial denaturation step at 98ºC for 2 min, followed by 5 cycles of denaturation at 98ºC for 10 sec, annealing at 66ºC for 15 sec, and extension at 72ºC for 1 min. After this step, another 35 cycles of denaturation at 98ºC for 10 sec, and annealing/extension at 72ºC for 1 min were performed. A final extension step at 72ºC for 10 min terminated the PCR reaction. For the *α-actin* fragments (300bp and 640bp), the following PCR program was used: initial denaturation step at 98ºC for 2 min, 5 cycles of denaturation at 98ºC for 15 sec, annealing at 66ºC for 15 sec, and extension at 72ºC for 30 sec. Then, 35 cycles of denaturation at 98ºC for 15 sec, and annealing/extension at 72ºC for 1 min were performed. The PCR reaction was concluded with a final extension step at 72ºC for 10 min. A fragment from the Kanamycin resistance gene (540bp) was PCR amplified and used as control for detecting potential off-target effects.

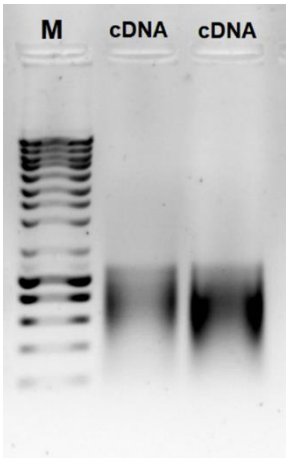



**Figure 4 -** Lanes 1 and 2: cDNA synthetized from the RNA extracted from RPW larval gut using First-strand cDNA synthesis Kit (Nzytech). M, NZYDNA ladder III.

**Table 2 -** Primers used to amplify the target genes.

| PRIMER NAME | PRIMER SEQUENCE (5'-3') | PRODUCT LENGTH (BP) |
|---|---|---|
| ACTIN (KT748776) | | |
| ACT-SF | GGGTTAAGCTTACCACTTACAACTCCATCATGAAA | 300 |
| ACT-SR | GGTTAAGATCTTTAGAAACACTTCCTGTGGACGA | |
| ACT-LF | GGGTTAAGCTTGATGAAGCCCAAAGCAAAAG | 640 |
| ACT-LR | GGTTAAGATCTTACCCAAGAAGGATGGTTGG | |
| TUBULIN (KT748814) | | |
| TUB-SF | GGGTTAAGCTTTGCAAGGTTTCCAACTCACA | 334 |
| TUB-SR | GGTTAAGATCTCTGAGGCAGGTGGTTACTCC | |
| TUB-LF | GGGTTAAGCTTGCCGAGGTACCCCGTGCCATCC | 613 |
| TUB-LR | GGTTAAGATCTCCCGGGCAGGTACCATGTTG | |
| KANAMYCIN RESISTANCE GENE | | |
| KAN-F | GGGTTAAGCTTAAATCACCATGAGTGACGACTG | 540 |
| KAN-R | GGTTAAGATCTGCGATTAAATTCCAACATGGAT | |

## Bacterial strain and construction of dsRNA expression vectors

Plasmid L4440 (Addgene Plasmid #1654) was selected for the construction of the dsRNA expression vectors because of two T7 promoter sequences opposite to each other that flank the multiple cloning site region. After PCR amplification, the target gene fragments were digested with restriction endonucleases *Hind*III and *Bgl*II and ligated into vector L4440 digested with the same restriction enzymes. The recombinant plasmids were introduced into competent *Escherichia coli* HT115 (DE3) [F-, mcrA, mcrB, IN(rrnD-rrnE)1, rnc14::Tn10(DE3 lysogen: lacUV5 promoter -T7 polymerase] cells (**Fig. 5**) via heat shock transformation protocol (https://sharebiology.com/heat-shock-transformation-e-coli-protocol/) and confirmed by molecular analyses such as colony PCR, restriction enzyme digestion, and Sanger sequencing. Strain HT115 has a mutation in the RNAse III (*rnc*) locus, which encodes an endoribonuclease that cleaves dsRNA molecules into small fragments (Dasgupta et al., 1998; Takiff et al., 1989). This means that this strain can be used to produce large amounts of full-length dsRNA. This

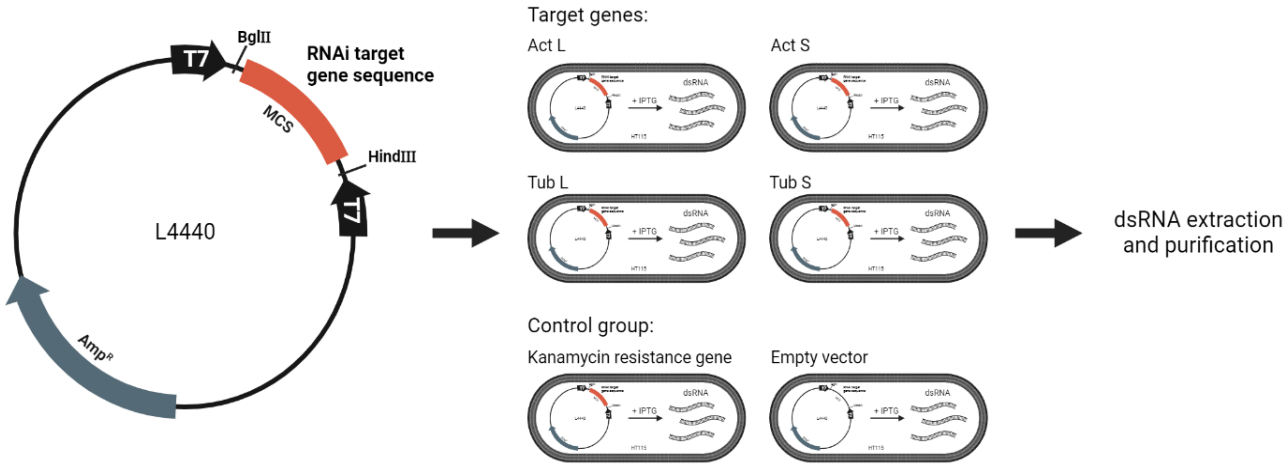



**Figure 5 -** Diagram of the dsRNA production system in bacteria. The RNAi target gene fragment is inserted in the multiple-cloning site which is flanked by two T7 promoters in opposing directions. Then the transformation is performed in *Escherichia coli* strain HT115 (DE3) cells. IPTG is used to induce RNA transcription by the T7 promoter. dsRNA is then extracted and purified.

strain can also express the T7 RNA polymerase via an IPTG inducible promoter (Ongvarrasopone & Panyim, 2007).

