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Development of potential microbial agents with two new entomopathogenic fungal strains to control the red palm weevil *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae)

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Abstract

Background Entomopathogenic fungi, representing a class of microbial agent, have been widely used in the field of pest management. The objective of this work was to isolate different species of fungi and to evaluate their virulence against the destructive and invasive red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae).

Results Two new entomopathogenic fungal strains isolated from dead diseased RPWs were identified as *Metarhizium anisopliae* ZZ-A1 and *Fusarium oxysporum* ZZ-L1 using growth characteristics, morphology, and rDNA-ITS sequence amplification. Bioassays showed that *M. anisopliae* ZZ-A1 strain exhibited significantly higher corrected mortality than *F. oxysporum* ZZ-L1 strain (90.92 vs. 77.28%) in fourth instar RPW larvae 12 days after treatment with a concentration of 1.0×10^{10} conidia/ml, as well as low median lethal concentration (LC_{50}) and median lethal time (LT_{50}) values.

Conclusions The results suggest that both fungal isolates can potentially be developed as effective and persistent a microbial agent against this widespread pest, RPW. However, *M. anisopliae* ZZ-A1 showed relatively higher insecticidal activity than *F. oxysporum* ZZ-L1.

Keywords *Rhynchophorus ferrugineus*, Biological control, *Fusarium oxysporum*, *Metarhizium anisopliae*, Pathogenicity

Background

Rhynchophorus ferrugineus (Olivier) (Coleoptera: Curculionidae), commonly known as the red palm weevil (RPW), is a serious threat to palm plantations in tropical and subtropical zones (Dembilio et al. 2009). More than 29 palm species, including well-known and widespread species such as *Phoenix dactylifera* L., *Cocos nucifera* L., *Elaeis guineensis* Jacq., *Areca catechu* L., *Archontophoehix alexandrae* (F. Muell.), and *Phoenix canariensis* Chaubaud (Arecales: Arecaceae), are susceptible to infestation by these weevils (Hussain et al. 2013). Since the 1980s, increases in international trade have contributed to the unintentional introduction of numerous exotic pests into new areas where these invasive species lack natural enemies, often resulting in successful

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establishment. This has often been the case with *R. ferrugineus* (Wan et al. 2015). Although the RPW is native to southern Asia (Lefroy 1906), it has now become established in China, Japan, the United States, the Middle East, Mediterranean regions and many other global locations (Peng and Hou 2017).

Feeding by the weevil ultimately leads to the death of the infested palms (Kharim and Krishnan 2022). Since RPWs are highly destructive, the economic and ornamental values of these palm trees may potentially be seriously affected by the establishment of this invasive species, causing huge annual economic losses (Hou et al. 2011).

The management of RPWs faces enormous challenges. The reasons for this situation are many, including characteristics of the hosts, as well as the pest itself: (1) the height of the palms, (2) the diversity of host species, (3) the strong adaptability of the weevil to altered environments, (4) the ability of the weevil to fly long distances and its tendency to migrate to new environments, (5) the long life cycle of the weevil, along with generational overlap, (6) the nature of the boring activity of the larvae and their concealed lesions on their host plants, and (7) the high reproductive potential of female adult weevils (Qin and Yan 2013). Legal, ideological, physical, chemical and biological methods have been used to limit the uncontrolled dispersal of this forest pest (Hussain et al. 2013). To date, suppression of RPWs in the field is still primarily based on conventional integrated pest management (IPM) measures consisting of training and education, strengthened surveillance and strict quarantine procedures, pheromone trapping, and especially the frequent application of synthetic insecticides (Peng and Hou 2017). Over the past few decades, there has been a strong reliance on the use of chemical pesticides in attempts to control RPWs, including general spraying, tree fumigation, trunk injection, and other application technologies (Hussain et al. 2013). In addition, the development and deployment of pheromone traps have recently shown a degree of success in managing RPW (Abdel-Moety et al. 2012). However, many adverse factors, such as environmental pollution, increases in resistance to pesticides, possible occurrence of deleterious effects on non-target organisms, issues involving human health, efficacy of residues, and high production cost of lures, etc., have motivated scientists to search for more environmentally friendly approaches (Al-Ayedh et al. 2016).

An attractive alternative is the use of biocontrol agents, but information on the biological control of RPWs remains scarce, especially in productive practice, with focus on looking for indigenous natural enemies (Mazza et al. 2014). In recent years, attempts to use bacteria [primarily *Serratia marcescens* Bizio (Enterobacterales: Enterobacteriaceae) (Pu and Hou 2016), *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) (Pu et al. 2017b) and gut bacteria (Liu et al. 2021)], fungi [mainly *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) (Hussain et al. 2015) and *Metarhizium anisopliae* (Metschn.) (Hypocreales: Clavicipitaceae) (Yasin et al. 2019)], viruses (e.g., polyhedrosis virus) (Mahmoud et al. 2018), and nematodes [e.g., *Steinernema carpocapsae* (Weiser) (Rhabditida: Steinemematidae) (Triggiani and Tarasco 2011)] (Troccoli et al. 2015) to control RPWs have become increasingly common in the literature. These pathogens have all been shown to possess varying degrees of pathogenicity against RPW larvae and adults. Although multiple pathogens, as described above, are useful in controlling RPWs, when the diversity of isolates, convenience, security, horizontal transmission and relatively higher insecticidal activity of entomopathogenic fungi (EPF) are considered, fungi are often thought to be the best option for controlling RPW among all of the above control agents (Qayyum et al. 2020).

Generally, the initial step in the development of mycoinsecticides would involve laboratory evaluations to grade the virulence of the tested isolates (Hussain et al. 2014). However, due to the lack of available fungal strain resources in the past, there is still an urgent need for isolating and identifying new entomopathogenic fungal (EPF) strains that are effective in controlling RPWs. In the present study, two new distinct strains of EPF were identified and isolated from dead, fungous diseased RPW larvae and adults. Additional bioassays on the strains were conducted. The study consisted of evaluating and comparing the virulence of these two different isolates in RPWs. This research may provide a basis and facilitate the selection of novel suitable isolates of EPF for the control and management of RPWs.

