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CAROLINA JORDAN

NOVAS POSSIBILIDADES PARA O CONTROLE MICROBIANO DE *Gonipterus platensis* (MARELLI) (COLEOPTERA: CURCULIONIDAE)

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Orientador: Prof. Dr. Carlos Frederico Wilcken

Coorientador: Dr. Gabriel Moura Mascarin

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TÍTULO DA DISSERTAÇÃO: NOVAS POSSIBILIDADES PARA O CONTROLE MICROBIANO DE *Gonipterus platensis* (MARELLI) (COLEOPTERA: CURCULIONIDAE)

AUTORA: CAROLINA JORDAN
ORIENTADOR: CARLOS FREDERICO WILCKEN
COORIENTADOR: GABRIEL MOURA MASCARIN

Aprovada como parte das exigências para obtenção do Título de Mestra em CIÊNCIA FLORESTAL, pela Comissão Examinadora:

Prof. Dr. CARLOS FREDERICO WILCKEN (Participaçao Virtual)
Proteção Vegetal / Faculdade de Ciências Agronômicas de Botucatu - UNESP

Pesquisador Dr. JOSÉ EDUARDO PETRILLI MENDES (Participaçao Virtual)
Pesquisa e Desenvolvimento / Bracell

Prof.ª Dr.ª NÁDIA CRISTINA DE OLIVEIRA (Participaçao Virtual)
Agronomia / Centro Universitário Integrado

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Aos meus amados pais, Paulo Roberto Jordan
e Antônia Cristina Romani Jordan.
Por serem refúgio e acolhimento,

dedico

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RESUMO

O Brasil é considerado, atualmente, uma potência florestal, possuindo a maior produtividade e uma das menores rotações do mundo em seus plantios de eucalipto. Todavia, a presença de grandes maciços florestais favorece a ocorrência e o estabelecimento de insetos-praga. O gorgulho-do-eucalipto, *Gonipterus platensis* (Coleoptera: Curculionidae), é o principal besouro desfolhador do eucalipto no mundo e um dos mais antigos a ser detectado fora da Austrália. Essa praga possui alto potencial destrutivo, tanto na fase adulta quanto na fase larval e o seu controle no Brasil se dá principalmente pela utilização de agentes de controle biológico, destacando-se os micopesticidas à base de *Beauveria bassiana,* como também o parasitóide de ovos, *Anaphes nitens* (Hymenoptera: Mymaridae). Por outro lado, existem somente dois produtos compostos por *B. bassiana* registrados e disponíveis comercialmente para o controle desse inseto. Ademais, desde 2012 as taxas de parasitismos vêm oscilando, com níveis de intenso a baixo. Diante disso, há necessidade de se explorar novas estratégias de controle, visando o manejo integrado de pragas. Consequentemente, o presente trabalho visa aprofundar o conhecimento sobre o controle microbiano desse inseto-praga, dividido em quatro diferentes objetivos, sendo eles; *i e ii)* conhecer e identificar os possíveis microsporídeos e vírus associados ao *G. platensis; iii)* selecionar isolados de *Bacillus thuringiensis* que expressem alta toxicidade a larvas neonatas de *G. platensis,* como também; *iv)* rastrear e selecionar isolados brasileiros de fungos entomopatogênicos com alta virulência a adultos de *G. platensis* com a maior produção de inóculo secundário em cadáveres de insetos. Obtivemos resultados pioneiros com essa pesquisa, indicando alternativas viáveis para o controle biológico desse inseto-praga, fornecendo assim subsídios ao manejo integrado e produção massal.

Palavras-chave: Controle microbiano. Entomopatogenos. Bioprospecção. Gorgulho-do-eucalipto.

ABSTRACT

Brazil is currently considered a forestry power with leading productivity and having one of the shortest rotations in the world in its *Eucalyptus* plantations. However, the extensive forest planted areas favour occurrence and establishment of insect pests. The eucalyptus snout beetle (ESB), *Gonipterus platensis* (Coleoptera: Curculionidae), is the major defoliating weevil in the world and one of the oldest to be detected outside Australia. This pest has a high destructive potential, both in adulthood and in larval phase, and its principal control in Brazil relies mainly on the use of biological control agents, especially mycopesticides based on *Beauveria bassiana*, as well as an egg parasitoid, *Anaphes nitens* (Hymenoptera: Mymaridae). Nevertheless, there are only two products based on *B. bassiana* commercially available for its control. In addition, since 2012, parasitism rates have varied with levels from intense to low. Therefore, there is an urgent need to explore new control strategies, aimed at integrated pest management. Consequently, the present work aims to expand the knowledge about the microbial control of this insect pest, split into four different objectives, namely: *i and ii)* to know and identify the possible microsporidian and virus pathogens associated with *G. platensis*: *iii)* to select *Bacillus thuringiensis* isolates that express high toxicity to neonate larvae of *G. platensis*, and *iv)* to screen and select Brazilian isolates of entomopathogenic fungi with high virulence against *G.platensis* adults coupled with the highest secondary inoculum production in insect cadavers. Pioneering results were obtained with this research, indicating viable alternatives for the biological control of this insect pest, thus providing subsidies for integrated management and mass production.

Keywords: Microbial control. Entomopathogens. Bioprospecting. Eucalyptus snout beetle.

SUMÁRIO

INTRODUÇÃO GERAL

O Brasil é reconhecido mundialmente pela alta produtividade florestal, em comparação com os demais países, com uma das menores rotações entre plantio e a colheita de eucalipto. Esse reconhecimento se dá principalmente devido à adoção de boas práticas de manejo, melhoramento genético e condições edafoclimáticas. Em 2019, o Brasil apresentou produtividade média de 35,3m^3.ha.ano^{-1}, e as suas áreas de plantio de eucalipto vêm se expandindo ao longo do tempo, ocupando atualmente 6,97 milhões de hectares (IBA, 2020).

Geralmente as florestas plantadas são compostas por uma base genética restrita de espécies, o que predispõem o estabelecimento e dispersão de pragas, ocasionando perdas de produção (SCHUHLI *et al*., 2016). Entre as pragas exóticas mais importantes se destaca o gorgulho-do-eucalipto, *Gonipterus platensis* (Coleoptera: Curculionidae) o qual voltou a ter importância desde o final de 2012 (IPEF, 2015). Essa espécie, nativa da Austrália, possui alto potencial destrutivo, se alimenta principalmente de folhas jovens, tanto na fase adulta quando na fase larval (TOOKE, 1955). Porém, é no estágio larval que ocorrem os maiores danos, uma vez que se alimentam exclusivamente de folhas jovens e brotações no ápice da copa (SOUZA *et al*., 2016).

Atualmente essa praga se encontra distribuída no Sul e Sudeste do Brasil (WILCKEN & OLIVEIRA, 2015). Quando ocorre em plantios novos, pode ocasionar desfolha completa do ponteiro, redução da produtividade, bifurcação do fuste e dificuldades no momento da colheita (TOOKE *et al.*, 1955; SANCHES *et al.*, 1993; SOUZA *et al.,* 2016).

Em 2016 foi realizado levantamento dos danos ocasionados pelo gorgulho-do-eucalipto no Estado de São Paulo e foi verificada redução do incremento médio anual (IMA) de aproximadamente 20%, variando de 10,4% a 42,8%, dependendo da espécie ou clone de eucalipto. Ao traçar cenários com a área atacada pelo inseto nos anos de 2012 (3,5 mil ha); 2013 (6,5 mil ha); 2014 (19 mil ha) e 2015 (26 mil ha), os autores concluíram que as perdas econômicas poderiam ser entre R$ 1,5 milhão (no melhor cenário) e R$ 6 milhões (no pior cenário) apenas nesse Estado (SOUZA *et al.,* 2016).

Em estudo recente realizado em Portugal, a desfolha por *G. platensis* resultou em danos econômicos por volta de 648 milhões de euros nos últimos 20 anos. Tais perdas econômicas ocorreram apesar do parasitismo por *A. nitens* (Hymenoptera: Mymaridae) apresentar variações, sendo bem-sucedido em algumas regiões e permanecendo pouco eficaz em outras. Sem parasitismo, as perdas teriam variado de 2,45 bilhões de euros, em um cenário onde as populações de *G. platensis* eram controladas com inseticidas, para quase 7,2 bilhões de euros se as perdas de madeira fossem compensadas pela madeira importada. Portanto, o benefício do controle biológico com *A. nitens* na área de estudo durante as duas últimas décadas foi de, pelo menos, 1,8 bilhão de euros (VALENTE *et al*., 2018).

No Brasil, a primeira detecção de *G. platensis* (ex- *G. scutellatus*) ocorreu em 1979 em Curitiba, PR (FREITAS *et al.*, 1979), estando distribuído atualmente nos estados do Rio Grande do Sul (WILCKEN & OLIVEIRA, 2015), Santa Catarina (FENILLI *et al.,* 1982), Paraná, São Paulo (ROSADO-NETO *et al.*, 1993) e Espírito Santo (WILCKEN *et al*., 2008). Já a espécie *G. pulverulentus* (ex- *G. gibberus*) foi detectada pela primeira vez no Brasil na década de 1950 em Pelotas, RS, restringindo-se aos estados da região Sul do país (BARBIELLINI, 1955; KOBER, 1955; FREITAS, 1979; FENILLI, 1982).

Atualmente as plantações florestais certificadas ocupam 4,4 milhões de hectares, com aumento de 1,7% de 2018 para 2019. Essas certificações são concedidas por organizações como o *Forest Stewardship Council* (FSC) e o *Nation Forest Certification Program* (Cerflor) (IBA, 2020), as quais possuem uma política de restrição de uso de pesticidas, exigindo que as organizações certificadas utilizem o manejo integrado de pragas (MIP) e se adequem ou sigam a legislação local de pesticida (FSC, 2019). Até o presente momento nenhum inseticida químico foi registrado pelo Ministério de Agricultura e Abastecimento (MAPA) para essa praga (AGROFIT, 2020).

Portanto, o controle biológico vem sendo a principal estratégia de manejo adotada a *G. platensis,* destacando-se o parasitoide de ovos *A. nitens* e produtos comerciais à base do fungo entomopatogênico *Beauveria bassiana,* os quais são compatíveis com o parasitoide (SCHRODER *et al.,* 2020*)*. Além disso, há também a utilização de outros inimigos naturais, como percevejos predadores (*Podisus nigrispinus* (Hemiptera: Pentatomidae) (NASCIMENTO et al., 2018) e nematóides entomopatogênicos (DAMASCENA *et al.,* 2020*).* Em 2005, a utilização conjunta de

produtos à base de fungos entomopatogênicos e de seu parasitoide de ovos, *A. nitens,* possibilitou o controle da praga que atingia 50.000 ha de plantios no Estado do Espírito Santo (WILCKEN et al., 2008). Porém, apenas dois produtos à base de fungos entomopatogênicos (*Beauveria bassiana*), são registrados e disponíveis comercialmente para seu controle (AGROFIT, 2020). No entanto, existem outras linhagens potenciais de fungos que foram negligenciadas no controle dessa praga.

Atrelado a isso, o controle pelo seu parasitoide pode variar entre níveis de intenso a baixo parasitismo (REIS et al., 2012). As causas para as baixas taxas de parasitismo pelo seu parasitoide *A. nitens* não estão totalmente esclarecidas, porém, podem ser atribuídas a fatores como: condições climáticas, densidade do hospedeiro, altitude e incompatibilidade entre parasitoide e seu hospedeiro (CORDERO RIVERA; SANTOLAMAZZA-CARBONE; ANDRÉS,1999; TRIBE, 2005; LOCH, 2008; REIS et al., 2012; MAPONDERA et al., 2012). Diante deste cenário, surge a necessidade de novas estratégias, favorecendo o manejo integrado de pragas.

A descoberta de novos patógenos infectivos e auto-disseminados em besouros (vírus, bactérias, fungos, protozoários ou nematoides) seria o primeiro passo para aperfeiçoar o manejo interado dessa praga, os quais devem ser eficientes e de fácil multiplicação, visando à produção de bioinseticidas. Os micoinseticidas comerciais, ou inseticidas à base de fungos, têm repercutido em grande sucesso no Brasil, onde milhões de hectares por ano são tratados com *B. bassiana* e *Metarhizium anisopliae* para controlar artrópodes-praga em sistemas agrícolas e florestais (MASCARIN *et al*., 2019).

A bactéria *Bacillus thuringiensis* var. *tenebrionis* se destaca também como uma alternativa promissora para o controle de larvas de *G. platensis,* como já verificado para outros coleópteros na Austrália e Nova Zelândia (ELEK & BEVERIDGE, 1999; WITHERS, 2013). Pouco se sabe também sobre os parasitas intracelulares, como microsporídeos, em populações de *G. platensis,* que podem causar efeitos deletérios ao seu desenvolvimento, como já relatado em outros insetos hospedeiros, como aumento na mortalidade das larvas, atraso no desenvolvimento, redução na fecundidade e longevidade do adulto e aumento na suscetibilidade a condições de estresse (SOLTER & BECNEL, 2000). Ademais, há também poucos relatos de vírus infectando besouros (SHUKLA et al. 1984; KIM & KITAJIMA, 1984; KITAJIMA et al. 1985), sendo os vírus importantes agentes de controle biológico.

Desta forma, este trabalho está dividido em quatro diferentes objetivos, sendo eles; *i e ii)* conhecer e identificar os possíveis microsporídeos e vírus associados ao *G. platensis; iii)* selecionar isolados de *Bacillus thuringiensis* que expressem alta toxicidade a larvas neonatas de *G. platensis,* como também; *iv)* rastrear e selecionar isolados brasileiros de fungos entomopatogênicos com alta virulência a adultos de *G. platensis* com a maior produção de inóculo secundário em cadáveres de insetos, fornecendo assim subsídios a programas de controle biológico mediante a exploração de novos biopesticidas microbianos sob a ótica do manejo integrado desse inseto-praga.

CHAPTER 1

First record of a new microsporidium pathogenic to *Gonipterus platensis* in Brazil

Journal: Scientific Reports

1.1 Abstract

Microsporidia are naturally occurring fungal-related parasites that can infect nearly all animal hosts, but their biocontrol potential of insect pests is routinely overlooked in agriculture and forestry. This research brings the first report describing the natural occurrence of a microsporidium causing disease in field-collected populations of the invasive eucalyptus snout beetle, *Gonipterus platensis* (Coleoptera: Curculionidae), a major destructive pest of eucalyptus plantations in Brazil. Adult beetles were collected during field surveys in commercial eucalyptus plantations in southern Brazil to be examined and dissected with typical symptoms to verify presence of microsporidian spores in haemolymph. From 14 plantations in different sites, the natural infection occurrence in these populations ranged from 0 to 65%, while a lab colony exhibited an infection incidence of 70%. Spore density in haemolymph of symptomatic insects averaged 2.1 (±0.4) × 10^7 spores/mL. Symptoms in infected adults were identified by an abnormal abdomen with malformation of the second pair of wings, impairing their flight activity. Electron transmission microscopy of the pathogen showed morphological features similar to species belonging to the genus *Nosema* or *Vairimorpha.* Phylogenetic analysis of the full-length small subunit ribosomal RNA gene suggests this pathogen's placement in the genus *Vairimorpha,* but with a sequence identity of ~94% with the nearest neighbours. The low level of sequence identity suggests this pathogen may represent a novel taxon in the genus and further requires whole genome sequencing for definitive taxonomic resolution. These findings provide insights on the natural occurrence of this novel pathogen of this invasive pest in *Eucalyptus* plantations in Brazil. Further studies are needed to determine potential of this microsporidium in the design of conservative or augmentative biological control programs for these invasive pests.

Key words: Invasive pest, natural mortality, *Gonipterus platensis*, entomopathogen, eucalyptus snout beetle, *Vairimorpha*.

1.2 Introduction

Various environmental, biological and genetic factors can influence performance and fitness during an insect's life cycle. A relevant factor is the occurrence of pathogens such as bacteria, viruses, fungi, and parasites that are responsible for about 80% of the diseases in insect populations[1]. Recent research has highlighted several cases of success in insect invasions facilitated by microorganisms[2,3,4,5,6], including some microsporidia.

Microsporidia have been reported to cause substantial deleterious effects on host fitness in host insects. These effects include malformations in infected pupae, increased larval mortality, developmental delay of immatures, reduced fertility and longevity of adults, and increased susceptibility to stress conditions[7]. These stress factors cause biological changes in the host insect and may be associated with a decrease in its rate of parasitism[1]. As microsporidian pathogens generally display efficient transmission mechanisms and moderate virulence, these traits may make them more effective agents in establishing enzootics in host population[8], as evidenced by the use of a microsporidium to control locusts[9]. In this context, microsporidian entomopathogens hold a great potential as long-term biocontrol agents of numerous arthropod pests, but their natural incidence and pathogenicity in populations of forest pests have been underexplored[10].

In the past, microsporidia were considered to be spore-forming protozoa, but in the light of modern taxonomy, this group was relocated to in or near the Fungi kingdom and are now called non-flagellate, single-celled fungi, and obligate intracellular parasites[11]. Several studies suggest a new classification for microsporidia within or near the fungi group, and the majority of entomopathogenic microsporidia belong to the genus *Nosema*, with more than 150 insect host species described in 12 insect orders, notably Lepidoptera, Hymenoptera, Diptera, Orthoptera and Coleoptera are among them[12].