## Transformed bacteria preparation and dsRNA expression

Recombinant bacteria carrying the different L4440-derived plasmids were cultured in Luria-Broth (LB) media containing 100 µg/mL ampicillin (LB-AMP) at 37ºC and 200 rpm for 16 h. Then, 50 µL of each culture was re-inoculated into separated tubes containing 5 mL of fresh LB medium. The cells were then allowed to grow at 37ºC and 200 rpm for about 3 hours until they reached an optical density at 600 nm of circa 0.5. Production of dsRNA was induced by adding IPTG (1 mM) to the media and the bacteria were incubated for 4 hours under the same conditions. The expressed dsRNA was extracted using RNazol RT (NZYTech), following the protocol provided by the manufacturer. The synthesized dsRNA was confirmed by electrophoresis on a 1.5% agarose gel.

## Delivery of recombinant bacteria expressing dsRNA to RPW larvae

To evaluate the insecticidal activity of bacteria expressing the target genes, IPTG-induced cultures were pelleted by centrifugation and resuspended in phosphate-buffered saline (PBS). Cell concentration was checked using an improved Neubauer chamber, and cells were diluted to $10^8$ and $10^4$ cells/mL in PBS. Cells were sonicated at a power of 5 W with 5 cycles of 10 seconds pulse and 15 seconds pause on ice and mixed with the larval diet. Small holes were opened into the diet to facilitate larval drilling.

## Bioassay of the bacteria expressing dsRNA

For the bioassays, larvae were placed on the treated diet and the control diet and were kept in an incubator under the same conditions described above for rearing laboratory insects. Six larvae were used per treatment. A control (recombinant bacteria with “empty” vector) was prepared for each treatment. Each bioassay was carried for 17 days and the diet was refreshed once a week. Larval weight was measured every two to three days and RNAi-induced mortality was also scored.

## Gene expression analysis with quantitative real-time PCR (qPCR)

To monitor the mRNA levels of target genes in larvae fed with diet containing bacteria expressing dsRNA, total RNA was extracted from experimental and control larvae using the NZY Total RNA Isolation Kit (Nzytech). Each RNA sample (0.5 µg) was used for cDNA synthesis in a 20 µL reaction mixture following the reaction conditions specified in the NZY First-Strand cDNA Synthesis Kit: 25ºC for 10 min, 50ºC for 30 min, 85ºC for 5 min. After addition of 1 µL of NZY Rnase H, the mix was incubated at 37ºC for 20 min. For the PCR reactions, 2 µL of cDNA was used as template in 20 µL reactions with the respective target gene primers. Ribosomal protein S6 was used as a reference gene (RP6F – 5’-GCAGATGCTTTAGGCGATGA-3’; RP6R – 5’-AAACGGACCCTGCTGTTAGT-3’) (Laudani et al., 2017). Reactions were performed under the following conditions: 40 cycles of 95ºC for 3 min, 95ºC for 10 seconds, 55ºc for 10 seconds, and 72ºC for 20 seconds. The relative expression of the target genes was normalized using the ΔΔCq method and compared with the reference gene (RPS6) and the negative control.

## Statistical analysis

All statistical analyses were performed using the Graphpad Prism 8.4.2 (Graphpad Software). RNAi-induced mortality and weight increment were analysed daily until day 17. Survival curves were analyzed using the Kaplan–Meier method and the log-rank test was used to evaluate the significance of differences in means. Bioassay results and qPCR data were analysed using One-way ANOVA. A value of $p<0.05$ was considered significantly different.

# RESULTS

## Insect rearing and diet optimization

Of the two diets tested, the first (Diet A) kindly provided by Professor Denis J. Wright (Imperial College London, Faculty of Natural Sciences, Department of Life Sciences) (Pickett, 2009), resulted in 100% mortality of insects at the early larval stage (**Fig. 6**). In the second and last diet (Diet B), the larvae showed vigorous growth. Some larvae completed their life cycle by undergoing metamorphosis and reaching the adult stage. Ongoing work in our laboratory is trying to determine the life history traits of RPW on this diet.

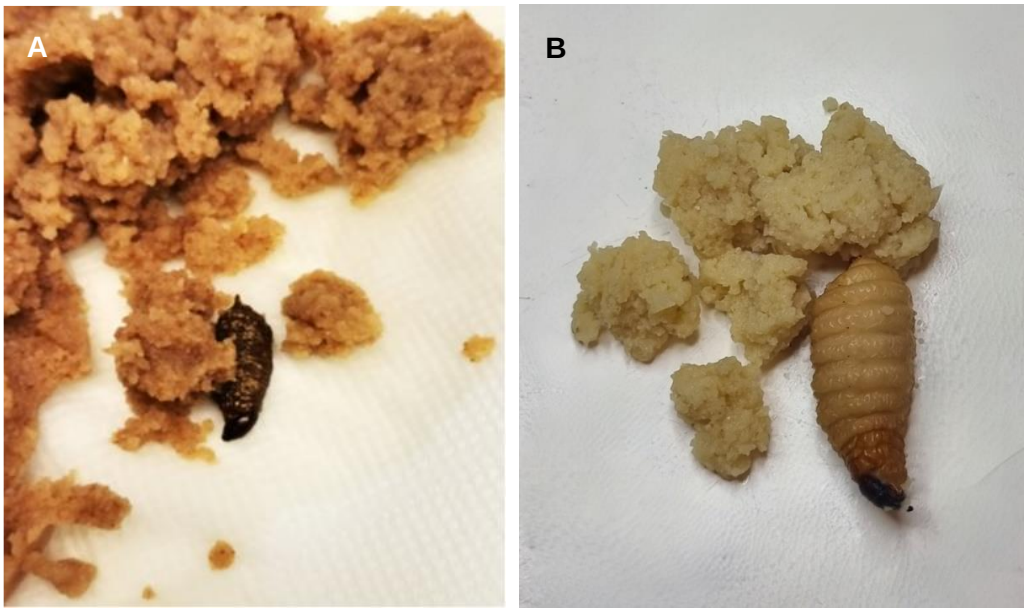



**Figure 6 -** (A) Larva in the first stages dead after feeding on the Diet A. Length: 1.8cm, Width: 0.4cm.; (B) Larva in a more advanced stage feeding on the Diet B. Length: 4cm, Width: 1.5cm.

During the experiment a total of 240 eggs were collected from RPW pairs. One hundred and twenty eggs were placed on apple slices and another 120 eggs were placed on cotton pads soaked in a 20% honey solution. Overall hatching rates of 40% (48 eggs) and 62% (74 eggs) were observed for each condition, respectively. Although a faster hatching rate (**fig. 7**) and a higher percentage of eggs hatching were observed for the eggs on cotton pads with a 20% honey solution, the differences between the two treatments were only significant ($p>0.05$) in the second and third days.