Methods

Collection and rearing of experimental insects

A laboratory population of RPWs was initially established by collecting adults from infested palm trees in Zhangzhou City, Fujian Province, China (117.65° E, 24.52° N), with the help of pheromone trapping. In the laboratory, adults were reared in a climatic chamber (Kesheng Experimental Instrument Co., Ltd., Ningbo, China) maintained at 28 ± 1 °C, 75 ± 5% relative humidity (RH), and a 12 h: 12 h (L: D) photoperiod. One male and female pair was placed in a clean, perforated plant tissue culture bottle (330 ml, 70 mm Ø, 107 mm height; Jiafeng Horticultural Products Co., Ltd., Chongqing, China) and supplied with sugarcane for feeding and oviposition. Subsequently, eggs were transferred to moist cotton within a Min Bo® Petri dish (90 mm Ø). Upon hatching, the larvae were individually transferred to Min Bo® Petri dishes

(60 mm Ø) and fed sugarcane. After the sixth instar larvae molted, they were moved individually to a transparent 330 ml perforated plant tissue culture bottle, and sugarcane was provided as food until pupation. All eggs, larvae, and pupae were kept in a darkened climatic chamber at 28 ± 1 °C with a RH of 75 ± 5%.

Isolation and culture of entomopathogenic fungal strains

Cadavers of RPWs at all life stages that had been killed by EPF infection were uniformly collected from the above laboratory population. The diseased RPWs were soaked in 75% ethanol for 1 min and subsequently surface-sterilized using 0.1% mercury chloride for 30 s, followed by three rinses in sterile distilled water. Parts of the tissues were then cut and separately inoculated on potato dextrose agar (PDA; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) medium plates sealed with parafilm. Purification of the fungal isolates was achieved using a monospore culture technique. In this study, a total of 13 fungal isolates were obtained from RPW cadavers at different stages of life history. All fungal isolates were grown separately on PDA medium and incubated at 25 °C for 7 days in a Blue Pard® biochemical incubator (Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China) for preliminary pathogenicity screening against RPW larvae. To further select the most promising EPFs, each fourth instar larva was placed on one PDA plate and contacted to the fungal growth directly for 10 min as one replicate. Each isolate was tested with 5 replicates. Based on the results of the preliminary pathogenicity survey, 2 EPF isolates sufficient for killing 5 tested RPW larvae within 12 days were selected for subsequent bioassays. These two fungal isolates were assigned a code based on their origin (ZZ for Zhangzhou), source of isolation/host developmental stage (A for adult and L for larva) and order, designated respectively ZZ-A1 and ZZ-L1. It meant that the code ZZ-A1 strain was isolated from infected adults and the code ZZ-L1 strain was isolated from infected larvae.

Morphological identification of isolates under a microscope

Optical microscopy (Nikon SMZ745 T, Japan) at 40× magnification connected to a digital camera (Digital Slight DS-Ri3, Nikon, Japan) for photography was performed to study the morphological characteristics of ZZ-A1 and ZZ-L1, including mycelia and conidia. The digitalized images of conidiophores were then processed and analyzed using NIS Elements D software (version 4.30, Nikon, Japan). The length, width, shape and other characteristics of conidiophores were calculated and recorded through measuring tools attached to the software.

Molecular biological identification of isolates

Extraction of total genomic DNA of fungi

Fungal total genomic DNA (gDNA) was extracted following the procedure described by the Fungi Genomic DNA Extraction Kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), and then gDNA products were stored at − 20 °C

Amplification of rDNA-ITS region sequences

A fungal DNA fragment of the internal transcribed spacer (ITS) region was amplified by polymerase chain reaction (PCR) using the universal primer set (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China) ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCC TCCGCTTATTGATATGC-3′). Each PCR (50 μl) contained 2 μl of gDNA template, 2 μl of each primer (ITS1 and ITS4, 10 μM), 25 μl of 2× Taq Plus MasterMix (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) and 19 μl of double-distilled water (ddH_2O). The PCR protocol for amplification of ITS regions was conducted with initial denaturing at 94 °C for 10 min, 25 cycles for denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and renaturation at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min.

The size and quality of ITS1-5.8S-ITS2 amplified products were determined by gel electrophoresis. The PCR products (5 μl) were later separated on a 1% agarose gel stained with the nucleic acid dye GelStain (10,000×) (Beijing TransGen Biotech Co., Ltd., Beijing, China) and visualized under ultraviolet light from a JS-680D Fully Automatic Gel Imaging Analyzer (Shanghai Peiqing Science and Technology Co., Ltd., Shanghai, China). The amplicons from the PCRs were run on a DYY-6C Electrophoresis Apparatus (U = 150 V, I = 200 mA, t = 28 min; Beijing Liuyi Biotech Co., Ltd., Beijing, China) using DL 2000 DNA Marker (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) to detect target bands. The ITS1-5.8S-ITS2 amplified products were further purified using a PCR purification kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) and sequenced by Invitrogen Trading (Shanghai) Co., Ltd., Shanghai, China.

Sequencing and phylogenies analysis

The DNA sequences obtained from this study were blasted against the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) GenBank database with the Basic Local Alignment Search Tool (BLAST) for homology searches to compare similarity with data of over 10 species represented in GenBank. The sequences of ZZ-A1 and ZZ-L1 were aligned with those of related reference strains and at least one out-group downloaded from GenBank by the Clustal X

2.0 program (Thompson et al. 1997). A phylogenetic tree was constructed, and evolutionary distance analyses were performed with the maximum likelihood (ML) method using MEGA 5.2 software (Tamura et al. 2011) to determine phylogenetic relationships. The reliability of the dendrogram was evaluated via bootstrap analysis with 1000 replicates. The sequences of the two entomopathogenic fungal strains were concurrently submitted to GenBank and assigned accession numbers.