Microsporidia generally cause sublethal and chronic deleterious effects in infected hosts and, as a result, are a serious problem in insects that are massively rearing in laboratories and bio-factories[1,7]. Spores are generally small and most entomopathogenic microsporidia are approximately 2 to 6 µm in length. Spores can have different morphologies, including rounded, oval, or pyriform, and less frequently reniform, long ovals to almost tubular shape and refringent appearance when examined under phase contrast microscopy[7].

The Eucalyptus snout beetle, *Gonipterus platensis* Marelli 1927, also known as the eucalyptus beetle, is currently the primary coleoptereus pest in commercial eucalyptus forestry in Brazil. *Gonipterus platensis* is native to Australia, has a high destructive potential as adults and larvae feed mainly on young leaves[13], and are distributed across the South and Southeast of Brazil[14]. In a study carried out in Portugal, defoliation by *G. platensis* resulted in wood losses of 648 million euros in the last twenty years[15].

Despite microsporidiosis being reported in a variety of different insect populations, there are no previous studies reporting the presence of this pathogen in populations of *G. platensis* in the literature. Recently, microsporidiosis symptoms observed in infected adult beetles (males and females) included typical morphological malformations comprising an abnormal abdomen with the second pair of wings displaced, which impairs flight activity in a laboratory colony. This promptly motivated us to identify the pathogen and determine its incidence in natural populations. Identifying the pathogen would also allow us to evaluate the potential of the organism to serve as a potential biological control agent, as it can both cause slow mortality and more pronounced reduction in host fitness and deformations in adults, or at least an ecological factor in a biological control program developed towards this insect pest in forest plantations. The knowledge of a microsporidium associated with *G. platensis* is of great importance to understand the ecology of this insect. It may also contribute to the development of a conservative biological control program with this entomopathogen that can negatively impact on this pest population. This knowledge is also important to prevent the pathogen from infecting laboratory colonies of this insect.

1.3. Results

1.3.1 Morphological characterization and spore density of microsporidium

Field-collected *G. platensis* adult beetles were observed with visible symptoms of microsporidiosis disease, as they exhibited notable malformations characterized by spread pair of wings with abnormal and shrivelled abdomen, and the membranous pair of wings completely extended from the elytra (Figure 1A). In this study, oval spores were identified as resistance structures of intracellular parasites isolated from the body content of *G. platensis* adults. In slides prepared with the beetle's body content, a large

amount of spores of the pathogen was easily and clearly observed, which was also used as a quick and easy diagnosis parameter to confirm microsporidiosis in field-collected beetles from different localities, as described later in this study (Figure 1B). Microscopy is an inexpensive and routine technique for the conformation of microsporidiosis, but it is not an accurate method for species identification since the morphological structures of some pathogens are similar[16].

Light microscopy revealed that fresh *Vairimorpha* sp. spore were generally elongated ovoid or oval shapes. Spore dimensions (n = 10) averaged 2.07 (± 0.136) µm in length and 1.20 (± 0.066) µm in width, respectively (Figure 1B). Ultrastructure of microsporidian spores were examined under transmission microscopy and revealed spore wall consisted of an electron-dense exospore. The coiled region of the polar tube comprised 8 turns, and the diplokaryotic nuclei were slightly separated from each other (Figure 1C). However, this does not discard that this pathogen also forms uninucleate spores as well. All the above-mentioned cellular features corresponded to the basic characteristics found in the genus *Vairimorpha*. Microsporidian spores from 10 beetles with similar size and weight were collected in *Eucalyptus* plantation located at São Jerônimo da Serra, SP, Brazil, and presented a sex ratio of 1:1. As result, the averaged of spore load of symptomatic beetles was determined at 2.15 (± 0.402) × 10^7 spores per adult with (Figure 1D).

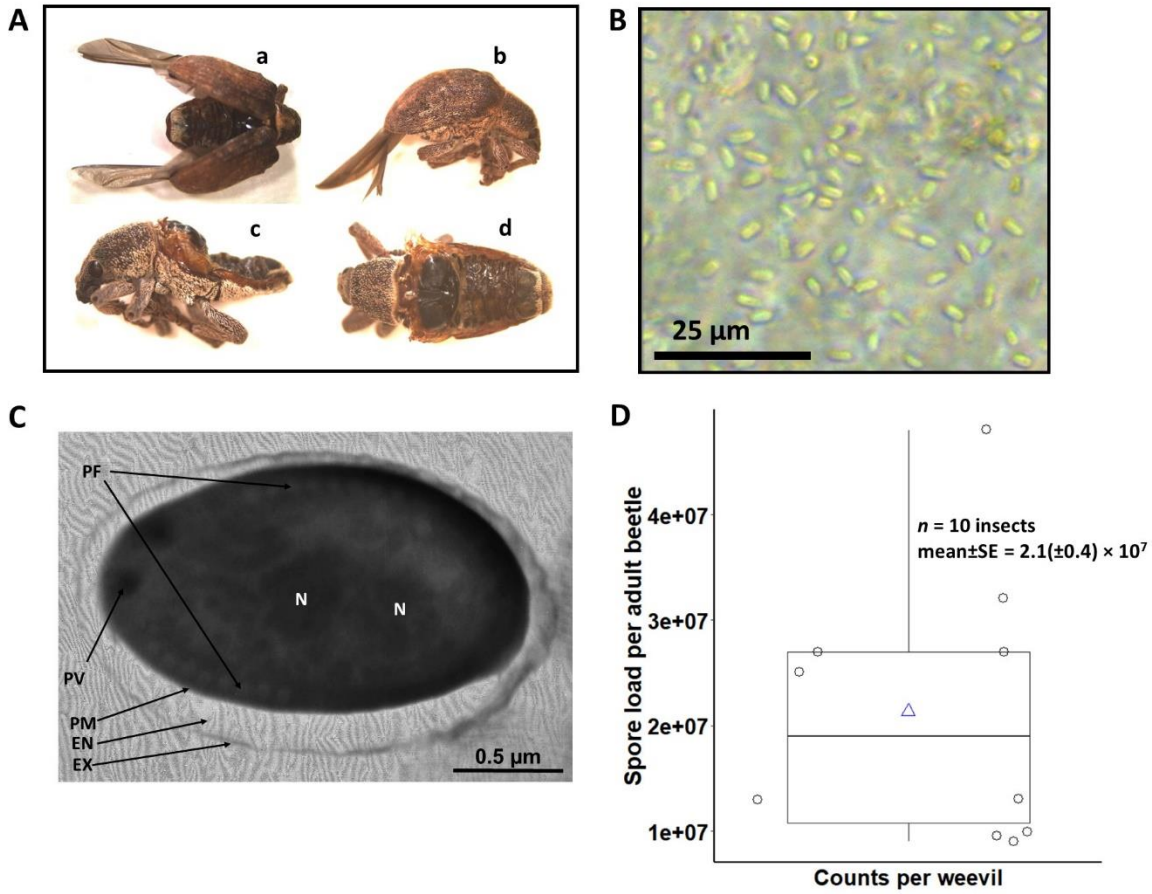

Figure 1. Disease diagnosis for the new microsporidiosis in symptomatic adult beetles and spore load in infected beetles. **A)** Field-collected *Gonipterus platensis* adult beetles depicting visible symptoms of microsporidiosis disease. **a** - spread pair of wings exposing the abnormal and shrivelled abdomen; **b** - Side view of a dead insect showing the membranous pair of wings extended from the elytra; **c** – Wingless adult with abnormal abdomen. d: Dorsal view of the abnormal abdomen. **B)** Microsporidian spores found in the haemolymph of *G. platensis* adults, observed under a phase contrast microscope. Scale bar = 25 µm. **C)** Electron micrograph of a longitudinal section of a mature microsporidian spore. The nucleus (binucleate, N), exospore (Ex), endospore (En), plasma membrane (PM), polar filament (PF) coiling posterolaterally around central diplokaryon showing 8 coils, and posterior vacuole (PV) are visible. Scale bar = 1µm. **D)** Density of spores per insect determined by enumerating the spore load from smeared symptomatic beetles and performing counts under a phase contrast microscope using a Neubauer chamber at 400× magnification.

1.3.2 Molecular identification and phylogenetic construction

Sequencing of the SSU rRNA gene was performed to confirm the identification of the pathogen found infecting populations of *G. platensis*. The PCR results confirmed the isolate was closely related to *Microsporidia* species, with the highest sequence identity (98%) to a sequence submitted to Genbank as *Microsporidia* sp. MB-2008

(GenBank accession no. EU589246). A phylogenetic analysis of the strain and closely related strains of *Microsporidia* species was conducted (Figure 2). The closest phylogenetic neighbor of the isolate found in *G. platensis* was found to be *Microsporidia* sp. MB-2008, which was isolated from another weevil, *Otiothynchus sulcatura* (Coleoptera: Curculionidae), followed by *Vairimorpha apis,* isolated from *Apis cerana* (Hymenoptera: Apidae). Based on the recent formal redefinition of the genera *Nosema* and *Vairimorpha* (Microsporidia: Nosematidae), the strain belongs to the *Vairmorpha* genus. Based on these findings, the name *Vairimorpha curculionidae* is proposed, when the species is formally circumscribed in the future.

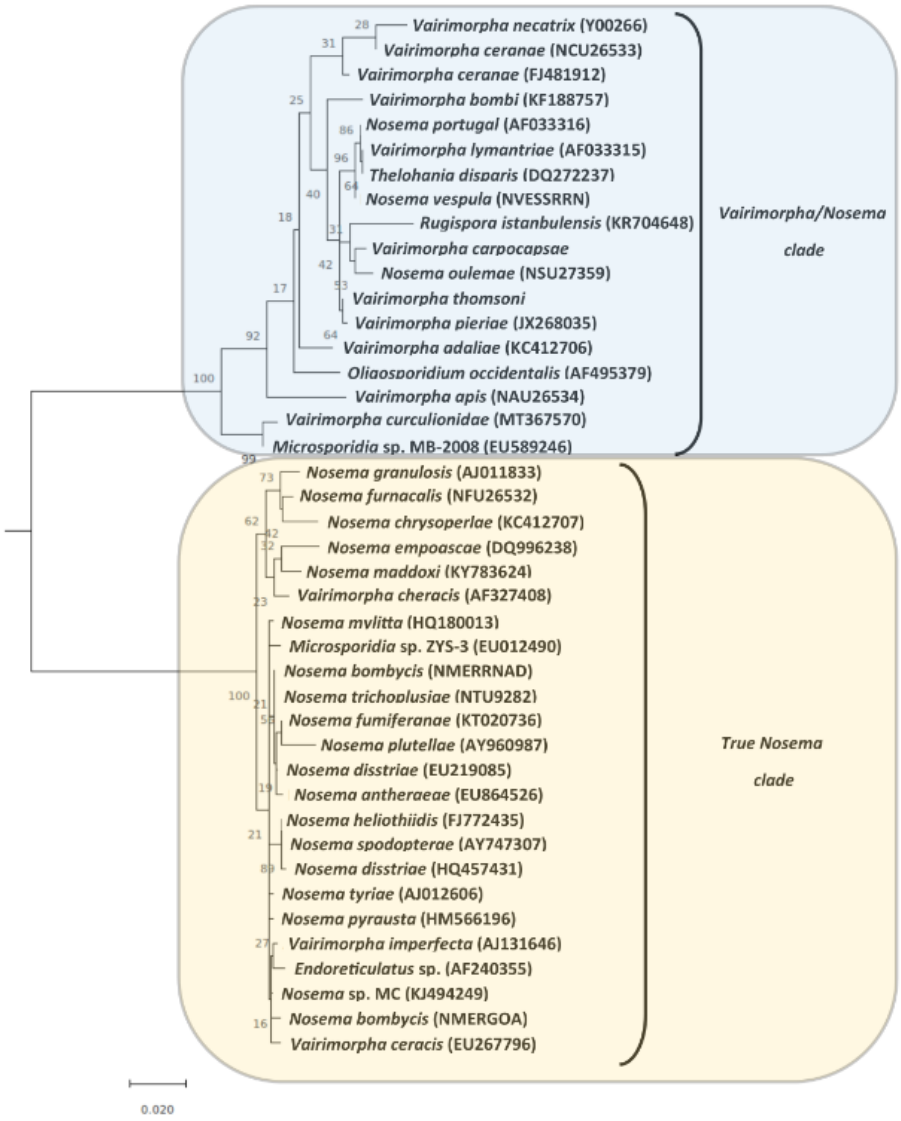

Figure 2. Phylogenetic tree based upon a small subunit rRNA gene alignment of 1068 positions in MEGA X with the Maximum Likelihood method using the Tamura 3-parameter model with a gamma distribution model. Values at branches indicate bootstrap support. The new microsporidium isolate is assigned to the *Vairimorpha* genus with a proposed name of *Vairimorpha curculionidae*. The outgroup was *Ordospora colligata* and is not shown.

1.3.3 Prevalence of microsporidiosis in different field populations of *Gonipterus* spp.

To determine the presence of microsporidium in other populations of the host insect, collections were made at 13 different sampling points distributed in three states as shown in the Figure 3A. Identification of infected adult beetles was based on visual diagnosis of the microsporidiosis symptoms and by checking for spores in haemolymph. Positive results were obtained for microsporidium in most surveyed field

sites, including beetles sampled from a mass reared insect colony up to the 2nd generation in Botucatu-SP. There was a significant variation in natural infection caused by microsporidium across different regions or sites of collection in South and Southeast Brazil (χ^2 = 62.87, df = 13, P < 0.0001). The prevalence of natural infection in these field-collected beetle populations sampled at different locations under field conditions ranged from 0 to 65%, but the highest pathogen incidence was observed in the insect colony maintained at the laboratory corresponding to 70% infection in adults confirmed by microscopic examination of the haemolymph sample, and these infected beetles were generally associated with typical symptoms of microsporidiosis previously described here (Figure 3B). The percentage of mortality of beetles were not calculated in these field populations, because these insects were collected alive and then immediately frozen prior to taking to the laboratory. Interestingly, it was the first time that we found microsporidium infecting *Gonipterus pulverulentus* (26.7% infection), despite the lower frequency of this insect species compared to *G. platensis* throughout Brazil.

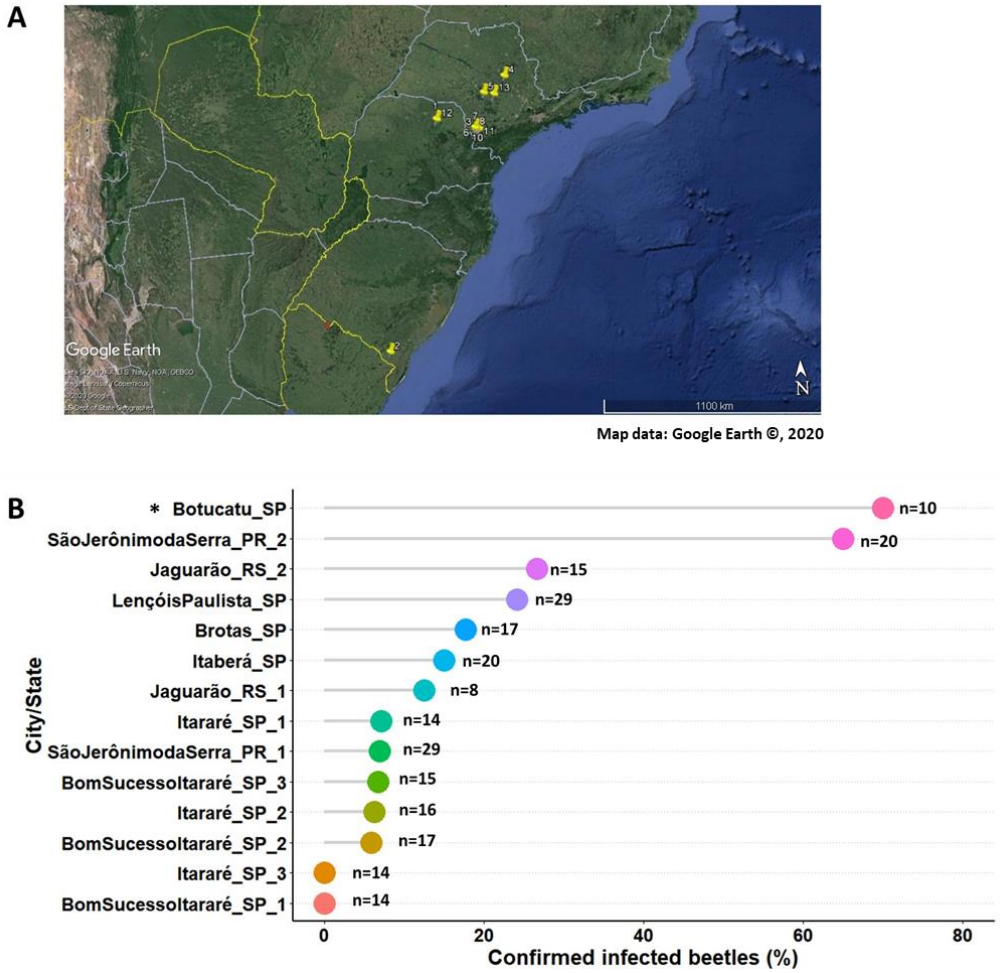

Figure 3. Natural prevalence of the new microsporidian pathogen in different field populations of the *Eucalyptus* snout beetle. **A)** Collection field sites (marked with yellow pins) mapped with Google Earth showing where *Gonipterus platensis* adults were sampled in South and Southeast Brazil from commercial *Eucalyptus* plantations. **B)** Proportion of infected beetles (number of infected versus number of healthy adults) by location. Survey of 14 distinct geographical points were performed to collect adult beetles (where n = number of total beetles collected in each site) for diagnosis based on typical microsporidiosis symptoms and presence of microsporidian spores in the haemolymph expressed as percent of confirmed infected beetles: one collection point was made in Botucatu, SP (*from insect colony kept in the lab); three samples from Bom Sucesso do Itararé, SP; three from Itararé, SP; one from Lençóis Paulista, SP; one from Brotas, SP; one from Itaberá, SP; two from Jaguarão, RS (*Gonipterus pulverulentus* was collected only in sample 2); and two from São Jerônimo da Serra, PR.