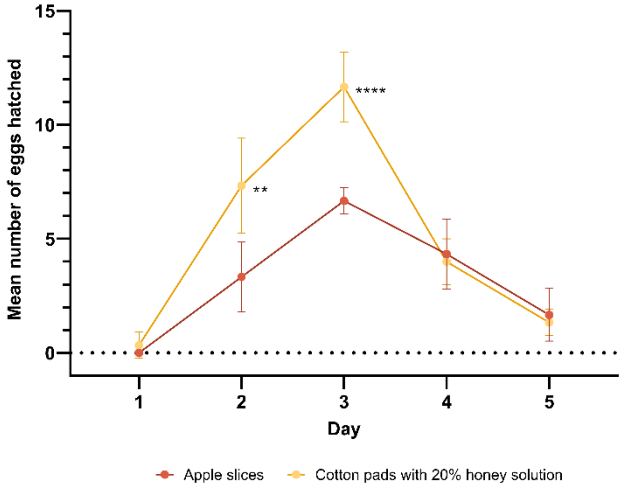



**Figure 7 -** Mean number of eggs hatched per day while on different substrates. It is possible to observe a greater daily hatching rate in eggs placed in cotton pads embedded in a solution of 20% honey.

## Bacterial transformation and dsRNA expression

To feed the insects with dsRNA, *E. coli* HT115 (DE3) bacteria were transformed with vector L4440 carrying fragments of one of the two target genes α-actin and β-tubulin (**Fig. 8**). Furthermore, two variants of different sizes were cloned for each target gene: for the α-actin, a long fragment of 640bp (ActL) and a short fragment of 300bp (ActS); for the β-tubulin, a long fragment of 613bp (TubL) and a short fragment of 334bp (TubS). Finally, a 540bp fragment of the kanamycin resistance gene was cloned into L4440 and used as an off-target control. These recombinant bacteria produced the corresponding dsRNA molecules when induced with IPTG (**Fig. 9**).

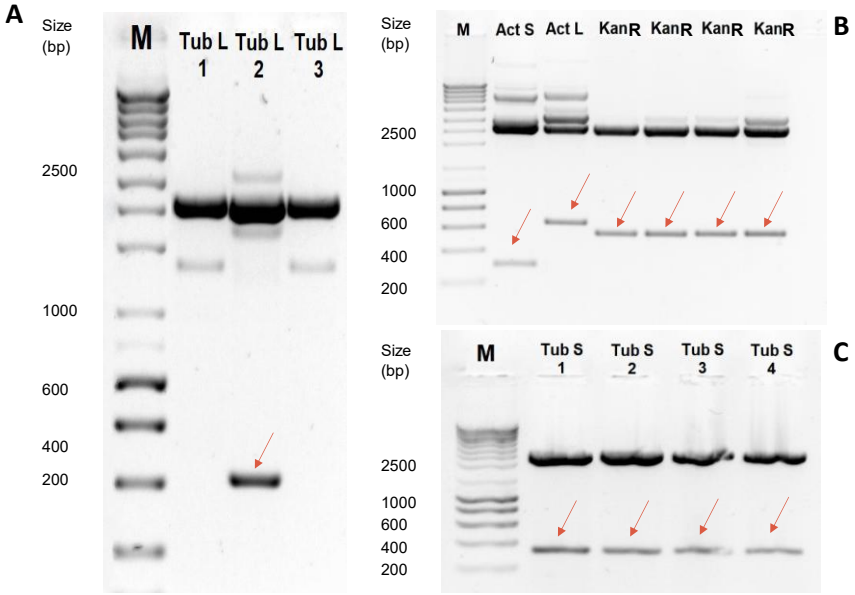



**Figure 8 -** Results of the bacterial transformation (positive colonies marked with the orange arrows): (A) positive colony containing the *β-tubulin* long fragment (TubL) (613 bp); (B) positive colonies containing the *α-actin* short (ActS) (300 bp) and long (ActL) (640 bp) fragment, and colonies containing the Kanamycin resistance gene (KanR) (540 bp); (C) positive colonies containing the *β-tubulin* short fragment (TubS) (334bp). M, NZYDNA Ladder III.

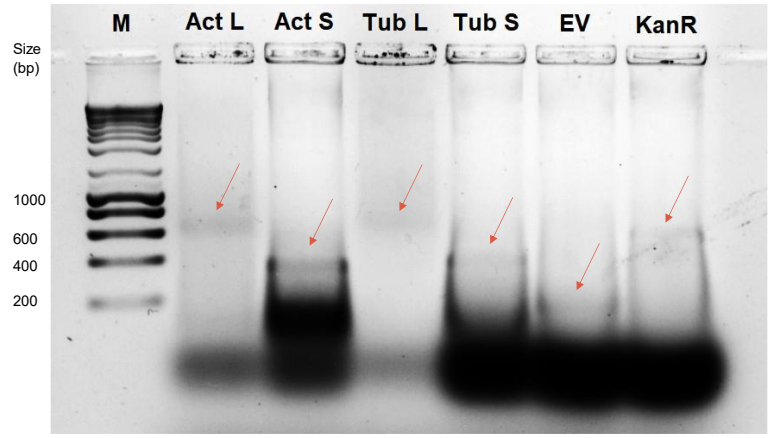



**Figure 9 -** Detection of dsRNA produced by recombinant bacteria. Arrows indicate dsRNA fragments. M: Ladder; ActL: *α-actin* long fragment; ActS: *α-actin* short fragment; TubL: *β-tubulin* long fragment; TubS: *β-tubulin* short fragment; EV: empty vector; KanR: Kanamycin resistance gene fragment.

## Gene expression analysis

RT-qPCR was used to verify the effects of feeding larvae with bacteria expressing dsRNA of RPW genes on the expression of the corresponding genes. As for the *α-actin* gene (**Fig. 10A**), ingestion of bacteria expressing dsRNA-ActL resulted in a 70.4% decrease in its expression in treated larvae compared to those fed the negative control (bacteria with empty vector L4440). When treated with dsRNA-ActS, feeding resulted in an 85.4% reduction in the expression of the *α-actin* gene. Both treatments were statistically significantly different to the negative control. As for the *β-tubulin* (**Fig. 10B**), gene expression suppression due to the ingestion of specific dsRNA was also detected in larvae treated with both gene fragments. Larvae from the dsRNA-TubL treatment showed a 61.7% reduction in *β-tubulin* expression, while the reduction was 96.76% for larvae exposed to the dsRNA-TubS treatment. Both treatments showed significant differences with the negative control. Since the high percent of sequence identity (50.5%) between the *α-* and *β-tubulin* of the tubulin genes superfamily (**Supplementary figure 1**), we determined the expression of the former in the cDNA of dsRNA-TubS-treated insects and found a 98.4% reduction in *α-tubulin* mRNA (**Fig. 10B**). Finally, the expression of the *α-actin* and *β-tubulin* genes did not differ between the dsRNA-KanR treatment and the negative control, indicating that there were no off-target effects in the insects due to the ingestion of dsRNA (**Fig. 10 A and B**).