Bioassays of entomopathogenic fungi against red palm weevils

To develop potential microbial agents with new EPF strains to control RPWs in future pest management programs, the difference in virulence levels of fungal isolates screened in this study were compared. Various concentrations of conidial suspensions of each isolate were applied to RPWs, and their effects were evaluated.

The purification of isolates that germinated rapidly was performed at least 10 times through PDA medium to enhance the performance of the isolates. Conidia were produced from the ZZ-A1 isolate and ZZ-L1 isolate cultivated on PDA plates for 14 days, after which they were respectively harvested by scraping the inoculum. The obtained conidial clumps were then suspended in a sterile distilled water solution containing 0.01% Tween 80, and the mixture was vortexed to attain homogenization. The concentration of the spore suspension was determined by counting the number of conidia per milliliter using a QIUJING® 25 × 16 hemocytometer.

Healthy fourth instar RPW larvae of similar size were selected for laboratory bioassays. A dilution series of conidial suspensions, standardized at 1.0×10^6, 1.0×10^7, 1.0×10^8, 1.0×10^9, and 1.0×10^{10} conidia/ml, was prepared for each isolate. Sterile distilled water solution containing 0.01% Tween 80 was used as the control. In the dosage-dependent bioassay, a spore suspension of 2 ml was sprayed on each tested insect to complete the inoculation of specific pathogens. The treated larvae were held for 10 min and moved separately to a new Petri dish (90 mm Ø), followed by supplying with sugarcane under darkened controlled conditions (28 ± 1 °C, 75 ± 5% RH). There were 3 replicates performed in this trial and 30 larvae in each replicate. Thus, a total of 90 larvae were treated with each concentration of the EPF isolate. After the treatment, the infection symptoms of each insect were observed, and the number of dead insects was recorded at 24 h intervals for 12 days following exposure.

To avoid cross-infection resulting from spore propagation, the dead larvae were removed in a timely fashion, surface-sterilized in 75% ethanol, and kept in moistened filter paper for 2–3 days to confirm whether the death was caused by fungal infection. Confirmation was accomplished by checking mycosis and observing fungal growth. Finally, Koch's postulates were used to verify the pathogenicity of the two EPF isolates, ZZ-A1 and ZZ-L1. To be specific, the fungi from those infected RPW larval cadavers in bioassays were further re-isolated, and then re-infected healthy sensitive RPW larvae again using the same method as described above. If the symptoms after inoculation are identified as being identical to the original diseased individuals, specific causative agent of RPW larvae can be confirmed.

Data statistical analysis

All data processing, statistical analysis and graph generation were performed using IBM SPSS Statistics 21.0 software (SPSS Inc., Chicago, IL, USA) and the SigmaPlot 12.0 program (Systat Inc., San Jose, Calif., USA). All data were expressed as the mean ± standard error (SE). Two-way analysis of variance (ANOVA) were employed using the univariate process of the generalized linear model (GLM) mode and Tukey's honestly significant difference (HSD) procedure for multiple comparison tests at $P = 0.05$ to analyze the diameter of fungal colonies at different culture times. The percentage of larval mortality in the bioassays was Abbott-corrected based on the mortality in the control group (Abbott 1925). The difference in virulence between the ZZ-A1 strain and ZZ-L1 strain, as well as the differences in mortality among different treatment concentrations for each isolate, were evaluated using the chi-square (χ^2) test with the statistical level of significance fixed at $P = 0.05$. Probit analysis was used to determine the toxicity regression equation, minimal lethal concentration (LC_1), sublethal concentration (LC_{10} and LC_{25}), median lethal concentration (LC_{50}), high lethal concentration (LC_{90} and LC_{99}) and median lethal time (LT_{50}) of the ZZ-A1 strain and ZZ-L1 strain (Finney 1971). In toxicity regression analysis, concentrations were transformed using the base 10.000 logarithm, and mortality data were transformed to probit values. Kaplan–Meier survival analysis with the Mantel–Cox log-rank test was further used to compare the survivorship curves for the fourth instar larvae infected by EPF.

Results

The symptoms of RPWs infected by entomopathogenic fungi

After RPWs were infected by entomopathogens, they became obviously sluggish, with conspicuous shriveling of the body, decreased appetite, and reduced fecundity. With increased infection time, the activity levels of the insects were weakened, with the body appearing to have convulsions in situ and even regurgitation of their food. The bodies of severely infected insects only slightly quivered when touched, otherwise appearing stiff, similar

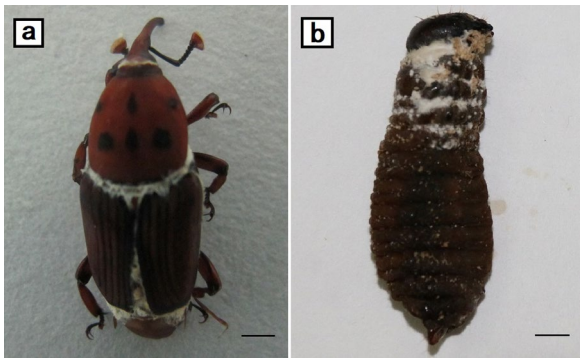

Fig. 1 Red palm weevils parasitized by entomopathogenic fungi **a** ZZ-A1 strain and **b** ZZ-L1 strain. Photographs from a Digital Slight DS-Fi2 camera (Nikon, Japan) connected to a Nikon SMZ745 T stereoscopic microscope (Nikon, Japan) at 0.67 × magnification. Scale bars, 10 mm