1.4 Discussion

The present work is the first to describe the occurrence of microsporidiosis in an insect colony from the second generation and in field populations of *G. platensis*

collected in commercial *Eucalyptus* plantations, with the disease being confirmed in two species present in Brazil, *G. platensis* and *G. pulverulentus* (Coleoptera: Curculionidae). Microsporidian infections have been reported in other Curculionidae[17,18] as well as other coleopterans[19,20,21,22,] which demonstrate that this fungal pathogen is more common than previously thought infecting this diverse insect order. Adult beetles collected across the three Brazilian states that tested positive for the presence of this pathogen. Prevalence of natural infection in these field populations varied from 0 to 65% and 70% infection was detected in the lab colony, indicating that this pathogen is spreading with expansion of the pest in Brazil and is established in mass reared lab colonies of *G. platensis*. However, additional research should still focus on the development of specific gene markers for rapid and accurate detection of this microsporidium in both asymptomatic and symptomatic *G. plantesis* beetles in complement to the traditional microscopic examination.

More than 1400 microsporidian species have been described so far and new species are being discovered each year[23,24]. There are several reports of these microorganisms infecting lepidopterans[25], hymenopterans[26] and orthopterans[27], demonstrating the high genetic plasticity of this group of pathogens. Even more important is the frequent occurrence of microsporidian epizootics in laboratory colonies, in which there is high aggregation and population density of insects facilitating pathogen spread and new infections[28].

Microsporidiosis is considered an important problem in the life cycle of insects[29] because of the reduction in pupal size, number and viability, along with a longer duration of the pupal stage[30]. We also described conspicuous morphological abnormalities in infected *G. platensis* and *G. pulverulentus* beetles, and these symptoms are good indicators of microsporidiosis diagnostic alongside the presence of spores in the haemocoel. Additionally, spore numbers in *G. platensis* adult beetles reached an average concentration of 2.15 (± 0.40) × 10^7 spores in symptomatic insects. This is similar to other microsporidia infections, *Nosema cerane* in honey bees yielded 1.15 × 10^7 spores/bee at 18 days post-inoculation[31]. However, diet had a considerable effect on the spore load observed in honey bees[31]. This difference illustrates the specific interaction between microsporidium and its host insect in regard to spore density for the development of microsporidiosis.

The morphological similarity between microsporidian species, particularly based on spore measurements in isolation, makes identification to species difficult. Therefore,

other methods are needed to confirm identification. Classification based on spore morphology can be difficult and inconsistent because some microsporidia have complex life cycles and form various types of spores. In some cases, different sporulation cycles occur at different stages of the host. Some species can also form different types of spores in the same host and sometimes in the same tissues[32]. Such evidence indicates high diversity of the spore dimension; hence, molecular analysis is essential in the identification of microsporidian species[16].

The SSU rRNA sequence has been widely used as a molecular marker to estimate phylogenetic relationships between microsporidia, because it is a highly conserved gene[12]. However, this gene alone cannot be used to distinguish closely related species. This is a limitation of this gene for a more refined phylogenetic separation between species of this pathogen[33,34]. However, they can be used in the taxonomic classification at the genus level[35,36]. In this case, the species is well resolved, even amongst members of the same genus. Future efforts are planned for whole-genome sequencing of this pathogen and then elucidate their key genes related to the infection process in *G. platensis*.

In general, the role of microsporidia as insect pathogens requires us to consider their ecological effects when developing a pest control program. This pathogen group could cause a chronic disease, which is debilitating to the host[37]. In addition, the transmission of microsporidia occurs by one or more means, including the ingestion of spores present in the environment, and parental transmission to offspring, which facilitates their multiplication and persistence in the target population[38.] This high transmissibility of microsporidia in host population coupled with low lethality are key for their enzootic and long-term prevalence, which may not be a suitable feature for an applied biological control agent, but it could be desirable for conservative biological control strategy towards forest pests. However, the method of transmission in *G. platensis* is currently unknown. Furthermore, we have observed that infected symptomatic *G. platensis* beetles under laboratory conditions survive shorter and females do not lay eggs (*i.e.*, impaired fecundity) in comparison to healthy beetles (data not shown).

Due to their low virulence, microsporidia act slowly in host death and are likely insufficient to control an insect pest when used alone[39]. However, when used in combination with another type of chemical or microbiological insecticide, it reveals

enormous potential and tends to be the most successful, as recently reported for the synergistic effect when combining *Metarhizium brunneum* with *Paranosema locustae* in the control of the migratory grasshopper *Melanoplus sanguinipes*[40], as well as improved grasshopper control achieved by combined application of *P. locustae* and an insect growth regulator, flufenoxuron[41]. However, there is a need to know in advance the environmental effects on the pathogen cycle in its host, in order to analyze the different strategies of release of the microsporidian pathogen in the field. In this sense, based on the historical use of microsporidia as biocontrol agents, especially in the case of *P. locustae* for management of locusts and grasshoppers, past lessons indicate that successful use of microsporidian pathogens for pest control is still a challenge in face of numerous drawbacks related to their production (they are obligatory pathogens), formulation, storage, and efficacy[42]. It also must be considered whether the microsporidium have a negative effect on control actions. It was recently reported that infection with a microsporidium reduced the efficacy of a granulovirus in larvae of *Phthorimaea* operculella[43]. While infection with *Bacillus thuringiensis* made *Galleria mellonella* more susceptible to infection by a microsporidium[44]. Because there is scarce information about the ecological and biological aspects in the host-pathogen interactions of this new microsporidium with *G. platensis* makes this obligatory pathogen an important potential natural enemy of this insect pest. Accordingly, the current study provides the first insight on the interaction between the eucalyptus snout beetle and its unreported microsproridium with a focus on the pathogen incidence in field and lab-reared beetle populations, molecular and morphological characterization, and description of typical microsporidiosis symptoms associated with infected adults.

Associated pathogens may also be present in other countries, so there is a need for a more in-depth study aimed at detecting the microsporidium in Australia, the native range of *Gonipterus,* and in other countries where *G. platensis* and other species of *Gonipterus* are present. We also need to investigate the host spectrum of this new microsporidiosis to different *Gonipterus* species as well as to non-target native beetles in Brazil, especially concerning predatory beetles, in order to find out if this pathogen could be lethal or harmless to non-target hosts.

In summary, this data indicates a probable new species of this pathogen, providing support for new studies on its biology and distribution, as well as identifying its potential to be a positive or negative factor in crop protection programs against *Gonipterus* spp. in Brazilian eucalyptus plantations.

1.5 Materials and Methods

The research was conducted in the Laboratory of Biological Control of Forest Pests and Molecular Biology Laboratory in Department of Plant Protection, School of Agricultural Sciences, São Paulo State University (UNESP), Botucatu, SP, Brazil.

1.5.1 Colony maintenance of *Gonipterus platensis*

Adults of *G. platensis,* collected during field surveys in *Eucalyptus* plantations located in Botucatu, SP, Brazil, were kept in wooden cages (40 x 45 x 80 cm) with glass roof and sides covered with voile fabric. These adults were fed on young leaves and buds of *Eucalyptus urophylla* (clone 433) (which leaves were cleaned with hypochlorite solution (0.01%) and neutral detergent prior to use), maintained in an air-conditioned room at 25 ± 1 ºC, RH 50 ± 10% and photoperiod of 12:12 h light:dark.

1.5.2 Genomic DNA extraction

For the extraction of genomic DNA, five insects with symptoms of infection (found dead with open wings and deformed abdomen) were washed in 0.85% (w/v) NaCl sterile solution (Figure 1). To extract genomic DNA from *G. platensis*, the abdomens of the five adults were macerated and added to a microtube with 160 µL of 10% Chelex solution (Sigma-Aldrich) and 8 µL of 20 mg/mL proteinase K. The samples were placed in a thermal block at 95ºC for 20 min, following a protocol for DNA extraction[45], with Chelex 100 resin (Sigma-Aldrich). The DNA was eluted and stored at -20 °C until use.

1.5.3 Sequencing of SSU rRNA

Polymerase chain reaction (PCR) was performed with Amplitaq Gold mastermix (Thermofisher Willington, MA) using the following parameters; 95°C, 10 min, 35 cycles of 95°C, 30 s; 58°C, 30 s; 72°C, 60 s. PCR primers targeting the SSU rRNA were: 18f, 5′-CACCAGGTTGATTCTGCC-3′ and 1537r, 5′-TTATGATCCTGC TAATGGTTC-3′ [46]. The resultant amplicons were prepared using a Nextera XT library preparation kit and indices (Illumina inc, San Diego, CA). The samples were sequenced using an Illumina MiSeq system with a MiSeq V3 2 x 300 bp sequencing kit. The demultiplexed reads were quality trimmed to Q30 and assembled using CLC genomics workbench v20.0 (Qiagen inc., Valencia, CA). The consensus sequences

for two full length SSU rRNA genes were accessioned in GenBank under MT367570-MT367571.

1.5.4 Phylogenetic Analysis

Phylogenetic analysis was conducted with MEGA X using Maximum Likelihood analysis and the Tamura 3-parameter model with a discrete gamma distribution (5 categories), as this model was found to be the best-fit using the maximum likelihood-based model selection algorithm implemented in MEGA X. The partial deletion (90%) option was used, and the level of bootstrap support was calculated from 1000 replicates.

1.5.5 Determination of microsporidian spore density in beetles

Microsporidian spores were isolated from typically symptomatic insects, which were originated from the field, and maintained at the Laboratory for Biological Control of Forest Pests (LCBPF/UNESP, Botucatu, SP, Brazil). The infected insects were homogenized in nuclease-free water in 0.2-mL microtubes. The suspension was subjected to three series of centrifugation: 2,000 rpm for 10 min followed by 2 cycles at 12,000 rpm for 1 min. After each centrifugation, the supernatant was discarded. The spores accumulated at the bottom of the tube forming a "pellet", which was later resuspended in nuclease-free water[7]. This procedure was performed individually for 10 insects, in order to determine the average concentration of spores per beetle (n = 10). The spores from each insect were purified and suspended in nuclease-free water and then immediately quantified with a Neubauer chamber at 400× magnification under a phase-contrast microscope (Leica DM 2500, Leica Microsystems, Heerbrugg, Switzerland). At the end of the counts, the microsporidium density per insect was determined.

1.5.6 Phase contrast microscopy

Spore immobilization and photomicrographs were performed according to the methods described in Vávra and Maddox[28]. Fresh spores were visualized from macerated body contents of infected insects after dilution in sterile 0.85% NaCl solution (w/v). A drop of this macerate was transferred to a glass slide and the spores were

examined under a phase-contrast microscope (Leica DM 2500, Leica Microsystems, Heerbrugg, Switzerland) at 400× magnification.

1.5.7 Transmission electron microscopy

The material was prepared at the Center for Electron Microscopy (Biosciences Institute - UNESP). Tissue samples from the digestive tract of *G. platensis* adults were fixed in Karnovsky's solution[47] modified (glutaraldehyde 25% paraformoldehyde 8% and 0.2 M monosodium / disodium phosphate buffer solution, pH 7.3). The samples were cut into small fragments of up to 2 mm^3 for better fixation and incubated for at least 3 h at room temperature. The samples were removed from the fixative and washed 3 times for 5 min in 0.1 M phosphate buffer with pH 7.3, followed by immersing the material in osmium tetroxide for 2 h. After, the material was washed 3 times for 10 min in distilled water and immersed in 0.5% uranyl acetate in distilled water for about 2 h, in order to have the block contrast, revealing / highlighting the nucleic acids. Dehydration was accomplished with a series of solution containing an increasing amount of acetone. The series consisted of 10 min in 50% acetone, 2 washes for 10 min in 70% acetone, 3 washes for 15 min in 90% acetone, and finally, 3 washes for 15 min in 100% acetone.

The infiltration was done slowly using a 1:1 mixture of Araldite resin + 100% acetone and left for approximately 12 h at room temperature. The fixed biological material was made in an appropriate form and further placed in an oven at 60°C for 2 to 3 days. Semi-thin cuts (0.5 µm thick) were made for choosing the region of interest and selected regions were trimmed again to further reduce the block surface allowing ultrathin cuts (60-90 nm) to be made and placed in appropriate grids. The cuts were contrasted with a saturated solution of uranyl acetate in 50% alcohol for about 20 min, followed by lead citrate for 10 min.

The slides were firstly examined under a light microscope (Zeiss Axioscop 2, Germany) to select the materials to be subsequently observed in the electron microscope. The analysis of the material was performed using the transmission electron microscope JEM 1011 (JEOL, Inc., Peabody, MA), operating at 40.60, 80 and 100KV JEO at the Electronic Microscopy Laboratory (ESALQ – USP, Piracicaba, SP, Brazil).

1.5.8 Detection of microsporidiosis in different field-collected ESB populations from Southern Brazil

In order to confirm the presence of the microsporidian pathogen in different field populations from South to Southeast Brazil, insects from three different states were collected in commercial *Eucalyptus* plantations (Table 1). After collection in the field, the insects were brought to the laboratory, examined for microsporidiosis symptoms in adult beetles to calculate the percent natural infection, and then stored in a -20°C freezer prior to dissection and microscopy to confirm the presence of the pathogen.

Table 1. Field sampling of *Gonipterus* spp. beetles retrieved from different localities in South and Southeast Brazil in 2019 for assessment of microsporidian pathogen incidence. Asterisk (*) indicates another species found during field surveys that was identified as *Gonipterus pulverulentus*.

Sample	City and State	Eucalyptus host	Geographical coordinates
1	São Jerônimo da Serra, PR	*E. grandis x E. urophylla*	-23.7516S /-50.7955W
2.1*	Jaguarão, RS	*E. dunnii*	-32.3760S /-53.3263W
2.2	Jaguarão, RS	*E. dunnii*	-32.3760S/ -53.3263W
3	Itaberá, SP	*E. grandis x E. urophylla*	-24.1403S/ -49.1091W
4	Brotas, SP	*E. grandis x E. urophylla*	-22.2117S/ -47.9985W
5	Lençóis Paulista, SP	*E. grandis x E. urophylla*	-22.8249S/ -48.8533W
6	Itararé, SP	*E. grandis x E. urophylla*	-24.1958S/ -49.2242W
7	Itararé, SP	*E. grandis*	-24.1768S/ -49.1959W
8	Itararé, SP	*E. grandis x E. urophylla*	-24.1175S/ -49.2479W
9	Bom Sucesso do Itararé, SP	*E. grandis x E. urophylla*	-24.1495S/ -49.0802W
10	Bom Sucesso do Itararé, SP	*E. grandis*	-24.1484S/ -49.1054W
11	Bom Sucesso do Itararé, SP	*E. grandis*	-24.1485S/ -49.0980W
12	Laboratory rearing (Botucatu, SP)	*E. grandis x E. urophylla*	-22.8456S/ -48.4348W
13	São Jerônimo da Serra, PR	*E. grandis x E. urophylla*	-23.7556S/ -50.7965W

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CHAPTER 2

Identification and genome sequencing of RNA viruses in Eucalyptus snout beetle *Gonipterus platensis* (Coleoptera: Curculionidae)

Journal: Archives of Virology (Published)

2.1 Abstract

The genome of two putative new RNA viruses (macula-like virus and bunya-like virus) were identified in total RNA extracted from dead eucalyptus snout beetles (*Gonipterus* spp) from a laboratory colony. However, only bunya-like virus was detected from field collected insects. The macula-like virus has a monopartite single-stranded RNA genome which encodes three open reading frames (ORFs): an RNA-dependent RNA polymerase (RdRp), a capsid protein (CP), and an ORFs with unknown functions (ORF3). The bunya-like virus genome was predicted as two RNA segments, a large fragment (L), encoding a single protein (RdRp), and a small fragment (S) encoding a putative nucleocapsid protein.