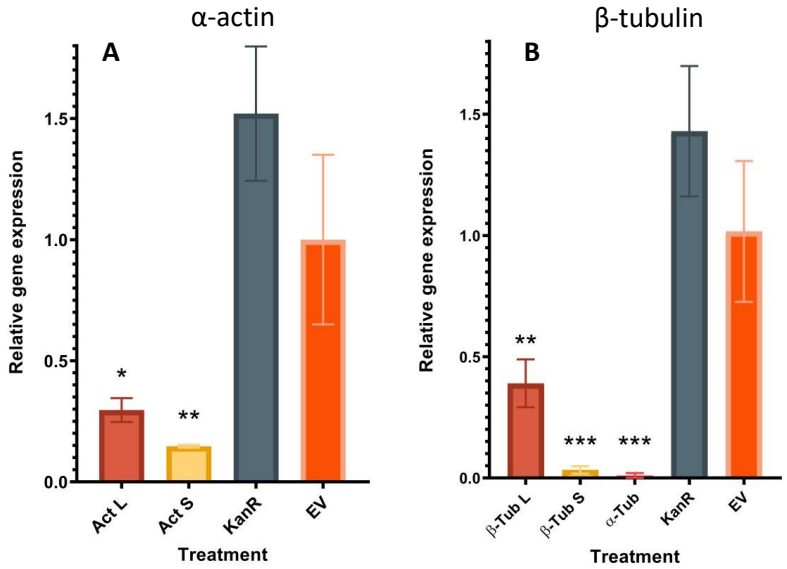



**Figure 10** - Reverse transcription quantitative PCR (RT-qPCR) analysis of the relative gene expression between treatments and control. (A) target: α-actin; (B) target: β-tubulin. EV: Empty vector (negative control). KanR: Kanamycin resistance gene. Gene knockdown was observed in the target genes actin and β-tubulin. An off-target effect in the α-tubulin was observed when targeting the β-tubulin gene. Neither target genes caused a downregulation of the Kanamycin resistance gene. Asterisks represent a statistical difference between the treatments and the negative control. * ($p<0.05$); **($p<0.05$); ***($p<0.001$).

## RNAi-induced insect mortality

In larvae exposed to a higher bacterial concentration ($10^8$ cells/ml), the survival curves of insects with knockdown of the *α-actin* and *β-tubulin* genes were significantly different from those of the control group ($p < 0.05$). The *α-actin* target resulted in 100% larval mortality on day 7, with the first dead insects found on day 5 for both sizes of dsRNA. In the case of the *β-tubulin* target, treatment with dsRNA-TubS resulted in 100% mortality on day 12, while mortality in dsRNA-TubL reached 66.7% on day 17 and remained unchanged until the end of the bioassays. Mortality in the dsRNA-TubS group was detected from day 5, while it was not detected in the dsRNA-TubL group until day 14.

In larvae exposed to a lower concentration of bacteria ($10^4$ cells/ml), significant mortality was observed only in larvae exposed to dsRNA-ActL ($p < 0.05$). This treatment resulted in 83.4% mortality at the end of the bioassays, but the first dead insects were recorded on day 7. In contrast, dsRNA-ActS resulted in 50% mortality at the end of the bioassay, with the first deaths observed on day 14. For the *β-tubulin* target, mortality by dsRNA-TubL and dsRNA-TubS was observed from day 14 onwards, with the former treatment resulting in mortality in 50% of the exposed larvae at the end of the bioassay and the latter in 33.4% (**Figs. 11 and 12**).

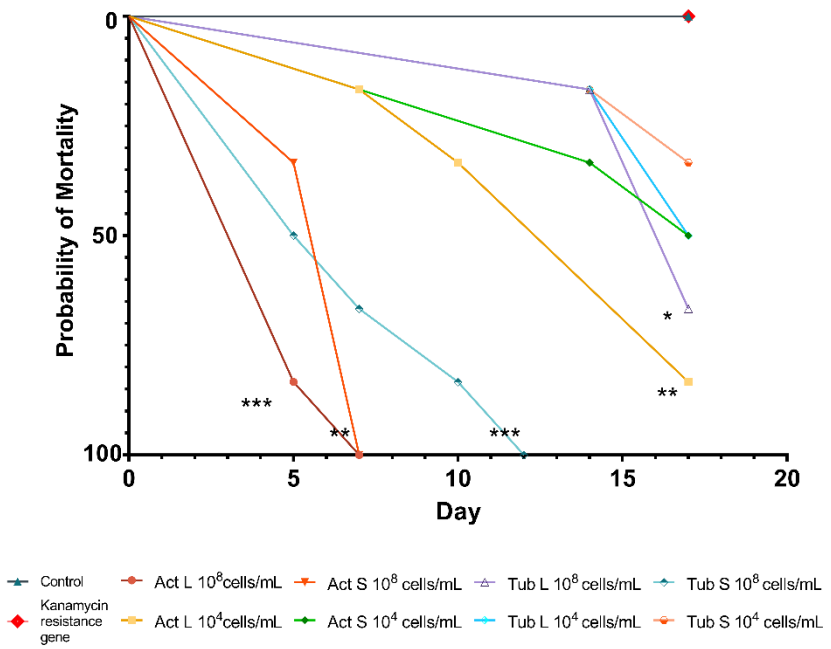



**Figure 11 -** Survival curve of larvae exposed to treatment and controls. Larvae exposed to the higher concentration of dsRNA of the *α-actin* fragments showed 100% mortality by the 7th day. The *β-tubulin* short fragment in the highest concentration induced 100% mortality by the 12th day. The other treatments induced some degree of mortality by the last day, but not quite as high. Asterisks indicate significant difference when compared to the negative control: * ($p<0.05$); ** ($p<0.005$); *** ($p<0.001$).