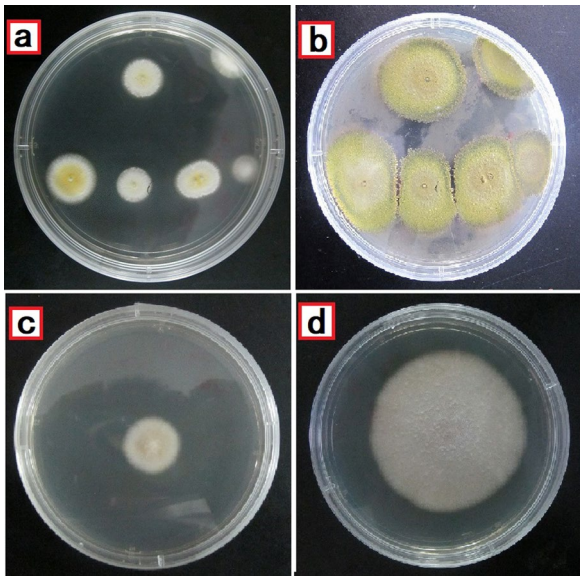

Fig. 2 The fungal colonies of entomopathogenic isolates **a** ZZ-A1 cultured for early phase, **b** ZZ-A1 cultured for later phase, **c** ZZ-L1 cultured for early phase and **d** ZZ-L1 cultured for later phase growing on PDA plates

to dead insects. Finally, the dead RPWs in all life stages exhibited conditions that were symptomatic of fungal infections, including the presence of mycelia and spores. Two different fungal species, provisionally named ZZ-A1 and ZZ-L1, were observed and obtained. The ZZ-A1 strain was isolated from adults (Fig. 1a), while the ZZ-L1 strain was isolated from larvae (Fig. 1b).

The symptoms of strain ZZ-A1 infection were as follows. In the preliminary stage of infection, white mycelia grew on the body surface, especially associated with the intersegmental membranes of the head, thorax and abdomen. After several days, the white coloration began developing into a greenish hue, after which green spores were produced (Fig. 1a).

The insects infected by the ZZ-L1 strain turned uniformly black and appeared slightly bulgy. The body began to harden, and a white floc developed on the surface. Colorless spores subsequently began to appear (Fig. 1b).

Morphological identification of the fungal isolates

Colony culture characteristics

Green sporulation is the most obvious characteristic of the ZZ-A1 strain. Round and opaque colonies with a raised center area on agar plates were white or cream, with villous and gossypine aerial hyphae on the surface. Punctiform olive green spores formed in the middle of the colony with a milky white margin, which turned darker (yellowish to dark-green) during growth and resulted in a tawny color at the back of the plates (Fig. 2a and b).

The colony characteristics of the ZZ-L1 isolate were different from those of the ZZ-A1 isolate. The shapes of the colonies were regularly round with flocculent apophysis, with the hyphae being white and dense. The color of the colonies varied from a white, pinkish-white, or carneose color. In addition, the colony took on a look of mealiness as a result of the large numbers of spores (Fig. 2c and d).

Regarding the growth characteristics of the fungi, the diameter of colonies on the PDA medium was significantly affected by the two strains ($F_{1,9}=6828.305$, $P<0.001$), interval ($F_{6,63}=7216.401$, $P<0.001$), and their associated interaction (two strains × interval: $F_{13,117}=428.250$, $P<0.001$). The ZZ-L1 isolate grew significantly faster on PDA plates than the ZZ-A1 isolate. At 7 days after incubation, the colony diameter of the ZZ-L1 strain averaged 45.6 mm, while the diameter of ZZ-A1 strain colonies averaged only 25.0 mm (Additional file 1: Fig. S1).

Microscopically characteristics

The morphology of hyphae and conidia of the ZZ-A1 strain from RPW cadavers was clearly observed under a microscope. The mycelium was smooth and hyaline, well-branched and separated, with major hyphae measuring 1.8–3.6 μm wide. Conidiophores were podgy, simple, or highly branched, with 2–5 sterigma at the branch top. Solitary or concatenate conidia were colorless, long, oval or cylindrical in shape, with obtuse ends. The measurements of size for the most conidia were 4.2–6.7 μm × 2.2–3.4 μm (Fig. 3). However, we observed that the distinctive characteristic of the ZZ-L1 strain was falciform conidia with septate, which measured 22.5–34.9 μm in length × 3.3–4.6 μm in width (Additional file 1: Fig. S2).

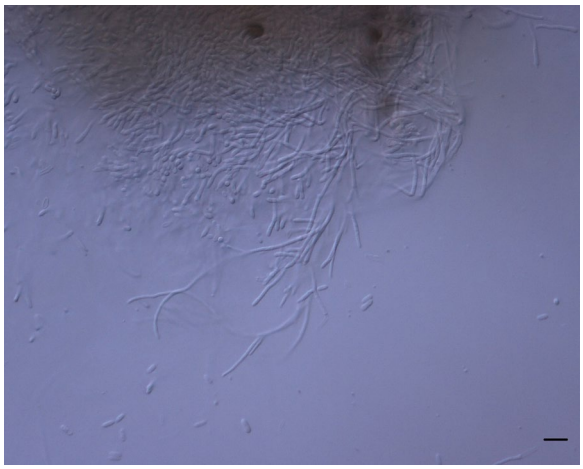

Fig. 3 Morphology of hyphae and conidia of the ZZ-A1 strain at 40 × magnification using optical microscopy. Scale bar, 10 μm

It was thus clear that the ZZ-L1 isolate phenotypically differed from the ZZ-A1 isolate in many aspects, including differently shaped conidia (Fig. 3 and Additional file 1: Fig. S2), different growth rate on PDA medium (Additional file 1: Fig. S1), and in various other colony culture characteristics (Fig. 2). These results demonstrated that the ZZ-L1 isolate was slightly larger and faster developing species.

Molecular identification of the fungal isolates

Approximately 708 bp and 715 bp fragments of ITS were amplified (ITS1-5.8S-ITS2) by PCR from strains ZZ-A1 and ZZ-L1, respectively (Additional file 1: Fig. S3). Sequencing results indicated that ZZ-A1 and ZZ-L1 could represent two new species or strains according to an NCBI Blast search. The ITS sequences of strain ZZ-A1 (accession No. MF467274) and strain ZZ-L1 (accession No. MF467275) were submitted to GenBank.