The genus *Gonipterus* (Coleoptera: Curculionidae) has around 20 species of defoliating insects of Australian origin widely distributed worldwide. Two species of this genus were introduced in Brazil in the last century, *Gonipterus platensis* and *G. pulverulentus*. *G. platensis*, also known as eucalyptus snout beetle, has emerged as a major pest of *Eucalyptus* spp, causing important losses in wood production [1, 2]. Chemical insecticides have been widely used to control agricultural pests, but this method is costly and dangerous for non-target organisms in forestry. The use of biological control strategies to control insects reduces pollution by chemical pesticides and consequently benefits environment and human health. Currently, the biological control of *G. platensis* is carried out by *Anaphes nitens* (Girault) (Hymenoptera: Mymaridae), a tiny wasp that parasitizes eggs of *G. platensis*, and by the entomopathogenic fungus *Beauveria bassiana*, but with limited effective control [2, 3]. There are few reports of viruses infecting beetles (Table S1).

An RNA-sequencing approach using next-generation sequencing (NGS) was used to investigate the presence of viruses in dead insects from a colony of *G. platensis* maintained at the Biological Control of Forest Pest Laboratory, São Paulo State University (UNESP), Brazil. Ten dead insects (200 mg) were macerated in

Phosphate Buffered Saline 1X (PBS-1X, 137.0 mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.4). The homogenate was filtered once through cheesecloth and centrifuged three times at 4,000 × *g* for 10 min for clarification. The supernatant was centrifuged at 63,000 × *g* for 75 min on 5 ml of 20% sucrose cushion in polyallomer ultracentrifuge tubes (Beckman) and the pellet was resuspended in PBS-1X. RNA was extracted using QIAamp Viral RNA Mini Kit from QIAGEN according to the manufacturer's instructions. RNA samples were then used to construct cDNA libraries followed by sequencing using 2 x 100 bp read length on the HiSeq™ 2000 platform (Illumina, San Diego, CA, USA) at Macrogen Inc. (Seoul, Republic of Korea). The generated reads were trimmed and *de novo* assembled using CLC Genome Workbench 6.5.2 (CLC bio, Qiagen). Contigs related to viruses were retrieved using BLASTx against an in-house viral RefSeq database. To extend the assembled sequences as far as possible generated/trimmed reads were mapped back to the respective viral genomes using software Geneious 11.1.5 [4]. Genome annotation was also performed using the same software, in which the open reading frames (ORFs) were confirmed using BLASTx search against the NCBI non-redundant protein database (06/2020).

The analysis of NGS data revealed presence of two putative viruses with sequence identity to members of the families Tymoviridae *(macula-like)* and Bunyaviridae *(*bunya-like), based on the ORF that encodes RdRp. In addition, 99% of the reads were found to be related to fungi and bacteria. The macula-like virus assembled consensus sequence (1,162 reads) has 4,857 nucleotides in length, with a genome coverage of 24X. This consensus codes for three putative genes, ORF1 (1,097 aa) with 53% amino acid identity (99% query coverage) to the RdRp protein of Tarnsjo virus (MK440641), ORF2 (202 aa) with 37.27% amino acid identity (77% query coverage) to the CP of the Guadeloupe Culex tymo-like virus (MN053827) and ORF3 (158 aa) with no similarity to any protein by BLASTx search in Genbank. Interestingly, the ORF that encodes the RdRp protein was shorter (1,158 aa) than the other RdRps of viruses of the same family deposited in Genbank, ranging from 1,600 to 3,300 aa. The RdRp lacks a viral methyltransferase domain at the N-terminus of the polyprotein whih is also not present in the genome of the phylogenetically (Fig.1) related virus Grapevine rupestris vein feathering virus (QIN93588). We have not used any specific method to determine the complete 5’ and 3’ putative untranslated sequences (UTRs).

The macula-like genome consists of monopartite single-stranded RNA of positive polarity containing three putative open reading frames encoding a RNA-dependent RNA polymerase (RdRp), a capsid protein (CP), an ORF with an overlap of 50 nucleotides at the 3´ end of the CP (ORF3) (Fig. 1A).

The bunya-like virus reads assembled into two contigs. The first contig consensus sequence (827,275 reads) has 1,356 nucleotides in length with coverage of 61X. This sequence (S segment) encodes an ORF of 317 aa that has 29.97% amino acid identity (99% query coverage) to the nucleocapsid protein of the Bunyavirus sp. (AOY18790). The second (L segment) contig consensus sequence (731,828 reads) has 6,830 nucleotides in length with coverage of 10X. This sequence (L segment) encodes an ORF of 2,198 aa that has 38% amino acid identity (95% query coverge) to the RdRp of Lampyris noctiluca bunya-like virus 1 (MH620814). Bunyavirus genome consists of single-stranded RNA of negative polarity divided into three segments: large (L), medium (M), and small (S). In this work, it was possible to assemble only two segments (S and L) of this putative new bunyavirus, whereas the M segment was not identified using the bioinformatics tools available (Fig. 2 A).

To confirm the presence of macula-like and bunya-like sequences in the pooled RNA to sequencing and in populations of *G. platensis* and *G. pulverulentus* collected from different fields and from the laboratory insect colony , we used Reverse Transcriptase (RT) reaction followed by Polymerase Chain Reaction (PCR) using specific oligonucleotide primers (macula R: 5'-AGTCACCACTGGCACAACAA-3' and macula F: 5'-GCTCATGACGCCTCGATGTA-3') to detect a fragment of 742 bp from the putative RdRp of the macula-like virus and (Bunyavirus R:5'-AGTATGCCTTTTCCCAGGGC-3' and Bunyavirus F: 5'-GGTAAAGGGCCCTGCTCATT-3') to detect a fragment of 666 bp from the putative RdRp of the bunya-like virus (Fig. S1 and Table S2). The two viruses (macula-like virus and bunya-like virus) sequences were detected in the samples that were submitted to sequencing. Out of eleven pools screened by RT-PCR using RNA extracted from the whole body of ten adult individuals collected in the field (randomly chosen) of the species *G. platensis* and *G. pulverulentus*, six were found to be positive for the presence of the bunya-like virus and no sample was positive for macula-like virus.

Phylogenetic trees were constructed using the amino acid sequences of the putative RdRps of Maculavirus and Bunyavirus (L segment) and sequences with the highest identity after BLASTx search (Fig. 1B, 2B and Table S3). Although the macula-

like virus was grouped with other viruses found in beetles (i.e. *Lampyris noctiluca* tymovirus-like virus 1) and others insects, it formed an evolutionary line independent and distant. The macula-like virus, based on phylogenetic analysis placed closest to Tarnsjo virus and Guadeloupe Culex tymo-like virus (Fig. 1B). Most arthropod-derived sequences clustered altogether, but some plant-derived sequences (Oat blue dwarf virus-OBDV, Eggplant mosaic virus-EMV and Grapevine fleck virus-GFkV) clustered with the arthropod-derived sequences. Even though *Tymoviridae* family is composed mainly of plant viruses, many viruses related to this family have been discovered in spiders, dragonflies or insect cells [5]. For instance, OBDV is a plant pathogenic virus that also able to replicate within leafhopper vectors[6].

According to the phylogenetic analysis, the bunyavirus-like sequence found in this work is most closely related to *Lampyris noctiluca* bunya-like virus 1, Wuhan insect virus 16, Hubei insect virus, Bunyavirus sp. and Hubei bunya-like virus 13, all of which have bipartite genomes. Wuhan insect virus 16 and Hubei bunya-like virus 13 were isolated from flea/ants and dragonflies, respectively [7–9], and *Lampyris noctiluca* bunya-like virus 1 was isolated from a glowworm [8] (Fig. 2B). A comprehensive characterization of new insect-specific bunyavirus strains with ancestral reconstruction showed that mammalian pathogenic bunyaviruses evolved from arthropod-specific parents [10].

The draft genome sequence of these new putative viruses, tentatively named *Gonipterus platensis* bunya-like virus (GPBLV) and *Gonipterus platensis* macula-like virus (GPMLV), were deposited into the NCBI GenBank database under the accession numbers: MT435496 for GPMLV, MT435497 to L segment and MT435498 to S segment of GPBLV. Identification of new viruses in insects helps us to increase our knowledge on their diversity and provides new information that can be used for the development of these insect pathogens for the control of important insect pests.

Fig. 1 (A) Diagram of the predicted genome organization of *Gonipterus platensis macula-like virus* (GPMV). The putative ORFs are indicated in yellow. (B) Phylogenetic analysis of *Gonipterus platensis macula-like virus* and other members of the family Tymoviridae using RdRP amino acid sequences. The phylogeny was based on the amino acid sequences deduced from viral gene sequences, using the sequences that gave the best hits in a BLASTx search against the NCBI non-redundant protein database (Table S3). The tree was inferred by the maximum-likehood method using PhyML method, using the best-fit substitution model inferred using jModelTest v2.1.10 and 1000 bootstrap replicates, both implemented in Geneious 11.1.5. The phylogenetic trees were visualized using Fig Tree v1.3.1 and are rooted at the midpoint. Official species names established by the International Committee on Taxonomy of Viruses (ICTV) are italicized.

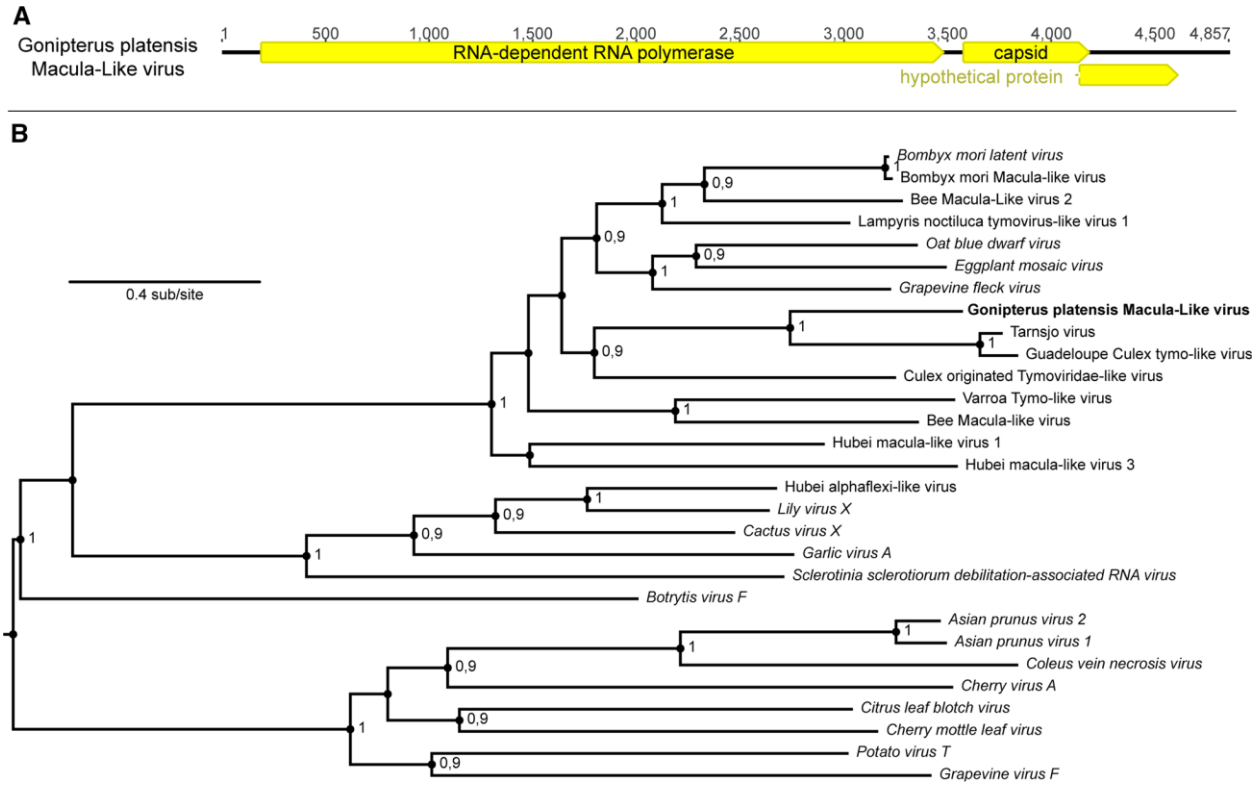
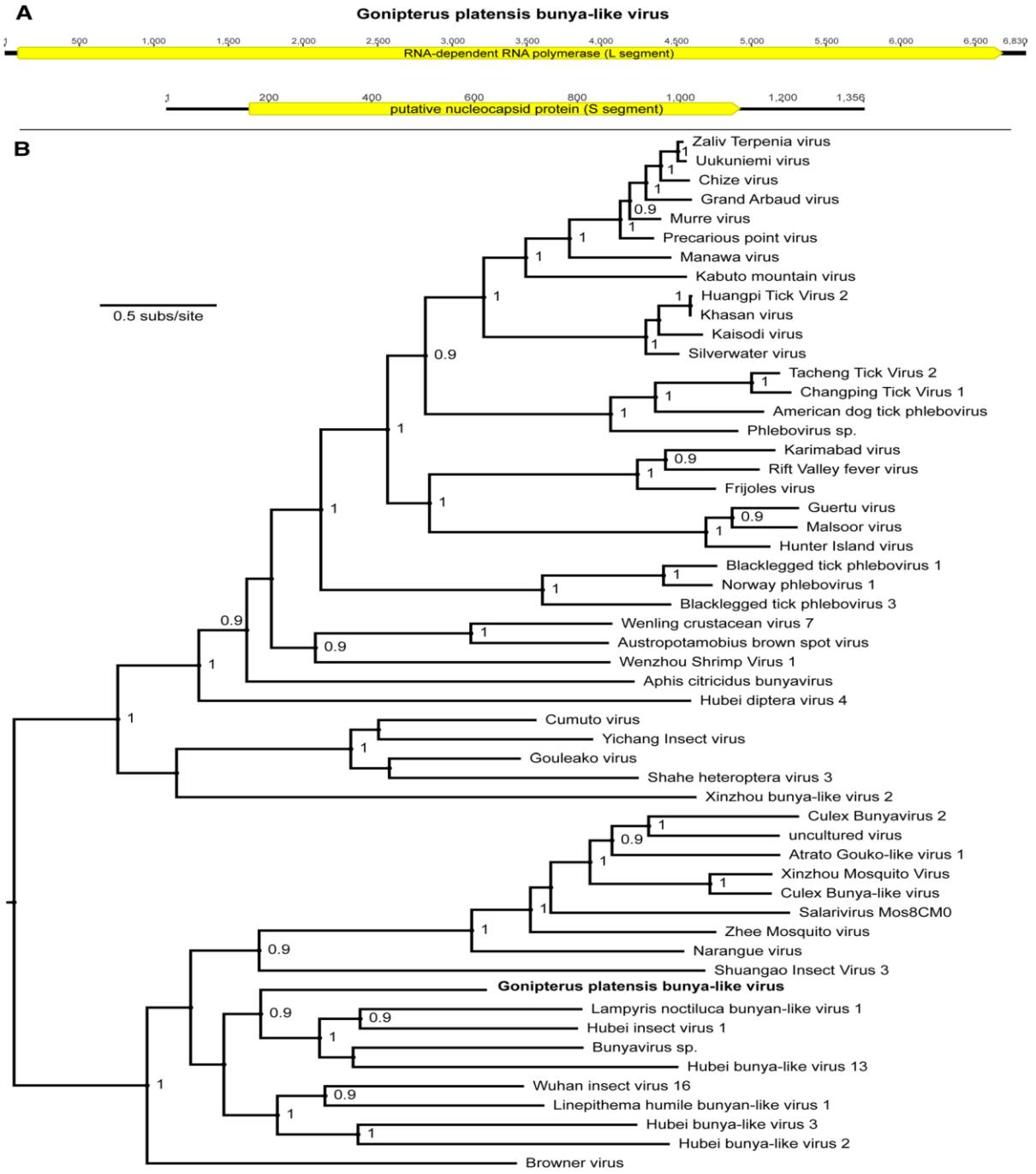


Fig. 2 (A) Diagram of the predicted genome organization of *Gonipterus platensis* bunya-Like virus (GPV). The putative ORFs are indicated in yellow. Phylogenetic analysis of the L segment (RdRp) of *Gonipterus platensis* bunya-like virus. The phylogeny was based on the amino acid sequences deduced from viral gene sequences, using the sequences that gave the best hits in a BLASTx search against the NCBI non-redundant protein database (Table S3). The tree was inferred by the maximum-likelihood method using the PhyML method and the best-fit substitution model using with jModelTest v2.1.10 and 1000 bootstrap replicates, both implemented in Geneious 11.1.5. The phylogenetic trees were visualized using FigTree v1.3.1 and are rooted at the midpoint. Official species names established by the International Committee on Taxonomy of Viruses (ICTV) are italicized.