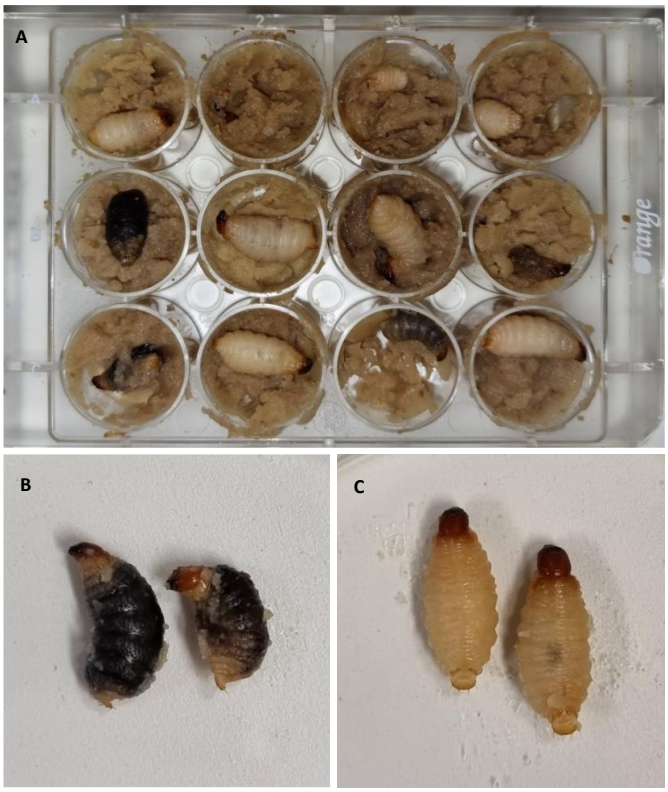



**Figure 12 -** RPW larvae subjected to diet containing recombinant bacteria. (A) 12 well plate with RPW larvae exposed to one of the treatments. (B) Dead larvae that resulted from the treatment with the target genes. (C) Alive larvae from the control group. Larvae exposed to the treatment started to show signs of melanization, which lead to death.

## RPW larval weight variation after fed diet containing recombinant bacteria expressing dsRNA of the target genes

In addition to mortality, a decrease in daily weight gain was observed in some of the larvae exposed to a diet containing recombinant bacteria expressing dsRNA from insect genes. We examined weight gain data from two groups of larvae per target gene - one group exposed to $10^8$ bacterial cells/ml (HCC) and the other group exposed to $10^4$ bacterial cells/ml (LCC) - and compared them to the weight gain of larvae exposed to bacteria transformed with an empty vector, i.e. the negative control.

The weight gain of larvae exposed to dsRNA-ActL showed significant differences compared to the control for both bacterial concentrations, HCC ($p < 0.0001$) and LCC ($p < 0.05$). In addition, a mean weight gain of 0.002±0.008 g and 0.016±0.014 g was measured for HCC and LCC, respectively, while a mean weight gain of 0.061±0.011 g was measured for the control group (**Fig. 13A**).

As with the dsRNA-ActS treatment, only larvae receiving HCC showed a statistically significant difference in weight gain ($p < 0.05$) compared to the control group. Larvae receiving the HCC-containing diet showed a mean weight gain of 0.008±0.019 g, while the mean weight gain of 0.032±0.017 g and 0.029±0.012 g for the LCC and control groups, respectively, showed no significant differences between them ($p > 0.05$) (**Fig. 13B**).

For dsRNA-TubL, only the larvae fed the HCC-containing diet showed a statistically significant difference ($p < 0.05$). Larvae exposed to HCC had a mean weight gain of 0.015±0.042 g, compared with 0.055±0.035 g in the LCC group and 0.053±0.035 g in the control group (**Fig. 14A**).

No statistical difference was observed in the weight gain of larvae exposed to dsRNA-TubS at any of the tested concentrations ($p > 0.05$). The mean weight gain was 0.043±0.044 g, 0.045±0.019 g and 0.048±0.020 g for the HCC, LCC and control groups, respectively (**Fig. 14B**).

Finally, larvae exposed to dsRNA-KanR (off-target control) and the negative control showed no significant differences in weight gain ($p < 0.05$), with mean weight gain values of 0.046±0.016 g for KanR and 0.042±0.029 g for the negative control (Fig. 15).

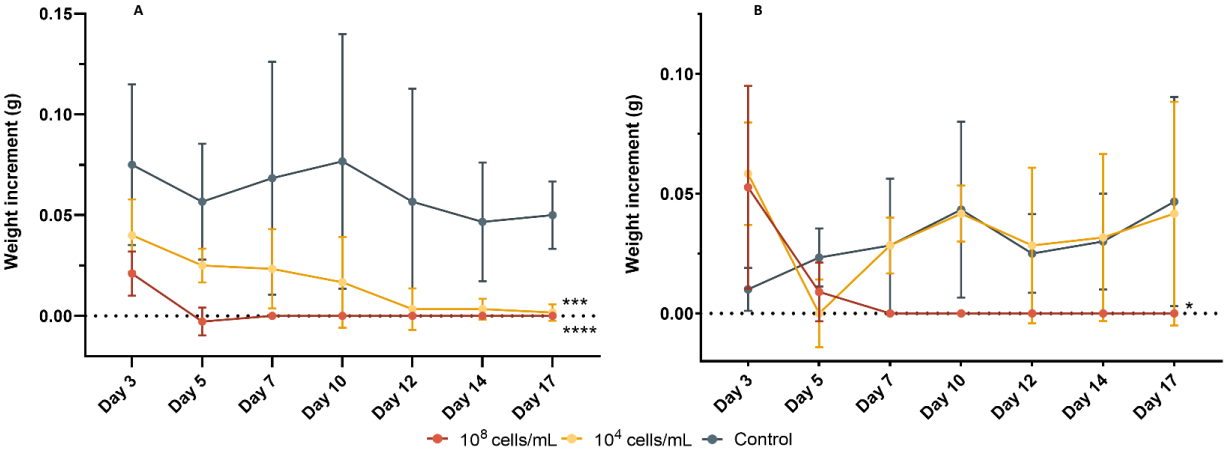



**Figure 13 -** Weight increment of RPW larvae exposed to diet containing recombinant bacteria expressing dsRNA of the actin gene. (A) □-actin long fragment; (B) □-actin short fragment. Asterisks represents significant difference in means when compared to the control group: *** ($p<0.001$); **** ($p<0.0001$). Lack of asterisk means no significative difference.

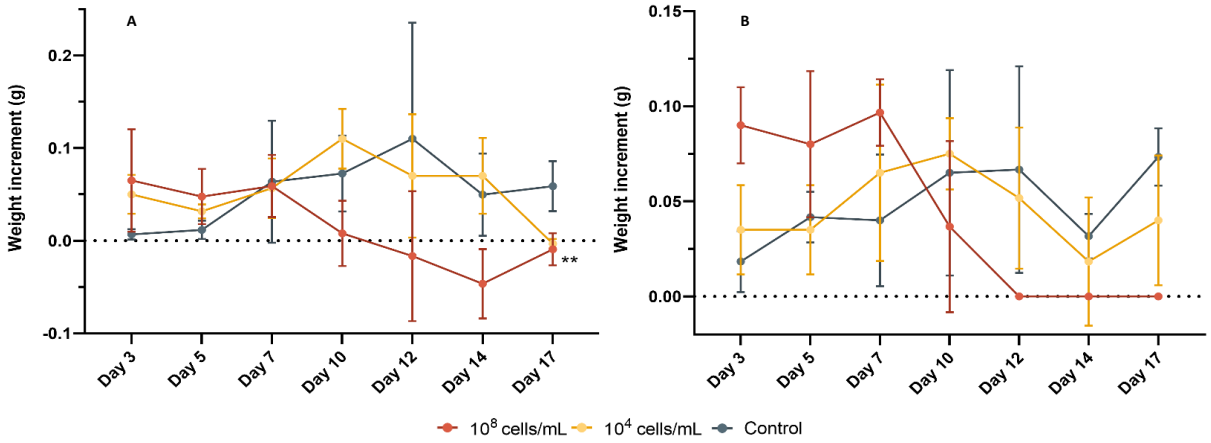



**Figure 14 -** Weight increment of RPW larvae exposed to diet containing recombinant bacteria expressing dsRNA of the β-tubulin gene. (A) β-tubulin long fragment; (B) β-tubulin short fragment. Asterisks represents significant difference in means when compared to the control group ($p<0.005$). Lack of asterisk means no significative difference.