The dendrogram of the ZZ-A1 isolate (Fig. 4) bifurcated into two distinct clades with a dissimilarity of 0.02. The ZZ-A1 sequence bore more than 93% homology to that of partial *Metarhizium* type strains shown in clade 1 according to an NCBI Blast search. Clade 1 consisted of four additional clusters, in which the ZZ-A1 isolate and *M. anisopliae* were identical (cluster I); *M. lepidiotae* (Driver and Milner) (Hypocreales: Clavicipitaceae) separated from the other clusters (cluster IV); and the remaining *Metarhizium* spp. showed a high similarity (cluster II and cluster III). Clade 2 was represented by two large clusters (cluster V and cluster VI, but these sequences showed the lowest similarity to the ITS sequence of the ZZ-A1 isolate used in this investigation.

The phylogenetic tree of the ZZ-L1 isolate (Fig. 5) also formed two clades, one of which had only one member, *B. bassiana*, which was used as an out-group. Another clade dominated by *Fusarium* can be further divided into a number of small clusters. However, the ZZ-L1 isolate was closely related to *F. oxysporum* Schlecht (Moniliales: Tuberculariaceae) (98% similarity).

Phylogenetic analyses of ITS genes highly supported the ZZ-A1 isolate as *Metarhizium* sp. and the ZZ-L1 isolate as *Fusarium* sp. However, molecular methodology is often hindered by the many difficulties encountered

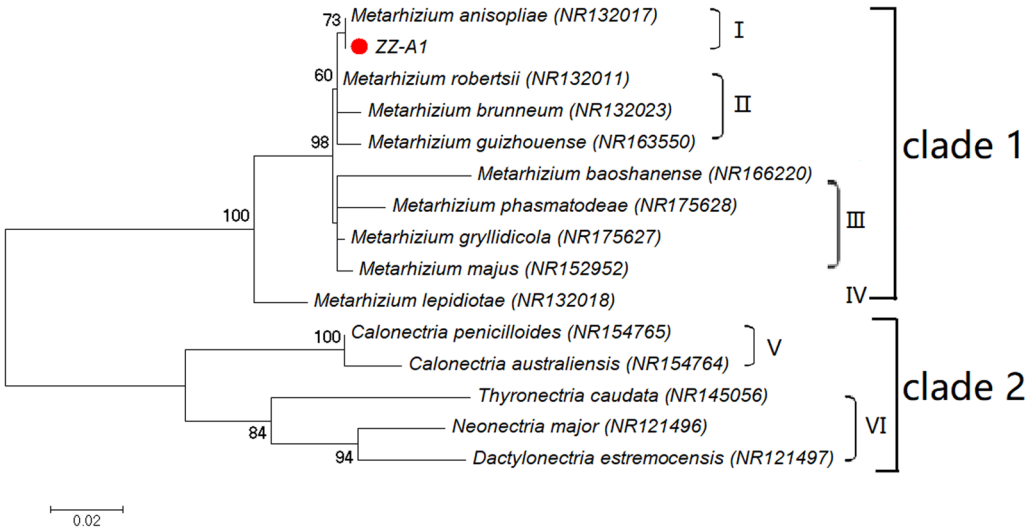

Fig. 4 Phylogenetic analysis tree of the ZZ-A1 strain and closely related fungal species. The dendrogram was constructed using the MEGA 5.2 program with the maximum likelihood (ML) method based on ITS sequences. Bootstrap values shown next to nodes are based on 1000 replicates. Bootstrap values ≥ 50% are labeled. The scale on the base of the dendrogram shows the degree of dissimilarity

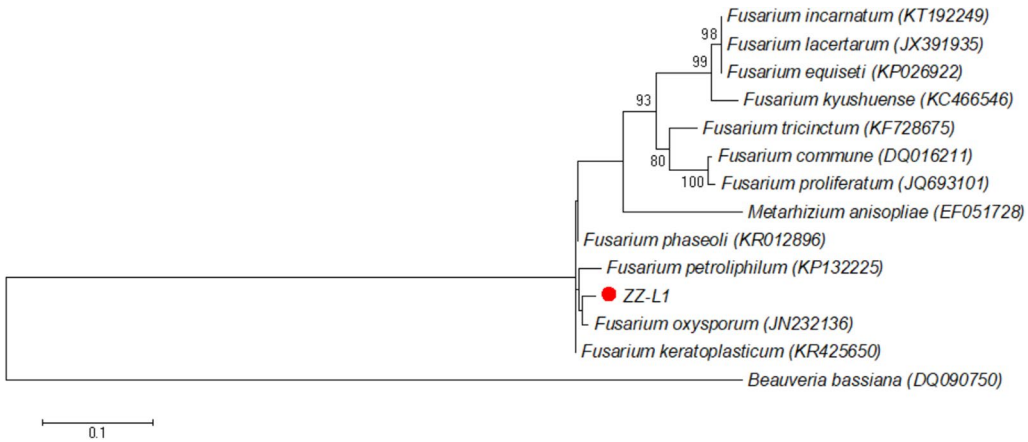

Fig. 5 Phylogenetic analysis tree of the ZZ-L1 strain and closely related fungal species. The dendrogram was constructed using the MEGA 5.2 program with the maximum likelihood (ML) method based on ITS sequences. Bootstrap values shown next to nodes are based on 1000 replicates. Bootstrap values ≥ 50% are labeled. The scale on the base of the dendrogram shows the degree of dissimilarity

in identifying microbes to the level of species. In many instances strains will be certainly identified to the level of genus, but the support rate for species usually cannot reach 100%. Identifying fungi to the species level often requires a combination of traditional morphology with a number of additional references. Accordingly, all the above results indicated that the ZZ-A1 and ZZ-L1 isolates were respectively typical of the species *M. anisopliae* and *F. oxysporum*, using multiple identifications, including colonial culture characteristics, microscopic characteristics and molecular marker sequences.