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2.2 Supplementary material

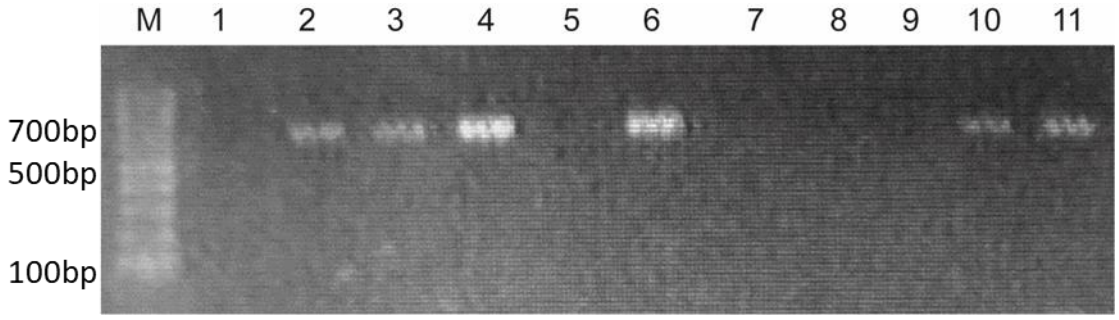

Fig.S1. Bunyavirus RT-PCR screening in adult of *Gonipterus* spp. collected from Brazilian eucalyptus fields. The rows presented a positive amplified band of 666 bp for the bunya-like virus and 742 bp for the macula-like virus. Five-microliter samples of PCR products were loaded into a 1.2% agarose gel. M, 100 bp molecular marker. Lanes 1-11, PCR samples from different Brazilian regions, according to Table S1. Complementary DNA sequences (cDNAs) were synthesized using SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, USA) and random hexonucleotides. After cDNA synthesis, a dilution of 1:50 was individually used as template for PCR with 0.4 µM of the primers (macula R: 5'-AGTCACCACTGGCACAACAA-3' and macula F: 5'-GCTCATGACGCCTCGATGTA-3') to detect a fragment of 742 bp from the putative RdRp of the macula-like virus and (Bunyavirus R:5'-AGTATGCCTTTTCCCAGGGC-3' and Bunyavirus F: 5'-GGTAAAGGGCCCTGCTCATT-3') to detect a a fragment of 666 bp from the putative RdRp of the bunya-like virus, 300 µM of dNTP mix (Promega), 1 U of GoTaq (Promega), and 1x of the supplied reaction buffer. The reactions were subjected to the following program: 95 ºC/2 min, 35 cycles of 95 ºC/ 30s, 53 ºC/30 s and 72 ºC/1 min with a final extension of 1 min at 72 ºC. This amplification should generate a product of 700 base pairs (bp) for both viruses.

Table S1. Viruses reported infecting insects of order Coleoptera worldwide

Virus	Host	Reference
Nudivirus	*Oryctes rhinoceros* (Dynastidae)	[1–4]
Entomopoxviruses	*Othnonius batesi* (Scarabaeidae: Melolonthinae), *Dermolepida albohirtum* (Scarabaeidae), *Aphodius tasmaniae* (Scarabaeidae:Aphodiinae)	[5–7]
Nudivirus	*Tenebrio molitor* (Tenebrionidae); *Scolytus scolytus* (Curculionidae: Scolytinae); *Oryctes rhinoceros* (Dynastidae)	[8, 9]
RNA vírus (unidentified)	*Heteronychus arator* (Scarabaeidae)	[10]
Alphanodavirus	*Costelytra zealandica* (Scarabaeidae)	[11]
Reovirus	*Epilachna varivestis* (Coccinellidae)	[12]
Nudivirus	*Sternochetus mangiferae* (Curculionidae)	[13]
Nudivirus	*Diabrotica undecimpunctata* (Chrysomelidae)	[14]
Entomopoxvirus	*Ips typographus* (Curculionidae: Scolytinae)	[15]

Table S2. RT-PCR detection of Maculavirus and Bunyavirus in *Gonipterus* spp. of different Brazilian regions. Presence (+) or absence (−) of different viruses are indicated. Insects from these regions were collected alive and were transferred to a 1.5 mL microtube and kept at 4 °C until the samples were processed. Eleven pools of the whole body of ten adult individuals collected in the field and from a laboratory colony (randomly chosen) of the species *G. platensis* and *G. pulverulentus* were macerated with Trizol™ Reagent (Thermo Fisher Scientific) and used for RNA extraction according to manufacturer's instructions. The total RNAs extracted were subjected to RT-PCR according to the same procedure used above. As positive controls, we used the cDNA produced from the RNA sequenced from dead *G. platensis* adults.

Samples	Municipality and State	bunyavirus	maculavirus
1	São Jerônimo da Serra, PR	−	−
2*	Jaguarão, RS	+	−
3	Jaguarão, RS	+	−
4	Itaberá, SP	+	−
5	Brotas, SP	−	−
6	Lençóis Paulista, SP	+	−
7	Itararé, SP	−	−
8	Itararé, SP	−	−
9	Bom sucesso do Itararé, SP	+	−
10**	Botucatu, SP	−	−
11*	São Jerônimo da Serra, PR	+	−

* adult *G. pulverulentus.* RS, Rio Grande do Sul, SP, São Paulo, PR, Paraná.

** Insect from a laboratory colony

Fig. S3. The list of the all viruses accession numbers used in this study.

GenBank Accession numbers	Tymoviridae family
YP_009160324.1	Bee Macula-like virus
YP_009551952.1	Bee Macula-Like virus 2
YP_009505642.1	Bombyx mori latent virus
YP_004464930.1	Bombyx mori Macula-like virus
YP_006843893.1	Culex originated Tymoviridae-like virus
QEM39127.1	Guadeloupe Culex tymo-like virus
QBP37021.1	Lampyris noctiluca tymovirus-like virus 1
QGA70915.1	Tarnsjo vírus
YP_009159826.1	Varroa Tymo-like virus
NC_011062	Potato virus T
KX883811	Hubei alphaflexi-like virus
KX883800	Hubei macula-like virus 3
NC_033254	Hubei macula-like virus 1
YP_009204561	Asian prunus virus 2
YP_009094347	Asian prunus virus 1
YP_006590065	Grapevine virus F
YP_001430021	Coleus vein necrosis virus
YP_325662	Sclerotinia sclerotiorum debilitation-associated RNA virus
YP_263303	Lily virus X
NP_624333	Citrus leaf blotch virus
NP_620106	Cherry virus A
NP_569126	Garlic virus A
NP_542612	Grapevine fleck virus
NP_148778	Cactus virus X

NP_068549	Botrytis virus F
NP_062428	Cherry mottle leaf virus
NP_044447	Oat blue dwarf virus
NP_040968	Eggplant mosaic virus
QIN93588	Grapevine rupestris vein feathering virus
MT435496	*Gonipterus platensis* Macula-Like virus

GenBank Accession numbers	Bunyaviridae family
AIE42674.1	American dog tick phlebovirus
QDW80893.1	Aphis citricidus bunyavirus
QHA33672.1	Atrato Gouko-like virus 1
QCO84579.1	Austropotamobius brown spot virus
ANT80544.1	Blacklegged tick phlebovirus 1
ANC97695.1	Blacklegged tick phlebovirus 3
QED21534.1	Browner virus
AOY18800.1	Bunyavirus sp.
AJG39235.1	Changping Tick Virus 1
AFH08728.1	Chize virus
AXQ04766.1	Culex Bunya-like virus
AXQ04881.1	Culex Bunyavirus 2
YP_009664615.1	Cumuto virus
QCI62741.1	Frijoles virus
YP_009664621.1	Gouleako virus
AFH08732.1	Grand Arbaud virus
YP_009666941.1	Guertu virus
YP_009293590.1	Huangpi Tick Virus 2
APG79273.1	Hubei bunya-like virus 13
APG79269.1	Hubei bunya-like virus 2
APG79267.1	Hubei bunya-like virus 3

YP_009330281.1	Hubei diptera virus 4
APG79218.1	Hubei insect virus 1
AHI10994.1	Hunter Island virus
YP_009449450.1	Kabuto mountain virus
YP_009551639.1	Kaisodi virus
AHK60947.1	Karimabad virus
AII79370.1	Khasan virus
QBP37023.1	Lampyris noctiluca bunyan-like virus 1
AXA52548.1	Linepithema humile bunyan-like virus 1
AHF71068.1	Malsoor virus
AFN73042.1	Manawa virus
AFH08736.1	Murre virus
QHA33858.1	Narangue virus
ASY03248.1	Norway phlebovirus 1
QDW81038.1	Phlebovirus sp.
AEL29680.1	Precarious point virus
ABD51503.1	Rift Valley fever virus
API61884.1	Salarivirus Mos8CM0
APG79318.1	Shahe heteroptera virus 3
AJG39251.1	Shuangao Insect Virus 3
AIU95032.1	Silverwater virus
AWK68110.1	Tacheng Tick Virus 2
AGW51765.1	uncultured virus
AIU95037.1	Uukuniemi virus
APG79349.1	Wenling crustacean virus 7
YP_009304989.1	Wenzhou Shrimp Virus 1
APG79216.1	Wuhan insect virus 16
APG79359.1	Xinzhou bunya-like virus 2
AJG39271.1	Xinzhou Mosquito Virus

YP_009305143.1	Yichang Insect virus
QCF29627.1	Zaliv Terpenia virus
AJG39275.1	Zhee Mosquito virus
MT435497	*Gonipterus platensis* bunya-like virus (L segment
MT435498	*Gonipterus platensis* bunya-like virus (S segment)

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CHAPTER 3

Potential of *Bacillus thuringiensis* isolates to manage *Gonipterus platensis* (Coleoptera: Curculionidae) larvae populations

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3.1 Abstract

The potential of the bacterium *Bacillus thuringiensis* var. *tenebrionis* to managing Coleoptera pests such as t *Gonipterus platensis* Marelli (Coleoptera: Curculionidae) without impact on its egg parasitoid, *Anaphes nitens* Girault (Hymenoptera: Mymaridae) is high. The objective of this work was to evaluate the pathogenicity of ten *Bacillus thuringiensis* (Bt) isolates to neonate *G. platensis* larvae. The genomic DNA of these isolates was extracted by the thermal lysis method to characterize, molecularly, their proteins Cry and Vip. Ten neonate *G. platensis* larvae per replication (three) received a standard concentration of$1x10^9$ spores/ml of each isolate. The concentration and the lethal time required to kill 50% of the neonate *G. platensis* larvae were determined for the best isolates in the selection bioassay. The isolates 107 and 178, at a concentration 10x lower than that recommended for the *Bacillus thuringiensis* var. *tenebrionis* (Btt) in commercial formulations against Coleoptera pests caused 100% mortality of *G. platensis* larvae. The Bt isolates, with coleopteran-specific genes, represent an effective tool for the biological control of the neonate *G. platensis* larvae.

Keywords: Biological control, entomopathogenic bacteria, Eucalyptus Snout Beetle.

3.2 Introduction

The reduced genetic base of planted forests facilitates the establishment and dispersal of exotic pests with production losses [1;2]. The period between planting and harvesting of the Brazilian *Eucalyptus* plantations is one of the shortest in the world and high productivity. This is mainly due to the good management practices, genetic improvement and edaphoclimatic conditions [3].

Damage by the eucalyptus weevil, *Gonipterus platensis* Marelli (Coleoptera: Curculionidae), an exotic pestin eucalyptus plantation has been reported from the late 2012 in Brazil. Damage by this pest, native to Australia and distributed in the South

and Southeast Brazil [4], is high because its larvae and adults feed mainly on young eucalyptus leaves [5]. *Gonipterus platensis* is, normally, managed with biological control agents, especially the egg parasitoid *Anaphes nitens* Girault (Hymenoptera: Mymaridae), products based on *Beauveria bassiana* [6] and there is no chemical insecticide registered to control this pest in Brazil [7].

The damage caused by *Gonipterus* spp. are increasing in different eucalyptus producing regions of the world, which has been attributed to the low parasitism rate on this pest and to inadequate climatic conditions and altitude for the parasitoid *A. nitens* [8]. Chemical control reduces populations of *Gonipterus* spp. and coleopteran pests are managed with the bacterium *Bacillus thuringiensis* var. tenebrionis in Australia and New Zealand [9].

New classes of insecticidal proteins are important to manage insect resistance to *B. thuringiensis* toxins [10]. The Cry proteins of this bacterium bind to different sites in the cells of the intestinal epithelium of target insects and its use, with Vip proteins, can expand the toxicity spectrum minimizing the risk of cross-resistance [10]. The objective of this work was to select *B. thuringiensis* isolates with high toxicity to neonate *G. platensis* larvae.

3.3. Materials and Methods

3.3.1 General information

The bioassays were carried out at the Biological Control of Forest Pests (LCBPF) and the molecular analysis at the Molecular Biology (Nematology) laboratories at the São Paulo State University (UNESP), School of Agriculture, Botucatu Campus, São Paulo, Brazil at 25 ± 1 °C, 50 ± 10% relative umidity and 12h photoperiod.

3.3.2 Rearing *Gonipterus platensis*

Gonipterus platensis adults were collected in eucalyptus plantations in the municipality of Lençóis Paulista, São Paulo, Brazil and kept in wooden cages (40 x 45 x 80 cm) with glass roof and voile fabric sides at room temperature (25 ± 1 ºC, 50 ± 10% RH and 12h photophase). These adults were fed with tender leaves and buds of *Eucalyptus urophylla*, previously cleaned with hypochlorite (0.01%) and detergent, which also served as a breeding and oviposition sites for *G. platensis*. Postures, performed on the plant pointers, were collected and stored in Petri dishes until the

larvae hatched and part of them used in the bioassays and another for the stock rearing.

3.3.3 Obtaining *Bacillus thuringiensis*

Ten *Bacillus thuringiensis* wild isolates, provided by the Laboratory of Bacteria Genetics and Applied Biotechnology (LGBBA) at UNESP - Jaboticabal Campus, São Paulo, Brazil and the *Bt* var*. israelensis*, *Bt* var. *morrisoni*, *Bt* var. *san diego*, *Bt* var. *tenebrionis* (Btt) and *Bt* var. *tolworthi,* from the collection of the Bacillus Genetic Stock Center, Columbus, Ohio, USA, were used (Table 1).

The *B. thuringiensis* isolates were cultured in test tubes with BHI liquid medium (Brain Heart Infusion Broth) and, subsequently, in Petri dishes with Nutrient Agar (NA) culture medium (meat extract 3 g/L, bacteriological peptone 5 g/L and Agar 15 g/L) and incubated at 28 ºC for seven days for the complete sporulation and release of crystals by the bacteria. The morphology of each isolate was characterized in phase contrast microscopy (1000x) to observe its vegetative cells, spores and crystals.

3.3.4 Extraction of genomic DNA for PCR use

The wild isolates and the standard strains of *B. thuringiensis* were grown in Petri dishes with NA culture medium. A 100 µL aliquot of the BHI stock was collected and inoculated in the culture medium, in these dishes, with the help of the Drigalski loop to obtain colonies incubated in B.O.D. for 12h at 28ºC. The colonies, of each isolate and of the standard strains, were resuspended in 200 µL of sterile water and their genomic DNA extracted by the method of thermal lysis, boiled for 10 minutes at 95ºC and subjected to thermal shock at -20ºC for 10 minutes. The DNA samples were stored in a freezer at - 20ºC.

3.3.5 Molecular identification of *Bacillus thuringiensis* isolates

The genes, which encoded insecticidal proteins for the order Coleoptera, were identified with primers for the proteins Cry and Vip (vegetative insecticidal protein), including Vip1 and Vip2 that form a binary toxin with activity against Coleoptera larvae [10;11]. Fourteen primer pairs were synthesized and the sequences of their base pairs described (Table 2).

The total volume used in the amplification reaction was 25 µL of Mix with 12.5 µl of Gotaq (Neobio), 5 U/µl Taq, 100 µM of each dNTP and 25 mM MgCl2, 10 mM of

each initiator, 3 µl of genomic DNA and nuclease-free water. PCR was performed in a thermocycler (Infinigen, model TC-96CG) and a negative control (nuclease free water) added.

Each primer pair was individually amplified (Table 3) and the PCR products visualized by electrophoresis in 1% agarose gel with a 1 Kb marker (Norgen) and photographed in an ultraviolet light transilluminator (Major Science).

3.3.6 Selecting the *Bacillus thuringiensis* isolates based on their virulence

The *Bacillus thuringiensis* isolates were cultured in Petri dishes with nutrient-agar culture medium incubated at 28 ºC for seven days, collected with a microbiological loop and diluted in autoclaved distilled water with 0.05% Tween-20. The spore/crystal suspensions were quantified in a Neubauer chamber and used at a standard concentration of $1x10^9$.

Young leaves from the apical branches of the hybrid eucalyptus clone were cleaned, immersed in the spore/crystal suspensions of the respective strains for one minute and left to dry in a unidirectional flow chamber for 15 minutes. The leaf petioles were inserted in floral foam saturated with distilled water to maintain their turgor in 250 ml plastic pots with a screened lid to allow aeration with ten *G. platensis* larvae each one. The mortality of these larvae was observed, daily, for seven days.

The experiment consisted of 16 treatments as ten isolates of *B. thuringiensis*, five standards strains; one of them is the *B. thuringiensis* var. *tenebrionis* as positive control besides autoclaved distilled water + Tween-80. Each treatment had three replications with 10 neonate *G. platensis* larvae each, totalling 30 larvae per treatment. The isolates were selected according to the percentage of total mortality of neonate *G. platensis* larvae evaluated during seven days.