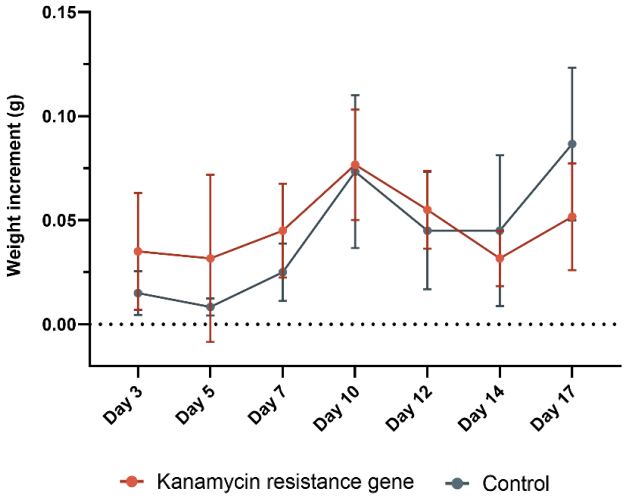



**Figure 15 -** Weight increment of larvae exposed to diet containing bacteria expressing dsRNA of the Kanamycin resistance gene (off-target control). Lack of asterisk means no significative difference

# DISCUSSION

Several methods have been assayed for the control of the RPW, since it is one of the most harmful pests to the palm trees worldwide. This weevil has a wide geographical distribution and has the ability to resist cold weather conditions (León-Quinto & Serna, 2022); however, as the RPW is native to warmer regions, it is expected to prefer the same conditions to reproduce and spread. With that being said, one of the major problems that we have to account for when dealing with this insect is the climate change (Omran, 2020). Studies have shown that this phenomenon may cause this species to colonize the northern regions in Europe and North America, where it has not yet been detected, and others in the Southern hemisphere. These predictions include countries such as Belgium, Norway, Sweden, Denmark, Finland, the United Kingdom, Colombia, Venezuela, Cameroon, Angola, and Botswana (Fiaboe et al., 2012).

To be able to test new methods to control RPW, this work established a colony of the insect in our laboratory at the CIIMAR - Faculty of Science of the University of Porto. The rearing of the RPW under laboratory conditions requires certain adequate parameters such as temperature, photoperiod, humidity, and a suitable diet. Since rearing the RPW in its natural host, palm trees, is quite costly, an artificial diet must be developed that provides all the nutrients required for the growth and development of the insect (Al-Ayedh, 2011), and also that contains ingredients readily available in a given region. For an artificial diet to be considered appropriate for insect development, it must contain a nitrogen source, carbohydrates, lipids, vitamins, preservatives, minerals, stabilizers, and fillers or attractants (Cohen, 2003; El-Mergawy & Ajlan, 2011). We tested two meridic diets for the rearing of the RPW. A meridic diet contains some well-characterised components (vitamins and preservatives) and other poorly defined ones, such as whole food-type ingredients (e.g. wheat germ and other raw grain flours) (Elshafie et al., 2013). The diet A we tested for RPW is commonly used for other species, such as *Heliothis virescens* (Fabricius) and *Spodoptera frugiperda* (J. E. Smith), where it has been shown to be suitable for insect growth and development under laboratory conditions (Prof. Denis J. Wright, personal communication). However, we found that this diet was not suitable for the RPW, so we made changes. The first change was to remove the flour from pinto beans and soybeans, which serves as a source of iron for the insects. Although iron plays a life-supporting role, it can be potentially destructive as a toxin and prooxidant, causing the formation of free radicals and the deterioration of other nutrients that are sensitive to oxidative degradation (Cohen, 2015). Insects of the order Lepidoptera usually require five times the amount of iron compared to other orders, such

as Coleoptera (Cohen, 2015; Keena, 2005; Keena, 2022), which may be why the two species mentioned above were able to develop while feeding on the Diet A and the RPW did not due to the increased iron concentration. Other components that were removed were the Wesson Salt Mix and casein, which are a source of minerals such as calcium and potassium. Although the redundancy of these two components does not necessarily lead to a harmful interaction, it is possible that an excess of aminoacids is supplied to the insect, which can lead to a metabolic burden of a toxic amount of ammonia or uric acid (Cohen, 2015). Finally, the use of hydrolysed components forces the insect to use a non-natural nitrogen source, which contributes to an increase in osmotic pressure due to the free aminoacids present. Therefore, the hydrolysed yeast was replaced by dried Brewer's yeast. All the above modifications resulted in a diet B that was very similar to diet made by ElShaffie (2013), except for the palm-derived ingredients, which allowed the author to achieve 93% adult emergence (Elshafie et al., 2013).

After completion of the larval period, RPW larvae pupate in a fibrous cocoon made of palm fibers, which can be replaced by sugarcane fibers in the laboratory (Kaakeh, 2017; Salama & Abdel-Razek, 2002; Salama et al., 2009). However, it has already been reported that RPW larvae can reach the adult stage in the laboratory without having to form a cocoon (unpublished results) (Hamadttu Abdel Farag & Jose Romeno, 2020). In our work, pupation started only after incubation of the larvae with sugarcane stalks and the photoperiod was set to complete darkness. However, we detected RPW pupae developing into adults inside and outside a cocoon (**Fig. 16**), suggesting that complete darkness is a very important factor to trigger metamorphosis in this species (Martín & Cabello, 2006). Some studies have shown that an excess of artificial light can be harmful to insects leading to developmental arrest, such as pupal diapause (Boyes et al., 2021). The latter would need to be further investigated in RPW.




**Figure 16 -** RPW larvae going through pupation. (A) Cocoon made inside of sugarcane, (B) Pupation occurring without cocoon, (C) Adult emerging from sugarcane.