Virulence evaluation and comparison of two new entomopathogenic fungal strains in controlling red palm weevils

The results showed that the difference in mortality between the two new EPF strains was non-statistically significant when factoring in the corrected mortality after treatment with low concentrations (1.0×10^6 conidia/ml: $\chi^2=0.997$, $df=1$, $P=0.318$; 1.0×10^7 conidia/ml: $\chi^2=0.588$, $df=1$, $P=0.443$; 1.0×10^8 conidia/ml: $\chi^2=1.422$, $df=1$, $P=0.233$; 1.0×10^9 conidia/ml: $\chi^2=1.659$, $df=1$, $P=0.198$; Fig. 6). However, conidia of *M. anisopliae* ZZ-A1 and *F. oxysporum* ZZ-L1 were found to be highly virulent to *R. ferrugineus* and killed 91.11 and 77.78% of test specimens, respectively, 12 days post-infection with 1.0×10^{10} conidia/ml, which exhibited a significant difference ($\chi^2=6.090$, $df=1$, $P=0.014$; Fig. 6). The formest value was the highest mortality recorded during this bioassay. In contrast, the control group showed significantly low natural mortality rates (2.22 ± 1.11%). There was an obvious tendency of the corrected mortality rate to significantly increase with conidial concentration ($\chi^2=87.029$, df = 4, $P<0.001$ for

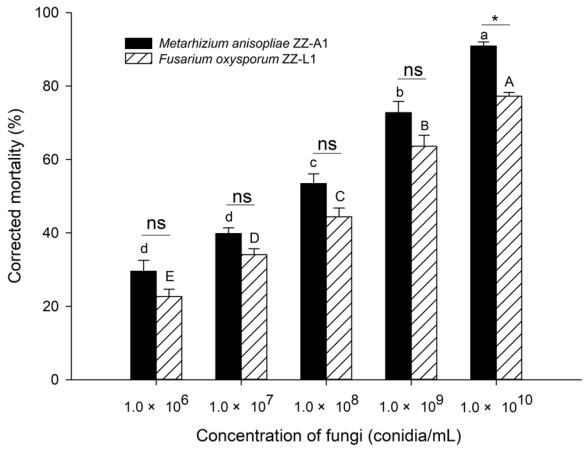

Fig. 6 Mortality of fourth instar red palm weevil larvae after exposure to entomopathogens 12 days post-inoculation. Columns with different lowercase letters differ significantly among different concentrations of *M. anisopliae* ZZ-A1, while columns with different uppercase letters differ significantly among different concentrations of *F. oxysporum* ZZ-L1 ($P=0.05$ level). "ns" and "*" indicate whether there is a significant difference between the two fungal strains at the same concentration (ns, no significance; *, $P<0.05$)

M. anisopliae ZZ-A1; $\chi^2=82.100$, df = 4, $P<0.001$ for *F. oxysporum* ZZ-L1; Fig. 6). In the highest concentration treatments (1.0×10^{10} conidia/mL), death occurred four days after *F. oxysporum* ZZ-L1 treatment, whereas death began on the second day after applying *M. anisopliae* ZZ-A1; when 12-day-old and RPW fourth instar larvae were inoculated with EPF, the survival rate was lower in the *M. anisopliae* ZZ-A1 treatment than in the *F. oxysporum* ZZ-L1 treatment (Additional file 1: Fig. S4).

In the concentration-mortality bioassays, toxicity regression equations of the two fungal strains

against RPW fourth instar larvae were estimated, and Pearson chi-squared goodness-of-fit tests were conducted for the *M. anisopliae* ZZ-A1 strain ($\chi^2=0.332$, $df=2$, $P=0.847$; $y=-3.44+0.46x$, $R^2=0.961$) and *F. oxysporum* ZZ-L1 strain ($\chi^2=0.293$, $df=2$, $P=0.864$; $y=-3.04+0.38x$, $R^2=0.990$). Probit analysis showed that *M. anisopliae* ZZ-A1 had higher LC_1, LC_{10} and LC_{25} values 12 days post-treatment compared to *F. oxysporum* ZZ-L1 (Table 1). In contrast, *M. anisopliae* ZZ-A1 exhibited low LC_{50} (3.2×10^8 and 4.8×10^8 conidia/ml, respectively), LC_{90} and LC_{99} values 12 days post-treatment compared to *F. oxysporum* ZZ-L1 (Table 1). In the time-mortality response, the LT_{50} values at the highest conidial concentration (1.0×10^{10} conidia/ml) for *M. anisopliae* ZZ-A1 and *F. oxysporum* ZZ-L1 were 5.9 and 8.4 days, respectively (Table 2).

The above results demonstrated that both of the new strains were highly and stably virulent to *R. ferrugineus* populations (Tables 1 and 2, Fig. 6 and Additional file 1: Fig. S4). However, both the LC_{50} value and the LT_{50} value of *M. anisopliae* ZZ-A1 were lower than those noted in *F. oxysporum* ZZ-L1, thereby indicating its greater pathogenicity (Tables 1 and 2). Koch's postulates were additionally used to prove the link between the fungal isolate and host and re-inoculated RPW larvae showed symptoms similar to the previously observed.

Discussion

Isolation of pathogens from naturally diseased dead RPWs is the initial phase for screening biocontrol resources that may be further developed into potential microbial agents and utilized in controlling these weevils (Verde et al. 2015). In particular, biological control with pathogenic fungi can offer long-term insect control without destroying the environment and non-target organisms (Pu et al. 2017a). The pathogenic fungi easily survive and spread to *R. ferrugineus* in dark and humid environments. In this study, morphological (including colony culture characteristics and microscopic characteristics) and molecular biology techniques were employed, resulting in two new EPF strains being isolated from RPWs and, ultimately, identified as *M. anisopliae* ZZ-Al and *F. oxysporum* ZZ-L1. Although a variety of *Metarhizium* isolates have previously been reported infecting RPW larvae and adults (Ishak et al. 2019), this is the first record of *Fusarium* and a new *M. anisopliae* strain from diseased dead RPWs.