3.3.7 Lethal Concentration (LC_{50}) and Lethal Time (LT_{50}) of the best isolates to neonate *Gonipterus platensis* larvae

The LC50 of the isolates 107 and 178 was based in the concentration-mortality of *G. platensis* in the bioassays with their multiple concentrations. Each treatment had three replications with 10 neonate *G. platensis* larvae each, totalling 30 larvae per treatment and the bacterial suspensions prepared and applied in the same manner as in the item 3.6. The isolates were applied at the concentrations of $7.5x10^7$, $1.5x10^8$,

$3x10^8$, $6x10^8$, $1x10^9$ and $4.5x10^9$ spores/Ml. The *Bt* var. *tenebrionis* (standard for Coleoptera) did not cause 100% mortality in the selection test at a concentration of $1x10^9$ spores/Ml and, therefore, $1x10^{10}$ spores/Ml were used to achieve it. The control had the application of sterile solution of Tween-80 (0.1%) on leaves of *E. urophylla* with three replications and 30 larvae per treatment. Larva mortality was observed daily, for seven days, and the isolates selected according to the total number of dead insects and their lethal LC50 concentration.

3.3.8 Experimental design

The experimental design was completely randomized with sixteen treatments with three replications with 10 larvae each.

3.3.9 Statistical analysis

A generalized linear model with binomial response and Probit binding function was adjusted to the mortality data of neonate *G. platensis* larvae per isolate, with time as factors. A structure of measures repeated over time using the Generalized Estimation Equation (GEE) technique was used with the genmod procedure (from the SAS statistical program - Free Statistical Statistical Software, SAS University Edition. The quality of the model fit was assessed by deviance analysis [12] and the treatment means compared by the Tukey-Kramer test [13].

Lethal concentration (CL50) and time (TL50), to kill 50% of the insects with a 95% confidence interval, were obtained by adjusting a logistic regression model with binomial distribution and probit link function [14]. The response variable was the proportion of insects killed per bacteria concentration. The Probit procedure (with the inverse cl option) of the SAS statistical program - Free Statistical Statistical Software, SAS University Edition was used.

Statistical analysis of data on mean mortality rate (ratio of total insects killed per day) was used to fit the generalized linear models with the gamma distribution and logarithmic link function. These models were adjusted to the data by maximum likelihood estimation, using the genmod procedure (SAS- Free Statistical Statistical Software, University Edition) and the treatment averages compared using the Tukey-Kramer test [13].

3.4. Results

3.4.1 Molecular characterization

The Cry35Ba gene presented in all isolates, except in the 115 and 116, and the Cry9 and Cry35Aa were not amplified in any isolate. Vegetative insecticidal proteins (Vip) were found in ten isolates and two other genes per isolate (Table 4). The Vip1/Vip2, Vip2, Vip1, Cry10, Cry1i, Cry3Ba and Cry3Bb genes were detected in six (40%), six (40%), five (33.3%), four (27%), four (27%), three (20%) and three (20%) isolates, respectively. The Cry35Ca (6%), Cry3Aa (6%), Cry7 (13%), Cry8 (13%) genes were the least common.

3.4.2 Mortality of *Gonipterus platensis* neonate larvae by the *Bacillus thuringiensis* isolates

The mortality of the neonate *G. platensis* larvae was 100% with the isolates 107 and 178 and medium (33,1% to 66%) or low (0% to 33%) with the others, even for those expressing specific genes for Coleoptera (Table 5).

The mortality of *G. platensis* larvae with the standard concentration of $1x10^9$ spores/ml of the isolates 107 and 178 was 100% (Table 5) after 2.92 ± 0.21 and 2.36 ± 0.14 days, respectively. The efficiency of the other isolates, with the Cry10, *B. thuringiensis israelense* and the isolate 115, was 60% ± 0.015 and 63% ± 0.029, respectively, of mortality of *G. platensis* larvae after 1.07 ± 0.1 days.

3.4.3 CL_{50} of the best isolates to *Gonipterus platensis*

The LC_{50} of isolates 178, 107 and *B. thuringiensis tenebrionis* were lowest at 5.15×10^8, 6.48×10^8 and 1.3×10^9, respectively (Table 7 and Figure 1).

3.4.4 Estimated Mean Lethal Time

The isolates 107 and 178, at the concentrations of 6.0×10^8 and 1.0×10^9, killed 50% of the neonate *G. platensis* larvae in shorter periods, 230.22 h and 232.5 h and 54.18 h and 82.83 h., respectively (Figure 2).

3.5 Discussion

Bacillus thuringiensis, an entomopathogenic and spore-forming bacterium, synthesizes a variety of insecticidal proteins, with potential and safety as a biocontrol agent because it is specific for a group of insect pests and does not affect humans and the environment [14; 15]. The *Bt* insecticidal proteins, with highly toxic crystals and spores, are widely used for the biological control of agricultural pests and represent 90% of the bioinsecticide market [16].

3.5.1 Molecular Characterization

The molecular characterization of the Cry3, Cry7, Cry8, Cry9, Cry35, Vip1 and Vip2 increases the potential of the *B. thuringiensis* isolates with these proteins because they are coleopteran-specific [17]. The lack of amplification of the coleopteran-specific Cry9 and Cry35Aa genes may be due to a gene interruption, mutation or under the control of a defective promoter, or expressed at very low levels, without contributing to the lethal effects of the isolates, as reported for Bt isolates from Colombia also with no amplification for the Cry gene fragments [18;19]. The presence of vegetative insecticidal proteins (Vip) in ten isolates and two other genes per isolate show insecticidal potential against insensitive or resistant pests to Cry proteins, as they act in different receptors in the insect intestine [20]. The PCR products, with single molecular weight, were expected to show at least one Coleoptera-specific gene in all strains and the Cry35Ba gene as the most abundant in 12 isolates (80%), confirms the fact that this insecticidal gene have been identified in strains toxic to other coleopteran pests, such as *Leptinotarsa decemlineata* (Coleoptera: Chysomelidae) [21]. The presence of the Cry35Ba gene in most isolates may explain their toxicity, mainly due to the expression of this Cry protein. The binary proteins Vip1 and Vip2, in ten isolates, are active against a wide range of Coleoptera pests [22]. The Cry and Vip genes, in *B. thuringiensis* isolates can be used together, since the Vip proteins bind to different sites in the cells of the intestinal epithelium of target insects, increasing the toxicity spectrum and minimizing the risks of cross resistance in organisms such as genetically modified insects [10]. The reduced gene frequency in the Bt strains, effective against Coleoptera larvae [23], increases the importance of seeking efficient ones and toxins to manage agricultural pests of this order [24;25;26]. The Cry9 and Cry35Aa genes are mainly associated with high activity against lepidopteran pests [27w]. The absence of the

tested strains for these genes also confirms reports in Argentina, Iran and China that the frequency of these genes varies between Bt strains [28].

3.5.2. Mortality of neonate *Gonipterus platensis* larvae by the *Bacillus thuringiensis* isolates

The 100% mortality of neonate *G. platensis* larvae by the isolates 107 and 178 may be associated with the high efficacy of the Cry10 gene, present in them as shown for the GMO cotton plant, expressing this toxin for the management of the cotton weevil *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) [29]. This increases the importance of studying bacteria expressing Cry genes to manage Coleoptera pests in eucalyptus plantations [30]. The average or low mortality with the other isolates, even for those expressing specific genes for Coleoptera may be due to their mutations interfering in the toxin-receptor interaction or the low action of toxins in the insect intestine as reported for the low to medium mortality of *Acyrthosiphon pisum* (Homoptera, Aphididae) [31;32].

The mortality of 100% of the *G. platensis* larvae, with the standard concentration of the isolates 107 and 178, may be associated to the molecular weight of the Cry10 gene, which was efficient against *Hypothenemus hampei* (Coleoptera: Scolytidae) [33] and *Anthonomus grandis* (Coleoptera: Curculionidae) [29; 34]. The efficiency higher than 50% of the other isolates with the Cry10 gene, *B. thuringiensis* israelenses and the 115, against *G. platensis* confirms the effects of toxins from this gene with specific action, possibly reaching the insect tissues and cells in a shorter period [35].

3.5.3. LC_{50} of the best *Bt* isolates applied on *Gonipterus platensis*

The LC50 of the isolates 178 and 107 at the concentrations 10 times lower than that of the standard strain, the *B. thuringiensis* tenebrionis (Btt), the basis of commercial formulations to control coleopteran pests [10], may be associated with their Cry10 gene and other specific genes for Coleoptera, such as the Cry35Ba and Cry7, the first efficient against *Sitophilus oryzae* (Coleoptera: Curculionidae) [36]. The distinct profile of the isolates increases the importance of seeking those with different modes of action and specificities to reduce the possibilities of insect pest resistance to pesticides based on *B. thuringiensis* [37;38]. The crystal morphology of the Cry35Ba

protein may be more associated with toxicity against beetles [39], that of Cry10 to Diptera and Coleoptera (ZORZETTI et al., 2018) and the Cry7 to Lepidoptera and Coleoptera [40].

The shorter median lethal time of the isolate 107 in both concentrations compared to the 178 is the inverse of the LC50 estimate and it is due to the Cry4 gene in the second, with possible synergistic interactions between proteins [41; 42]. Combinations of toxins increase toxicity and decrease lethal time [43] as reported for the synergistic effects between the Bt toxins against different insects such as *Earias insulana, Plutella xylostella* and *Chilo partellus* [41; 42].

The biological characteristics highlight the *B. thuringiensis* as the main bacterium used in the biological control of insects [10]. Their performance in the bioassays and molecular characterization are important to select isolates effective against insects. The toxic potential of wild isolates tested against neonate *G. platensis* larvae varied due to the different genes expressed by each and their toxicity to the larvae of this insect.

3.6. Conclusion

The *B. thuringiensis* isolates, with coleopteran-specific genes, represent an option for the biological control of neonate *G. platensis* larvae. The mortality of neonate *G. platensis* larvae was higher with the *B. thuringiensis* isolates 107 and 178 and, therefore, represents an alternative for the biological control of this eucalypt pest.

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Tables and Figures

Table 1. Identification (Ident.) of *Bacillus thuringiensis* isolates used in the experiments and respective isolation places.

Ident.	Isolation location	Ident.	Isolation Places
93	Cotia, São Paulo, Brazil	187	Jaboticabal, Sao Paulo, Brazil
107	Bela Vista, São Paulo, Brazil	706	Presidente Olegário, Minas Gerais, Brazil
115	Bonazópolis, São Paulo, Brazil	923	Teixeiras, Minas Gerais, Brazil
116	Bela Vista, São Paulo, Brazil	940	Uberaba, Minas Gerais, Brazil
178	Jaboticabal, São Paulo, Brazil	1100	Coqueiral, Minas Gerais, Brazil

Table 2. Primers (Init.), Sequences and Pb base pairs, used in the PCR reactions of genes encoding Cry and VIP insecticidal proteins for Coleoptera, references (R).

Init.	Sequences (5' – 3')	Pb	R
Cry3Aa F	ATGAATCCGAACAATCGAAGTGAACATGAT	641	44
Cry3Aa R	TTAATTCACTGGAATAAATTCAATTTTGTC		
Cry3Ba F	ACTTACGCAACAATACACTGAC	1300	16
Cry3Ba R	TAGGTTAGTGGTTGAAGCATAG		
Cry3Bb F	TACGCAACAATACACTGACC	690	16
Cry3Bb R	TCATCTGTTGTTTCTGGTGG		
Cry7 F	CTGAGGAGAATGGTTGGTATG	606	16
Cry7 R	TCGCTTGTGTTTCCGTTGTC		
Cry8 F	ATGAGTCCAAATAATCTAAATG	338	45
Cry8 R	TTTGATTAATGAGTTCTTCCACTCG		
Cry9 F	GGACCTTACAAACACGATTAC	412	16
Cry9 R	AATAAACTTCTTGACCAGC		
Cry35Aa F	CAAAGTGATAATGGAAAGG	496	16
Cry35Aa	TACCTCCTCCTACTTCTGG		
Cry35Ba	AACTGATGAAATACCTGAAG	585	16
Cry35Ba R	TCAACAATAAATCCTACAGC		
Cry3Ca F	TGACAAGAAAGGGAGGAAG	501	46
Cry3Ca R	TTCGTCCATCTCGTAAAGTC		

VIP1 F	TTATTAGATAAACAACAACAAGAATATCAATCTATTMGNTGGATHGG	585	11
VIP1 R	GATCTATATCTCTAGCTGCTTTTTCATAATCTSARTANGGRTC		
VIP2 F	GATAAAGAAAAAGCAAAAGAATGGGRNAARRA	845	46
VIP2R	CCACACCATCTATATACAGTAATATTTTCTGGDATNGG		
VIP1/VIP2 F	AAATTAGTGATCCGTTACCTTCTT	742	47
VIP1/VIP2 R	CAACTTGCTTTTCTTTCCCTTTAT		
Cry1i F	AACACTCAGTATATGAAT	378	48
Cry1i R	CACATGTGATGCTGAAAT		
Cry10 F	TCAATGCTCCATCCAATG	348	49
Cry10 R	CTTGTATAGGCCTTCCTCCG		

Table 3. Genes, denaturation, pairing, extension and number of cycles for gene amplification by PCR by primer used in the molecular analysis of the primers that encode Cry and VIP insecticidal proteins for the order Coleoptera.

Genes	Denaturation	Pairing	Extension	Nº of cycles
Cry3Aa	95ºC - 1 min	62ºC - 1 min	72ºC – 1 min	30 cycles
Cry3Ba	94ºC - 5 min	40ºC – 40 sec	72ºC – 1 min	35 cycles
Cry3Bb	94ºC - 5 min	40ºC – 40 sec	72ºC – 1 min	35 cycles
Cry7	94ºC - 5 min	40ºC – 40 sec	72ºC – 1 min	35 cycles
Cry8	95ºC - 2 min	49ºC - 1 min	72ºC – 1 min	30 cycles
Cry9	94ºC - 5 min	40ºC – 40 sec	72ºC – 1 min	35 cycles
Cry35Aa	94ºC - 5 min	40ºC – 40 sec	72ºC – 1 min	35 cycles
Cry35Ba	94ºC - 5 min	40ºC – 40 sec	72ºC – 1 min	35 cycles
Cry3Ca	94ºC - 2 min	50ºC - 1 min	72ºC – 1 min	30 cycles
VIP1	95ºC - 1 min	47ºC - 1 min	72ºC – 1.5 min	35 cycles
VIP2	95ºC - 1 min	47ºC - 1 min	72ºC – 1.5 min	35 cycles
VIP1/VIP2	94ºC - 1 min	48ºC - 1 min	72ºC – 1 min	30 cycles
Cry1i	94ºC - 1 min	50ºC - 1 min	72ºC – 1.5 min	35 cycles
Cry10	95ºC - 1 min	51ºC - 1 min	72ºC – 1 min	30 cycles

Table 4. Molecular characterization of species and isolates (*Bt.* sp. Isol.) of *Bacillus thuringiensis* tested against *Gonipterus platensis* (Coleoptera: Curculionidae) larvae using Cry3Aa (1), Cry3Ba (2), CryBb (3), Cry7 (4), Cry8 (5), Cry9 (6), Cry35Aa (7), Cry35Ba (8), Cry3Ca (9), VIP1 (10), VIP2 (11), VIP1 / VIP2 (12), Cry 1i (13) and Cry10 (14) pathogenic to Coleoptera.

Bt sp. Isol.	Genes													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
morrisoni	-	-	-	-	-	-	-	+	-	+	-	+	+	-
san diego	-	-	+	-	-	-	-	+	-	-	+	-	-	-
tenebrionis	+	+	+	-	-	-	-	+	-	+	-	+	-	-
tolworthi	-	-	-	+	+	-	-	+	-	-	+	-	+	-
israelensis	-	-	-	-	-	-	-	-	-	-	+	-	-	+
93	-	-	-	-	-	-	-	+	-	-	-	-	-	-
107	-	-	-	-	-	-	-	+	-	-	-	-	-	+
115	-	-	-	-	-	-	-	-	-	-	-	-	-	+
116	-	-	-	-	+	-	-	-	-	-	+	+	-	-
178	-	-	-	+	-	-	-	+	-	-	-	-	-	+
187	-	-	-	-	-	-	-	+	+	-	+	+	+	-
706	-	+	-	-	-	-	-	+	-	+	-	-	-	-
923	-	+	-	-	-	-	-	+	-	-	-	+	-	-
940	-	-	+	-	-	-	-	+	-	-	-	-	-	-
1100	-	-	-	-	-	-	-	+	-	+	+	+	+	-

Table 5. Identification code (Cod. LGBBA) and percentage (Mort.) and mortality time (Mort. Speed) of mortality (mean ± standard error) of neonate larvae of *Gonipterus platensis* (Coleoptera: Curculionidae) by *Bacillus thuringiensis* in standard concentration of $1x10^9$ after seven days. (Temperature: 25 ± 1ºC, RH: 50 ± 10% and photophase: 12 h).