There are several media that have been tested for hatching RPW eggs, such as cotton wools saturated with a honey solution, Petri dishes lined with moist filter paper, pieces of sugarcane and apple slices in plastic containers (Abdel-Hameid, 2019; Fayyaz et al., 2009). In this work, two strategies were tested to assess egg hatchability: apple slices in plastic containers and cotton wools saturated with a 20% honey solution. Egg hatchability in sugarcane pieces was not tested, as these are not so easily accessible in Portugal mainland. The percentage of hatched eggs was higher in cotton wools with the honey solution than in apple slices. These results are in agreement with those observed by other authors (Fayyaz et al., 2009; Kaakeh et al., 2001) and a possible explanation for this observation could be that the cotton wools with the honey solution keeps the eggs better moist, while the apple slices were observed to dry out when kept in an incubator.

The use of recombinant bacteria to orally deliver dsRNA molecules to an insect pest has been shown to be a good alternative to trigger an RNAi response that leads to knockdown of the target gene (Delgado-Martín & Velasco, 2021; Li et al., 2011; Zhu et al., 2011). In this work, HT115 *E. coli* was transformed with the L4440 vector cloned with the target gene sequences to express dsRNA specific for the selected insect genes. Although an RNAi response can be detected in treated insects, the production of dsRNA by recombinant bacteria is reportedly not as effective as dsRNA synthesized *in vitro* (R. S. Leelesh & L. K. Rieske, 2020). In addition, the L4440-HT115(DE3) system still needs to be improved, as it is not consistent in the production of dsRNA and can even produce non-specific effects.

One of the problems of the L4440-HT115(DE3) system is the production efficiency of dsRNA. Ma et al, 2020 compared the pET28 vector against the L4440 vector in two *E. coli* strains, i.e. BL21(DE3) and HT115(DE3). The authors found that the expression efficiency of the pET28 vector, which has a single T7 promoter, was higher than that of L4440 in both strains. The authors explain that the production of a single dsRNA molecule might be higher due to the need to consume less energy during the transcription of the dsRNA (Ma et al., 2020). Also, the pET28-BL21(DE3) system was able to achieve a higher dsRNA expression efficiency than all other combinations of plasmid-*E. coli* strain systems. Another problem with the L4440 vector is that it tends to cause non-specific effects. De-Souza et al. (2019) found that the dsRNA produced by the multiple cloning site (MCS) of this plasmid shares complementary sequences with some reporter vectors, inducing partial transgene silencing in *C. elegans* via the antiviral RNAi pathway. This means that RNAi induced by L4440-derived vectors can lead to unexpected results (De-Souza et al., 2019).

A critical step for a successful gene silencing is the selection of the target genes, and next generation sequencing (NGS) can be useful in providing guidance to the target gene selection (Wang et al., 2011). RNA-seq is a deep-sequencing technology that does not include a cloning or amplification step, requires less RNA sample, and is not limited to the detection of transcripts of available genomic sequences (Haas & Zody, 2010; Wang et al., 2009). In this work, two housekeeping genes of RPW (*α-actin* and *β-tubulin*) were selected to assess the bacterial feeding method for RNAi in this species. These two genes are present at each stage of the RPW life cycle and play essential biological functions. The protein actin is a major component for cell motility, cell division, and muscle contraction. The protein tubulin (α-tubulin, β-tubulin, and γ-tubulin) are structural proteins of the cytoskeleton and can be found in microtubules. These genes have been tested as RNAi target in other insect orders such as Hemiptera (Rosa et al., 2012; Upadhyay et al., 2011), Lepidoptera (Wang et al., 2018), and Orthoptera (Wynant et al., 2012), where it was shown to have a lethal effect. According to our results, both RNAi targets caused mortality in RPW during the duration of the bioassay, with dsRNA-ActL and dsRNA-ActS fragments causing mortality faster than dsRNA-TubL and dsRNA-TubS in the two bacterial concentrations tested. Our results are consistent with Baum et al. (2007), who described a lower lethal dose for *actin* dsRNA ($LC_{50}$: 2.86 ng dsRNA/$cm^2$) compared to *α-tubulin* dsRNA ($LC_{50}$: 5.20 ng dsRNA/$cm^2$) and *β-tubulin* dsRNA ($LC_{50}$: 51.98 ng dsRNA/$cm^2$) in another coleopteran species, the Western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) (Baum et al., 2007). Despite the fact that both targets caused mortality, the RT-qPCR of dsRNA-TubS treated cDNA showed a downregulation of *α-tubulin* gene in addition to the targeted *β-tubulin* gene. So, considering the mortality percentage, the mortality rate, and the probability of inducing off-target effects, targeting the *α-actin* gene seems to be a good option for insecticidal RNAi in RPW.

The exposure of RPW larvae to bacteria producing dsRNA molecules also caused impaired weight gain compared to control larvae. This effect has previously been observed in other insect species, such as *Leptinotarsa decemlineata* (Hussain et al., 2019), *Helicoverpa armigera (Kolge et al., 2021),* and *Tribolium castaneum (Agrawal et al., 2014).* In RPW, to our knowledge, no results have yet been demonstrated regarding weight alterations. Again, a higher concentration of bacteria expressing dsRNA had a better effect on silencing of both genes according to our results, but an earlier and significantly stronger effect was seen for the α-*actin* gene compared to the *β-tubulin* gene.

The reason why knockdown of α-*actin* had better effects on mortality and weight loss of RPW larvae than that of *β-tubulin* is not known with certainty. These two proteins are the main components of the cytoskeleton: actin forms polymers known as thin filaments, and tubulin polymerizes to form microtubules. The functions of the cytoskeleton are many. For instance, this structure gives shape and support to the cell, helps form vacuoles, holds various cell organelles in place, aids in cell signalling, and supports intracellular movements, such as migration of cell organelles and transport of vesicles in and out of the cell (Hohmann & Dehghani, 2019). The proteins α-actin and β-tubulin have different roles in these activities. One possible explanation for the results of RNAi could be that the cells are more sensitive to any change in the amount of α-actin than β-tubulin proteins for their proper functioning. Another reason could be that the turnover rate of proteins during larval growth is higher for α-actin than for β-tubulin, so there is a constant need for the former. A deficiency of α-actin can impair cell division in the intestinal tissue of the insect and create breaches for microorganisms to enter the hemolymph, where they multiply until they cause the insect's death by septicemia.

Our results show the potential of RNAi by feeding to develop a new bioinsecticide against RPW. Endophytic bacteria have been isolated and identified from many plants, including palms, and could be used as carriers of dsRNA to larval borers. Recombinant bacteria could be applied by drenching the roots so that they can be taken up by the plant and colonize it (R. S. Leelesh & L. K. Rieske, 2020; Singh et al., 2018). Another way to use insecticidal RNAi is to spray the palm trees with naked dsRNAs to target the adults or to spray it directly into the ash stems so that it can be ingested by the newly hatched larvae (Pampolini et al., 2020). However, studies have shown that naked dsRNA degrades very rapidly when exposed to environmental factors (Bachman et al., 2020), so several authors have tested different nanomaterials to shield it. Some of the materials tested involve chitosan (Kolge et al., 2021), agar, alginates, cellulose, poly(vinyl alcohol) (PVA), and poly(ethylene glycol) (PEG) (Sikder et al., 2021).