The phenotypic disparities between *F. oxysporum* ZZ-L1 and *M. anisopliae* ZZ-A1 strains are of significance. Notably, conidia morphology differs, with *M. anisopliae*'s conidia being smaller and cylindrical, while *F. oxysporum*'s conidia are larger and falciform. Additionally, *F. oxysporum* exhibited a substantially higher growth rate on PDA medium compared to *M. anisopliae*.

Table 1 Lethal concentration values of fourth instar *Rhynchophorus ferrugineus* larvae infected by fungi within 12 days post-inoculation

Fungal species	Lethal concentration (LC) values (95% confidence limit) (conidia/ml)					
	LC_1	LC_{10}	LC_{25}	LC_{50}	LC_{90}	LC_{99}
Metarhizium anisopliae ZZ-A1 strain	2.1×10^5 (6.9×10–4.7×10^6)	5.7×10^6 (2.7×10^4–4.7×10^7)	3.8×10^7 (8.2×10^5–1.9×10^8)	3.2×10^8 (3.4×10^7–9.3×10^8)	1.8×10^{10} (7.0×10^9–1.1×10^{11})	4.9×10^{11} (8.6×10^{10}–3.6×10^{13})
Fusarium oxysporum ZZ-L1 strain	6.9×10^3 (1.0×10^{-3}–2.5×10^6)	1.0×10^6 (6.0×10^{-3}-5.4×10^7)	1.9×10^7 (3.4×10–3.3×10^8)	4.8×10^8 (4.0×10^5–2.8×10^9)	2.3×10^{11} (4.7×10^{10}–7.9×10^{13})	3.4×10^{13} (1.3×10^{12}–1.6×10^{20})

Table 2 Lethal time for 50% mortality values of fourth instar *Rhynchophorus ferrugineus* larvae infected by fungi

Concentrations (conidia/ml)	*Metarhizium anisopliae* ZZ-A1 strain				*Fusarium oxysporum* ZZ-L1 strain			
	LT_{50} (days)	95% confidence limits (CLs)		R^2	LT_{50} (days)	95% confidence limits (CLs)		R^2
		Lower	Upper			Lower	Upper	
1.0×10^6	15.5	13.7	18.8	0.961	15.8	14.0	19.3	0.990
1.0×10^7	13.0	12.0	14.6		13.7	12.5	15.6	
1.0×10^8	10.3	9.8	11.0		11.7	11.0	12.7	
1.0×10^9	7.4	7.1	7.8		9.7	9.2	10.2	
1.0×10^{10}	5.9	5.6	6.2		8.4	8.1	8.8	

R^2, determination coefficient

The variations in conidia size and growth rates suggest potential ecological differences, with *F. oxysporum* possibly having adaptations for rapid development and different dispersal mechanisms. Further research is needed to explore the genetic and ecological factors underlying these distinctions.

Metarhizium anisopliae is an EPF microorganism that has been used for selective targeting of several insect pests in diverse pest management programs and has proven to be an effective microbial control agent (Leger et al. 1992). The finding that the cumulative corrected mortality was as high as 90.92 ± 1.10% in fourth instar RPW larvae, 12 days after exposure to a concentration of 1.0×10^{10} conidia/ml demonstrated that *M. anisopliae* ZZ-A1 exhibited obvious toxicity toward this invasive species. Similar to the results in this study, previous studies by several researchers have also shown that *M. anisopliae* was an effective control agent against RPW. As early as 2002, Ghazavi and Avand-Faghih (2002) isolated *Metarhizium* from infected RPWs for the first time and found that pupae and adults were successfully superinfected. It was indisputable that *M. anisopliae* var. *anisopliae* was again identified as the causal agent when Zhu et al. (2010) recorded 80% larval mortality resulting from a fungal infection in RPW larvae. Later, 13 different strains were isolated from infected RPW, with each of the strains producing differences in their pathogenicity in fourth instar weevil larvae. The highest corrected mortality of the 13 strains exceeded 90% (Zhang et al. 2012). Surprisingly, the average mortality of infected 1-day-old eggs was 100% for all of the tested *M. anisopliae* isolates (Sutanto et al. 2021), which was probably caused by the highest susceptibility of RPW eggs to pathogens. This result is also significantly differentiated from current findings in RPW larvae, pupae and adults.

Another EPF that has been proved to be highly toxic to RPW is *B. bassiana* (Hajjar et al. 2015). A total of 2 promising *B. bassiana* isolates JEF-484 and JEF-158 were screened by Yang et al. (2023). According to their bioassay results, JEF-484 showed the highest mortality (80%) and shortest LT_{50} (4.8 days) on the last stage of RPW larvae, and JEF-158 showed a significantly strong ovicidal effect (Yang et al. 2023). Except for eggs and larvae, *B. bassiana* can also basically cause more than 60% mortality in pupae and adults (Sutanto et al. 2021). Unexpectedly, this fungal species has not been isolated from diseased RPWs at any life stages in Zhangzhou in our study.

Some *Fusarium* species have been associated with insects, and a large number of these have been reported to be EPF (Teetor-Barsch and Roberts 1983). Studies on *Fusarium* pathogenicity to insects have shown that a number of *Fusarium* strains exhibited varying degrees of virulence in the following insect orders: Hemiptera, Diptera, Lepidoptera, Coleoptera, and Hymenoptera (Bai and Chen 1991). The present study, however, is the first to report *F. oxysporum* infecting RPW larvae. The results from the bioassay of *F. oxysporum* ZZ-L1 in this study along with the cumulative corrected survival rate of fourth instar RPW larvae (shown to be as low as 22.72% 12 days after exposure to 1.0×10^{10} conidia/ml) implied that *F. oxysporum* may play an important role in controlling RPWs in nature and effectively reducing the population density of the pests.