Cod. LGBBA	Per. Mort. (%)	Mort. Speed(days)
107	100 ± 0.093a	2,92 ± 0,21a
178	100 ± 0.071b	2,36 ± 0,14ab

B. thuringiensis tenebrionis	63 ± 0.049c	1,13 ± 0,16bc
115	63 ± 0.029c	1,13 ± 0,12bc
B. thuringiensis israelensis	60 ± 0.015c	1,07 ± 0,1bc
116	43 ± 0.011cd	0,77 ± 0,36cd
93	23 ± 0.009de	0,42 ± 0,16df
706	13 ± 0.008de	0,24 ± 0,06f
B. thuringiensis morissoni	13 ± 0.008de	0,24 ± 0,06f
B. thuringiensis tolworthi	10 ± 0.007de	0,18 ± 0,01f
1100	7 ± 0.007e	0,12 ± 0,06f
923	7 ± 0.007e	0,12 ± 0,06f
B. thuringiensis san diego	7 ± 0.007e	0,12 ± 0,06f
187	3 ± 0.005e	0,06 ± 0,06f

Averages followed by the same letter, per column, do not differ by the Tukey-Kramer test at a 95% confidence level.

Table 6. Lethal concentration (CL) required to kill 50% of the neonate *Gonipterus platensis* (Coleoptera: Curculionidae) larvae (CL50) by the isolates 107 and 178 and *Bacillus thuringiensis* tenebrionis (Bttenebrionis) after seven days (Temperature: 25 ± 1 ºC, ER: 50 ± 10% and 12 h photophase)

CL	**178 (%)**	**107 (%)**	***Btt tenebrionis* (%)**
1.0×10^{10}	-	-	100 ± 0.000
1.0×10^{9}	100 ± 0.033	100 ± 0.000	63 ± 0.088
6.0×10^{8}	67 ± 0.089	37 ± 0.089	50 ± 0.057
4.5×10^{8}	27 ± 0.176	17 ± 0.176	20 ± 0.115
3.0×10^{8}	23 ± 0.033	13 ± 0.033	23 ± 0.033
1.5×10^{8}	13 ± 0.088	3 ± 0.088	10 ± 0.058
7.5×10^{7}	7 ± 0.067	0 ± 0.000	7 ± 0.033

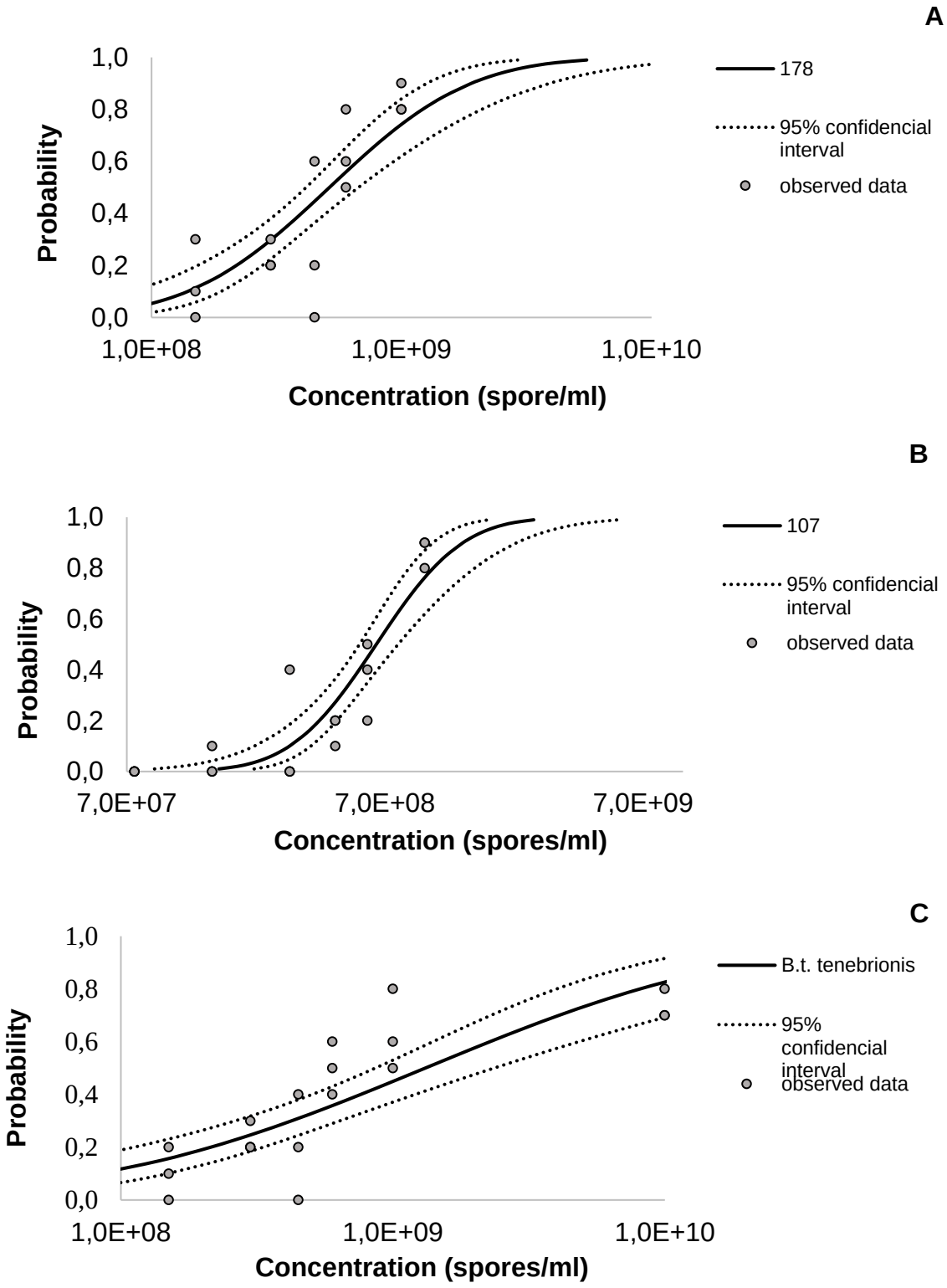
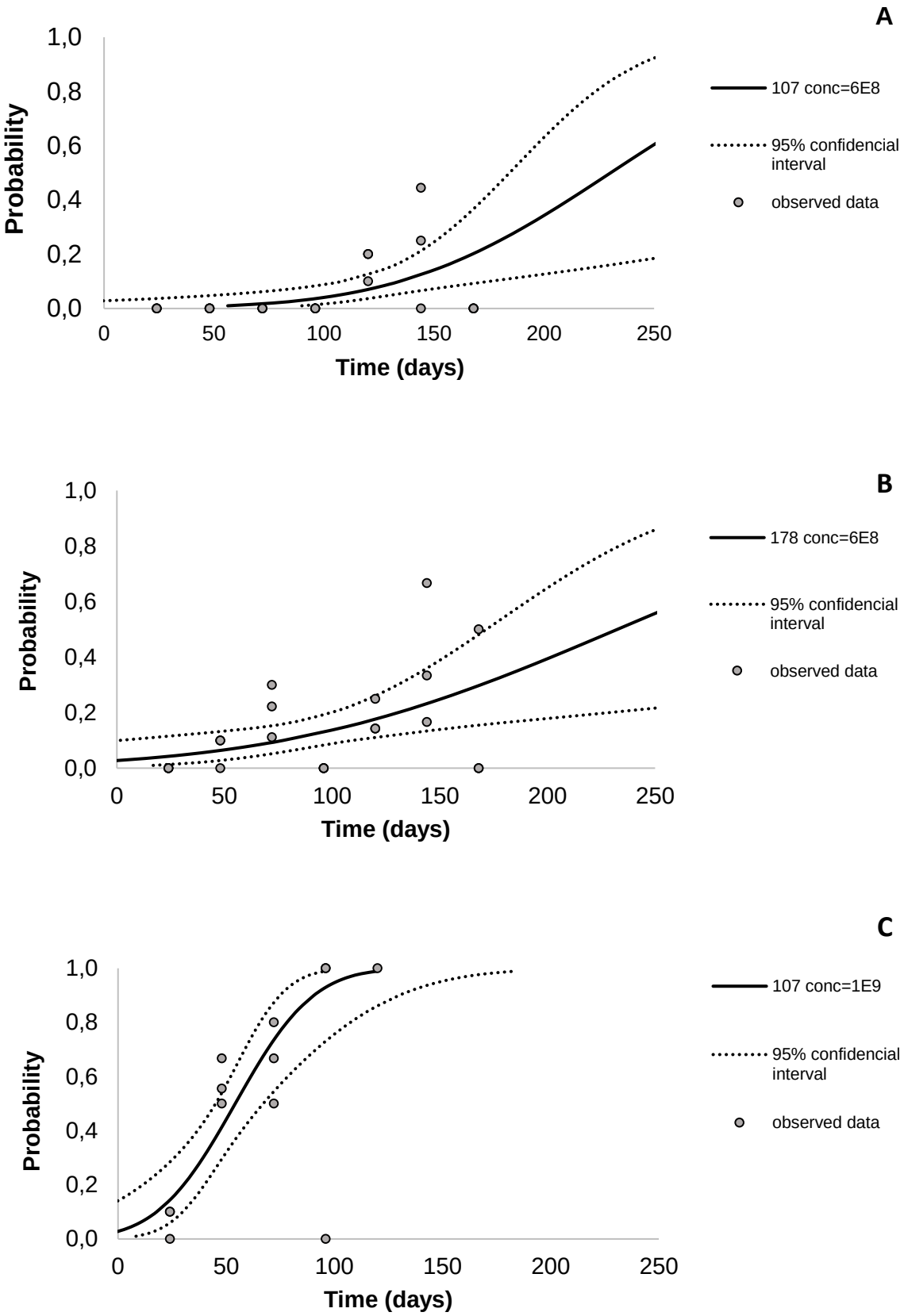


Figure 1. Lethal concentration 50% (LC_{50}) for isolates 178 (A) and 107 (B) and *Bacillus thuringiensis tenebrionis* (*Bt tenebrionis*) (C) for *Gonipterus platensis* (Coleoptera: Curculionidae) neonate larvae after seven days (Temperature: 25 ± 1ºC, RH: 50 ± 10% and 12 h photophase).


A
1,0
0,8
0,6
0,4
0,2
0,0
Probability
0
50
100
150
200
250
Time (days)
107 conc=6E8
95% confidencial interval
observed data
B
178 conc=6E8
95% confidencial interval
observed data
C
107 conc=1E9
95% confidencial interval
observed data

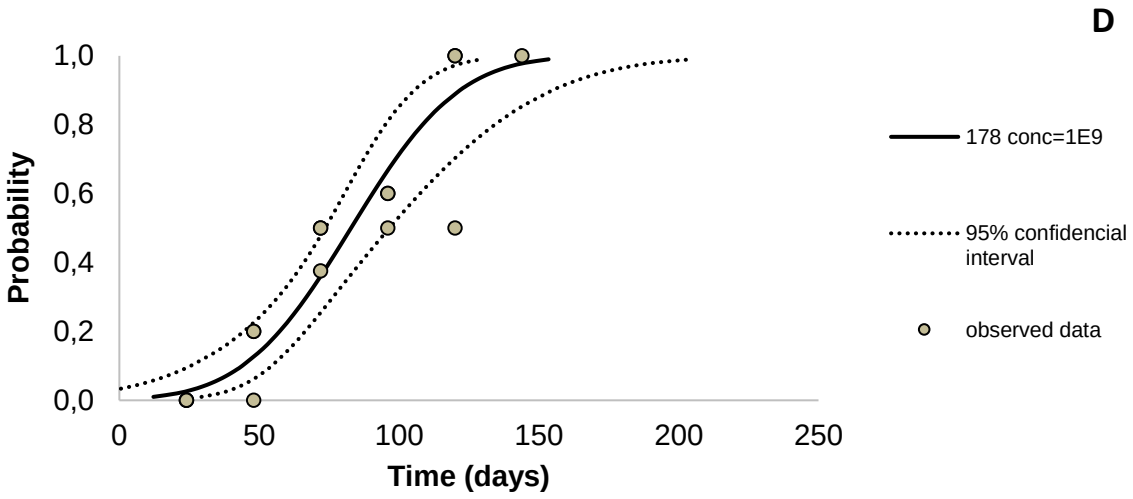


Figure 2: Median Lethal Time 50% (LT_{50}) of isolates 107 (A) and 178 (B), at a concentration of 6.0 x 10^8 and LT_{50} of 107 (C) and 178 (D), at 1.0 x 10^9 applied to *Gonipterus platensis* (Coleoptera: Curculionidae) neonate larvae after seven days (Temperature: 25 ± 1ºC, RH: 50 ± 10% and 12 h photophase).

CHAPTER 4

Entomopathogenic fungi as the microbial frontline against the alien *Eucalyptus* pest *Gonipterus platensis* in Brazil

Journal: Scientific Reports

4.1 Abstract

The eucalyptus snout beetle (ESB), *Gonipterus platensis*, is endemic to Australia but has become a major invasive, destructive pest of Brazilian eucalyptus plantations. Efforts to develop insecticides based on entomopathogenic fungi against ESB are limited by the lack of known virulent strains. We therefore explored the virulence of indigenous Brazilian strains of major entomopathogenic fungi–*Beauveria* spp. and *Metarhizium anisopliae*–against ESB adults. We found widely varying virulence and later capacities for conidial production on infected adult cadavers. Two strains stood out, *B. bassiana* IBCB-240 and *M. anisopliae* IBCB-364, as especially lethal for ESB adults under laboratory conditions, sporulated abundantly on infected insects, and also outperformed comparable strains used in commercial mycoinsecticides. Notably, *B. bassiana* IBCB-240 exhibited lower LT_{50} and LC_{50} values than *M. anisopliae* IBCB-364 at low inoculum levels ($\leq 10^7$ conidia mL^{-1}). Taken together, this study emphasizes natural variation in virulence among indigenous *Beauveria* and *Metarhizium* strains against ESB adults and identifies fungal strains with higher virulence than existing commercialized strains for managing this eucalyptus pest in Brazil.

Key words: *Eucalyptus* snout beetle, invasive pest, biological control, strain selection, virulence.

4.2 Introduction

In Brazil, *Eucalyptus* plantations provide wood production to many kinds of industries (pulp and paper, siderurgy, fiberboard panels, biomass, etc.), and are cultivated on 5.7 million hectares (ha) with an average productivity of 36 m^3 ha^{-1} annually[1]. The *Eucalyptus* snout beetle (ESB), *Gonipterus platensis* Marelli (Coleoptera: Curculionidae), is an Australian defoliating pest present in Brazil since 1979 that, in 2003, caused damage to 50,000 ha of *E. grandis* x *E. urophylla*

plantations[2,3]. Infestations of this pest can cause development of epicormic shoots, severe defoliation of the upper third of the tree crown and death of branches[4]. In Portugal, losses for wood productivity of *E. globulus* can reach 86%[5], and the economic losses due to ESB in the last twenty years were estimated to be 648 million euros[6].

Currently in Brazil, the forest plantations certified by such organizations as the Forest Stewardship Council (FSC) and National Forest Certification Program (Cerflor) occupy 3.5 million hectares[7]. These organizations recommend pesticide policies to reduce or eliminate uses of chemical pesticides and comply with local pesticide regulations. To date, no chemical insecticides have been registered in Brazil against this pest[8,9]. Therefore, biological control is the key management strategy adopted for *G. platensis* control, using natural enemies such as the egg parasitoid *Anaphes nitens* (Hymenoptera: Mymaridae)[10], the predator *Podisus nigrispinus* (Hemiptera: Pentatomidae)[11] and entomopathogenic nematodes[12]. In addition to these diverse biocontrol agents, entomopathogenic fungi are the largest commercially utilized microbial biocontrol agents in Brazil, but few effective fungal strains have been studied for use against ESB[9]. Based on their safety for humans and other non-target organisms[13], and their low risk to elicit insect resistance[14], entomopathogenic fungi are uniquely able to invade the host insects directly through the integument and thus can be used as an additional component in the integrated management toolbox against ESB in eucalyptus plantations. Indeed, these mycoinsecticides are environmentally friendly alternatives or even complementary insecticides where millions of hectares in Brazil are treated annually with *B. bassiana* and *M. anisoliae* to control forest and agricultural arthropod pests[9].

Commercial preparations of *B. bassiana* and *M. anisopliae* were first used to control *Gonipterus* sp. n. 2 in South Africa under laboratory conditions, with *Beauveria* showing higher efficiency by contact and ingestion[15]; *B. bassiana* was later reported to induce epizootics in *G. platensis* in Itararé, SP, Brazil[16]. This fungus is compatible with *A. nitens* releases due to its low infectivity for this parasitoid[17]. Field applications of mycoinsecticides require a set of environmental conditions suitable for inoculation and colonization in the host, so during the dry season the control efficiency drops to less than 20%[18]. To date, in Brazil, there are only two commercial mycoinsecticides based on *B. bassiana* officially available for ESB control: Boveril (ESALQ-PL63 strain) and

Ballvéria (IBCB-66 strain)[8]. However, these commercial fungal strains may not be suitable or virulent enough to control ESB effectively and are not so used.