Although there are still drawbacks with this technology such as public acceptance and regulatory issues (Mat Jalaluddin et al., 2019), biopesticides with this mode of action (MoA) are starting to be validated for use against insect pests. Ledprona is a sprayable dsRNA formulation that has been proven in field trials to be effective in inducing mortality against *L. decemlineata*, while ensuring dsRNA stability under long storage conditions, being easy and safe for the user to handle, and having no negative impact on dsRNA performance (Pallis et al., 2022; Rodrigues et al., 2021).

# CONCLUSION AND FUTURE PERSPECTIVES

In this work, methods for rearing RPW under laboratory conditions were evaluated. The diet developed to feed the larvae allowed this insect to complete its life cycle and proved to be a good substrate to mix recombinant bacteria and perform bioassays. It was found that cotton pads with a 20% honey solution had a higher hatching rate than apple slices. It also appears that complete darkness is required to induce pupation in this species, but this needs further investigation.

The results have shown that feeding RPW larvae with bacteria expressing dsRNA leads to downregulation of the selected target genes, i.e. $\alpha$-*actin* and *β-tubulin*, demonstrating that the use of recombinant bacteria expressing dsRNA could be a good alternative to existing methods of RPW control. It was found that $\alpha$-*actin* is a better target as it induces mortality faster and in higher percentages, while dsRNA-TubS not only affected the expression of *β-tubulin* but also caused downregulation of other Tubulin superfamily genes. Since dsRNA-Act induced RNAi response in RPW, and previously in some other insect species, it seems to be a good target for several insect orders.

In this work, a preliminary study was carried out to evaluate if this technique could also be applied to adult RPW. Apple cubes of similar sizes were soaked with recombinant bacteria expressing dsRNA-ActL (which worked better in larvae) and used as insect food. This study was conducted with seven adult RPW: three specimens were exposed to apple cubes soaked with dsRNA, two specimens were exposed to apple cubes soaked with a suspension of bacteria transformed with and empty vector (negative control), and two others were exposed to apple soaked in PBS. The results were promising as the adults exposed to dsRNA-ActL shortly showed a 100% mortality, while none of the control insects died (**Fig.17**). The RNAi of $\alpha$-actin in the exposed adults needs further confirmation at the molecular level, but it opens up the possibility of using this technology as a larvicidal and adulticidal, creating new tools for the complete control of this invasive pest.

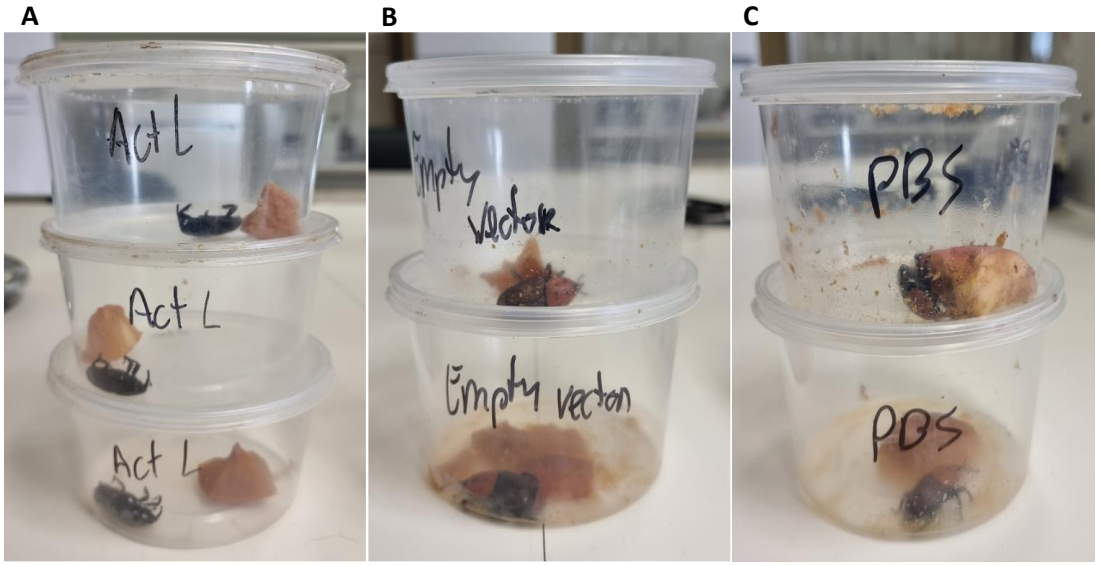



**Figure 17 -** RPW adults exposed to apple slices soaked in a dsRNA-expressing recombinant solution. (A) Adults exposed to *α-actin* long fragment (ActL) dsRNA, (B) Adults exposed bacteria containing an empty vector, (C) Adults exposed to apple cubes soaked in a PBS solution. 100% mortality was observed in adults exposed to recombinant bacteria expressing actin dsRNA, while adults exposed to both control showed no signs of phenotypic alterations or mortality.

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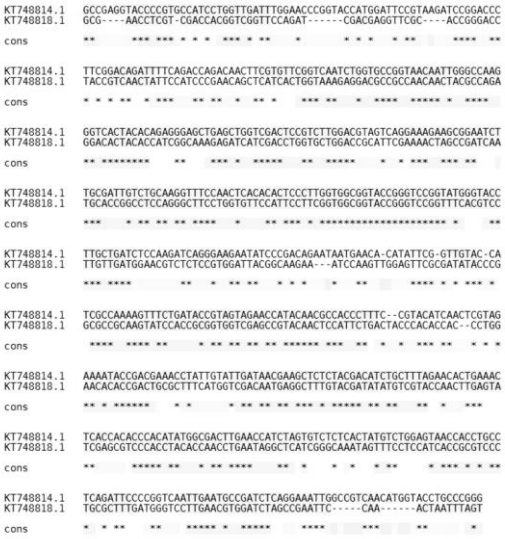
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# ANNEXES

```
#=======================================
#
# Aligned_sequences: 2
# 1: KT748814.1
# 2: KT748818.1
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 715
# Identity:      361/715 (50.5%)
# Similarity:    361/715 (50.5%)
# Gaps:          230/715 (32.2%)
# Score: 703.5
#
#
#=======================================
```



**Supplementary figure 1** – Alignment of the *β-tubulin* and *α-tubulin* sequences of RPW using Pairwise Sequence Alignment, EMBOSS. There is 50.5% identity between the two gene sequences. (https://www.ebi.ac.uk/Tools/psa/emboss_needle/)