In evaluating the virulence of the two new EPF strains, the LC_{50} of the *M. anisopliae* ZZ-A1 isolate (3.2×10^8 conidia/ml) was found lower than that of the *F. oxysporum* ZZ-L1 isolate (4.8×10^8 conidia/ml), although the LC_1, LC_{10} and LC_{25} values were high in *M. anisopliae* ZZ-A1. In addition, the LT_{50} of *F. oxysporum* ZZ-L1 (decreasing from 15.8 to 8.4 days) was not as low as it was in comparable *M. anisopliae* ZZ-A1 treatments (decreasing from 15.5 to 5.9 days), which killed the treated larvae rather quickly. The above results indicated that even though the *M. anisopliae* ZZ-A1 strain exhibited higher toxicity in the laboratory than the *F. oxysporum* ZZ-L1 strain and may be a more effective biocontrol agent in controlling RPW in the field, both EPF strains resulted in less than 25% survival of individuals at the highest concentration. This demonstrated that both have the potential to be developed and utilized as microbial agents in controlling RPWs.

The commercial production of traditional microbial pesticides is centered on a limited number of pathogenic microbes, including *Metarhizium* spp., *Beauveria* spp., *Bacillus* spp. and nuclear polyhedrosis virus (Zhang et al. 2011). Based on the results from this research, one alternative may be the use of *F. oxysporum* that had been screened out for controlling RPWs. A potential problem that may arise with some *Fusarium* strains, however, is that a number of species are also pathogens responsible for causing various wilt diseases in plants (Rep and Kistler 2010). Therefore, it is critical to thoroughly test these and other potential strains to evaluate any possible effects they may have on plants, in order to develop efficient, low toxicity and high specificity microbial agents that are able to not only control RPWs in their natural habitat but are effective in controlling other invasive species including other coleopteran pests.

Interestingly, acoustic sensors in terms of the rate of impulse bursts can be used to monitor the sound signal within the palm trunks produced by the movement and feeding activity of RPW infestations in a palm tree. The mean rates of bursts produced by the RPW larval activity decreased to zero two months after the trees were separately injected with *B. bassiana* or *M. anisopliae*

compared to the increased rates over time in the control treatment trees (Sutanto et al. 2023). Thus, it can be seen that the efficacy of the fungal isolates used in the field study using the injection system against RPWs is promising. Although the findings of our current study suggest that the isolates ZZ-A1 and ZZ-L1 could be developed as a biological control agent for the management of RPWs, field evaluations are needed to reach the sound conclusions and practical applications. The research of Sutanto et al (2023) provides us with some guidances' for further field trials, including the application technology of fungal isolates and the assessment method of control efficacy against RPWs.

Conclusions

Two EPF isolates (*M. anisopliae* ZZ-A1 and *F. oxysporum* ZZ-L1) selected for concentration-dependent bioassays were respectively isolated from diseased RPW adults and larvae collected from Zhangzhou. Different degrees of virulence of EPF isolates were found for the RPW larvae under laboratory conditions. It was concluded that the two new EPF strains may be potential microbial agents when used in biological control programs for RPW populations. Conidial suspensions of *M. anisopliae* ZZ-A1 were found to be more effective against RPW larvae than *F. oxysporum* ZZ-L1, because ZZ-A1 significantly showed the highest mortality and lowest LC_{50}. Therefore, the combination of these 2 promising EPFs might provide new microbial materials and an opportunity for the practical microbial control of RPW at different life stages in palm tree fields. However, the evaluation of the pathogenicity of the two fungal isolates in controlling RPWs in this study was conducted under strictly controlled laboratory conditions. Because field conditions are variable, complex, and uncontrolled, results obtained under these situations may be vastly different from the closely monitored circumstances present in the laboratory. In future researches, focus should be placed on the applicability of the laboratory-generated results to controlling RPWs in natural environments. It will be necessary to determine the efficacy, dosage, application technology, appropriate stage and safety assessment of EPFs in filed trails against RPW populations to establish a solid foundation for development into a field-ready product.

Abbreviations

RPW	Red palm weevil
IPM	Integrated pest management
EPF	Entomopathogenic fungi
RH	Relative humidity
PDA	Potato dextrose agar
gDNA	Genomic DNA
ITS	Internal transcribed spacer
PCR	Polymerase chain reaction
ddH_2O	Double-distilled water
NCBI	National Center for Biotechnology Information
BLAST	Basic local alignment search tool
ML	Maximum likelihood
SE	Standard error
ANOVA	Analysis of variance
GLM	Generalized linear model
HSD	Honestly significant difference
LC	Lethal concentration
LT	Lethal time
CLs	Confidence limits

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41938-023-00754-4.

Additional file 1: Fig. S1. Growth status of colonies of two fungal strains on PDA plates within 7 days. Circles represent the colony diameters after x-number of culture days (n=10). Different lowercase letters without parentheses indicate significant differences in colony diameter on different culture days for the same strain, while different uppercase letters within parentheses indicate significant differences in colony diameter between the ZZ-A1 strain and the ZZ-L1 strain at similar times (two-way ANOVA using univariate process of GLM mode; Tukey's HSD multiple comparison test at $P=0.05$). **Fig. S2.** Morphology of conidia of the ZZ-L1 strain at 40× magnification using optical microscopy. Scale bar, 10 μm. **Fig. S3.** Agarose gel electrophoresis (1%) of fungal rDNA-ITS PCR products of the ZZ-A1 and ZZ-L1 isolates. **Fig. S4.** Survival rates of fourth instar red palm weevil larvae treated with different concentrations of *Metarhizium anisopliae* ZZ-A1 and *Fusarium oxysporum* ZZ-L1. Kaplan–Meier survival analysis was used to determine larval survival at different concentrations of the two fungal species. Black solid symbols and white hollow symbols represent *M. anisopliae* ZZ-A1 and *F. oxysporum* ZZ-L1, respectively. Concentration of conidia/ml: circle, 1.0×10^6; diamond, 1.0×10^7; triangle, 1.0×10^8; square, 1.0×10^9; star, 1.0×10^{10}.

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Author contributions

YP conceived and designed research. ZZ and CD collected and reared experimental insects. YP, ZZ and CD conducted experiments. YP, ZZ and XC analyzed data. YP and XC wrote and reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated and analyzed during this study are indicated in the manuscript.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests
The authors declare that they have no competing interests.

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