This research screened indigenous Brazilian strains of *M. anisopliae* and *Beauveria* spp. for improved activity against ESB adults under laboratory conditions as a first and crucial step in developing more effective mycoinsecticides, and we compared the most promising strains to those in the commercial fungal strains registered for ESB control in Brazil. This is the first comprehensive fungal screening for the biological control potential against this invasive pest, and raises possibilities for much enhanced control of ESB in Brazilian eucalyptus plantations.

4.3. Results

4.3.1 Fungal strain selection against *G. platensis*

The screening revealed wide variation of pathogenicity and virulence among fungal strains and demonstrated a remarkable phenotypic plasticity of *G. platensis* (ESB) adults regarding fungal infection (χ^2 = 126.04, df = 25, P < 0.0001). The average mortality rates due to *B. bassiana* strains and another unidentified *Beauveria* strain ranged between 5% and 85%, while *M. anisopliae* strains inflicted mortalities in the range of 27.5–95% (Fig. 2A). According to the overall mortality analysis, the most pathogenic strains were *B. bassiana* IBCB-240 and *M. anisopliae* IBCB-364, inducing 85% and 95% deaths in ESB adults at 20 days post-spraying, respectively. Boveril and Metarril killed 60% and 52.1% of ESB adults, while their unformulated strains, ESALQ-PL63 and ESALQ-E9, promoted mortality rates of 65.9% and 32.5%, respectively. In this sense, *M. anisopliae* IBCB-364 outperformed Metarril and its unformulated strain, ESALQ-E9, whereas *B. bassiana* IBCB-240 did not statistically differ from Boveril or its strain ESALQ-PL63 (Fig. 2A). The lowest mortality rate, as low as 5%, was achieved with *B. bassiana* IBCB-66, the active ingredient of the commercial product Ballvéria.

The proportion of infected, sporulating cadavers varied but was significant only for treatments with 10^8 conidia mL^{-1} (χ^2 = 115.92, df = 24, P < 0.0001). The mycosis rate induced by *B. bassiana* and *Beauveria* sp. ranged from 2.5% to 85%, while *M. anisopliae* killed from 22.5% to 95% (Fig. 2B). The greatest numbers of sporulating cadavers were observed with *M. anisopliae* IBCB-364 (95% sporulated insects) and IBCB-364, *B. bassiana* IBCB-240 caused 85% mycosis. The sporulation rate was lowest with *B. bassiana* IBCB-66 (2.5%) and *M. anisopliae* IBCB-196 (22.5%), whereas the remaining strains exhibited moderate levels of mycosis. Control mortality averaged

12.5±8.4% and showed no evidence of fungal infection. A high percent mortality did not always coincide with a high *post-mortem* sporulation rate. In this sense, while 75% of weevils were killed by *B. bassiana* ESALQ-PL63, only 30% of those cadavers showed external sporulation, thus indicating that the fungus might not complete its life cycle in the host.

All strains of *Beauveria* spp. and *M. anisopliae* killed ESB adults, although the rate at which this happened differed significantly (Supplemental Fig. S1). The survival analysis indicted the susceptibility of ESB adults to different fungal strains

tested at a single concentration used as a discriminatory baseline ($\chi^2 = 250.59$, df = 25, $P < 0.0001$). All pairwise comparisons between ESB survival curves after exposure to fungal strains are described in Supplemental Table S1. Cox regression analysis based on hazard ratios (HR) comparing controls (untreated ESB) with all test strains revealed that most *M. anisopliae* and *Beauveria* strains significantly increased the risk of insects dying ($\chi^2 = 246.4$, df = 25, $P < 0.0001$) (Supplemental Fig. S2). Accordingly, weevils exposed to highly virulent strains exhibited mortality risks of 55.6 and 38.12 times greater than insects from control group when exposed to *M. anisopliae* IBCB-364 and *B. bassiana* IBCB-240. Conversely, the only exception noted for *B. bassiana* IBCB-66 that portrayed a similar hazard ratio and survival pattern to control (Supplemental Table S1 and Fig. S1-S2; log-rank $P = 0.988$). *B. bassiana* IBCB-6 and *M. anisopliae* IBCB-196 mortality rates were less than 50%, thereby treated as low virulence candidates (Supplemental Fig. S1, S2). These two strains significantly reduced ESB lifespan resulting in LT_{50}'s of 12.8 and 10.9 days and LT_{90}'s of 21.4 and 18.1 days post-treatment, respectively, with a concentration of 1×10^8 conidia mL^{-1} (Fig. 3A). In agreement with mortality and mycosis best results, insects exposed to both *B. bassiana* IBCB-240 and *M. anisopliae* IBCB-364 showed similarly sharp reductions in survival rates among all fungi tested (log-rank $P = 0.214$, Supplemental Table S1, Fig. S1). The remaining strains had low or moderate virulence profiles (Supplemental Fig. S1, S2). Accordingly, ESB adults from control groups and those exposed to the less virulent strains also survived longer with estimated LT_{50} and LT_{90} values exceeding 25 and 40 days, respectively (Fig. 3A).

Hazard ratios (HR) were estimated relative to the mortality pattern of the reference strain (ESALQ-PL63) using its commercial formulation Boveril. Using the classification proposed by Valero-Jiménez et al.[19], 17 strains were classified as lethal,

four strains fell into the HL category, and three were classified as SL strains (Fig. 3B). Virulence varied widely: strains *M. anisopliae* IBCB-364 and *B. bassiana* IBCB-240 were on average 3.8 and 2.6 times, respectively, more virulent, while *B. bassiana* IBCB-66 was on average 14 times (i.e., 1/0.069) less virulent than the reference Boveril.

4.3.2 Conidial production by sporulated cadavers

Conidial production on infected cadavers varied strongly among fungal strains (χ^2 = 308.40, df = 23, P < 0.0001), with *B. bassiana* and *Beauveria* sp. strains producing the most conidia per cadaver. The lowest post-mortem conidial production was by *M. anisopliae* ESALQ-E9 (2.5×10^5 conidia weevil^{-1}), whilst the highest yields were by *B. bassiana* strains IBCB-18, IBCB-240, IBCB-246, IBCB-329, IBCB-6, IBCB-35, IBCB-620, IBCB-87 and *Beauveria* sp. IBCB-634, which all produced more than 10^8 conidia per cadaver (Fig. 4). For *M. anisopliae* strains, IBCB-348 and IBCB-364 gave the highest conidial production on cadavers with a range of $5.8–9.3 \times 10^7$ conidia per adult. Moreover, *B. bassiana* IBCB-66 had only one individual that sporulated (2.8×10^7 conidia adult^{-1}) post-mortem but also showed poor virulence, and was not considered further in this analysis. Conidial production on cadavers by *B. bassiana* IBCB-240 and *M. anisopliae* IBCB-364, the two most virulent strains, did not differ statistically. Sporulated (mycosed) insects infected by either *M. anisopliae* or *B. bassiana* showed especially typical fungal outgrowth through the intersegmental membranes followed by a profuse conidiogenesis (Supplemental Fig. S3).

Strains depicting the highest virulence do not necessarily yield higher conidial production on infected cadavers. The higher overall mortality rates of the most virulent strains, IBCB-364 and IBCB-240, also showed higher mycelial growth/sporulation (mycosis) rates (Correlation coefficient r = 0.89, P < 0.0001) and lower LT_{50} and LT_{90} values (r = -0.98, P < 0.0001), yet no significant relationship was found with inoculum production in infected beetles (r = 0.36, P = 0.19), according to the Spearman's correlation test (Supplemental Fig. S4).

4.3.3 Virulence of the two strains most lethal to *G. platensis*

Virulence of the most virulent strains of *B. bassiana* and *M. anisopliae,* showed a strong concentration-time-effect against adult *G. platensis*, in which mortality rate

increased with concentration and time (Supplemental Fig. S5). The values for LC_{50} at 15 days post-infection and LT_{50}/LT_{90} obtained for different spore concentrations were determined. When examining the concentration-mortality data on 15 days post-spraying, *B. bassiana* IBCB-240 was more virulent than *M. anisopliae* IBCB-364, especially at the lower applied concentrations, as also supported by the significant difference between their concentration-mortality curves (χ^2 = 12.17, df = 2, P = 0.0023) (Fig. 5A). In addition, the slopes of the mortality curves of *B. bassiana* IBCB-240 significantly and *M. anisopliae* IBCB-364 differed significantly (t = 1.997, P = 0.046), suggesting that *G. platensis* adults displayed different susceptibilities to both pathogens (Table 2). In agreement with these results, *B. bassiana* IBCB-240 required a 3-fold reduction in conidial concentration to kill 50% of weevils in relation to *M. anisopliae* IBCB-364, indicating that the former is more virulent at lower inoculum level (t = 2.26, P = 0.026) (Table 2). With respect to mycosis (sporulation) rates, infected adults showed 100% confirmed mortality with either strain.

According to the survival analysis, the lifespan of *G. platensis* decreased as conidial concentrations increased (χ^2 = 72.73, df = 1, P < 0.0001) and was strongly reduced in the presence of these strains (χ^2= 19.34, df = 2, P < 0.0001). After 20 days of incubation, the control weevils exhibited a mortality rate of 12.5±6.5% without any signs of fungal mycelial growth sporulation. The Kaplan-Meier survival analysis revealed virulence differences among strains of *B. bassiana* and *M. anisopliae* (Fig. 5B). In addition, LT_{50}/LT_{90} values considerably decreased with increasing conidial concentrations (Fig. 5C). ESB adults exposed to lower concentrations of *M. anisopliae* IBCB-364 (5×10^6 and 10^7 conidia mL^{-1}) had survivorships similar to untreated control insects (log-rank P = 0.50 and P = 0.08, respectively, Supplemental Table S2), indicating that this strain was not virulent for this weevil (Fig. 5B). This finding is further shown by longer lethal times for *M. anisopliae* and the control group with no differences in their LT_{50}/LT_{90} values (Fig. 5C). On the other hand, at these same low dosages, survival curves differed among fungal strains, indicating *G. platensis* lifespan after exposure to *B. bassiana* IBCB-240 decreased faster than with *M. anisopliae* IBCB-364 (log-rank P = 0.016 and P = 0.033, respectively) (Fig. 5B, Table S2). This also means that LT_{50}/LT_{90} values differed significantly between both strains, with lower values attained by *B. bassiana* IBCB-240 (Fig. 5C). Nonetheless, similar adult survival curves were achieved with higher concentrations, 5×10^7 to 5×10^8 conidia mL^{-1}, for both

fungi (log-rank $P = 0.41$, $P = 0.63$ and $P = 0.40$, respectively) (Fig. 5B, Supplemental Table S2), in which LT_{50} values fell to 8.2–29.9 days while LT_{90} values were 21 to 36.6 days (Fig. 5C).

4.4 Discussion

Eleven of 15 *Beauveria* strains and one of eight *M. anisopliae* strains tested here (including IBCB-240 and IBCB-364) originated from curculionid host species, suggesting a pre-adaptation of these fungi to *G. platensis*. Overall, 21 of 23 tested fungal strains significantly reduced weevil survival starting at day 5, with *B. bassiana* IBCB-240 and *M. anisopliae* IBCB-364 having the lowest LT_{50}/LT_{90} values, between 11 and 21 days. Among all these strains, only four of them fell in the highly lethal category when compared to the reference Boveril, but our primary focus emphasized the two top strains that outperformed the others in all evaluated parameters, including *in vivo* conidial production. Other fitness parameters that may be affected by sub-lethal doses were not examined in the current study[20,21].

The cuticle is the primary physical and chemical barrier that fungal entomopathogens must overcome to infect the host[22]. Because *B. bassiana* IBCB-66 performance did not differ significantly from the untreated control group, we assumed this fungus either encountered difficulties penetrating the weevil or that infections were blocked by the host's immune system. Some antimicrobial cuticular secretions and the cuticular thickness of *G. platensis* might confer some natural resistance to penetration and/or infection by some strains of generalist fungal entomopathogens, as indicated here by the low virulence of *B. bassiana* IBCB-66, which may require higher fungal exposures to infect and to kill weevils. This observation agrees with the study of Ramirez et al.[23] that some *Cordyceps* (formerly *Isaria*) spp. strains with minimal pathogenicity to mosquitoes could not complete cuticular penetration or infection of these hosts. Such cuticular resistance is strongly related to detrimental effects to conidia of *B. bassiana* and *M. anisopliae* by cuticular lipids and aldehydes[24,25]. From a broader perspective, our results about the widely-varying virulence for ESB by a diversity of fungal entomopathogens may reflect a co-evolutionary host/pathogen 'arms race' and provide a useful model system to study the multifactorial complexities of interactions by between a fungus and its host on and in the cuticle, and, if penetration succeeds, between the pathogen and the host's immune system by allowing or blocking a lethal infection. The low virulence and low mortality rates of some *B.*

bassiana and *M. anisopliae* strains against *G. platensis* suggest that ESB may be able to resist many generalist fungal pathogens.

However, due to host phenotypic plasticity and natural genetic variation among strains of a single fungal species[19,26], host origin cannot guarantee strain virulence; *Beauveria* and *Metarhizium* possess famously broad host spectra and innumerable ecological adaptations[27]. This was especially true for the least pathogenic strains, including *B. bassiana* IBCB-66, IBCB-6 and IBCB-31, which all came from weevil hosts but produced slow infection and poor mortality rates in *G. platensis* (Fig. 2, 3).

The wide genetic plasticity and broad lifestyle options of these two fungi coupled with their large genomes (size ~ 33-39 Mb) provide diversity among their strains as well as highly variable degrees of virulence to different hosts, which represents a valuable resource of biocontrol candidates for use in pest control and comprising the majority of registered commercial mycopesticides worldwide[27,28]. One plausible explanation for the results obtained with the time-dose-mortality response bioassays between *M. anisopliae* and *B. bassiana* can be drawn from comparing their genomes for species-specific virulence genes and gene family expansions and contractions that might correlate with host ranges and complex multifaceted pathogenic strategies. It has been shown that *B. bassiana* contains many more gene products similar to or derived from bacterial toxins than most other fungi that may give some ecological advantages over other entomopathogenic fungal species to access a wide variety of hosts[29]. Accordingly, highly expressed virulence genes unique to *B. bassiana* strain (e.g., bacterial-like toxins) might provide some comparative advantage over *M. anisopliae* against *G. platensis,* even at low inoculum dosages.

Post-mortem production of conidia has not been investigated previously in *G. platensis* and plays an important role for fungal secondary infection through horizontal transmission, recycling in the host's environment, and disease establishment in host population. In our study, we demonstrated that strains of *Beauveria* spp. and *M. anisopliae* differed greatly in their capability to sporulate on host cadavers, and that this emergent sporulation from cadavers confirmed the successful infection and completion of the fungal life cycle in this host, with the majority of fungal strains being able to produce conidia on cadavers. Notably, the highest sporulation rates occurred in the highly lethal (HL) group of strains (Fig. 2). Exceptionally, only one individual infected by the low-virulence strain *B. bassiana* IBCB-66 exhibited profuse sporulation,

which seems that even after significant lethal infections the fungus was unable to complete the sporulation phase in the host for unknown reasons. Despite the remarkably variable capacity for *in vivo* conidial production among fungal strains, remarkably more conidia were produced on cadavers infected by *Beauveria* spp. than by *M. anisopliae*. On most infected *G. platensis* cadavers, subsequent fungal growth and sporulation was limited to intersegmental membranes, mainly those involving the mouthparts, antennae, compound eyes, legs, and under the elytra (Supplemental Fig. S3). It has been postulated that a fungus produces large numbers of infective propagules could enhance fungal population density in host environments and the likelihood of further dispersal in the host population[30]. Comparing both highly lethal strains in this study, *B. bassiana* IBCB-240 strain produced 4 times more conidia per cadaver than *M. anisopliae* IBCB-364; this, in turn, may indicate that this *B. bassiana* strain is better adapted to weevil's natural habitat and, because of its copious sporulation on cadavers can provide enough propagules for effective microbial control and dispersal to enable secondary infections in the host population. In agreement with our results, it was observed that *M. anisopliae* strains induced lower total sporulation on termite cadavers than *B. bassiana* strains, but the former exhibited abundant rapid sporulation, which emphasizes distinct sporulation patterns between these fungal species and also underscores their distinct ecological adaptations[31].

Our results revealed the highly virulent strains to be up to 3.8 times more effective–and to produce higher *in vivo* sporulation than the strain used in the commercial Boveril bioinsecticide registered for ESB control in Brazil (Fig. 2, 3, 4). The dose-dependent effects of spore concentration have already been established[32]. It is especially important that the highly lethal strain *B. bassiana* IBCB-240 yielded a stronger dose-effect and more rapid reduction of survival rates for *G. platensis* than *M. anisopliae* IBCB-364 at low concentrations ($\leq 10^7$ conidia mL^{-1}) (Fig. 5, Table 2). In this sense, reducing the required effective dosage would lessen the costs of production and application while also shortening exposures to non-target hosts.

Beyond the virulence traits addressed in the present study, additional efforts should be placed on determining the most tolerant fungal strains to heat, dehydration (osmotic stress), UV-tolerance, mass production of spores, as well as bioefficacy under field applications before developing a commercial mycoinsecticide against this insect pest. Taken all together, the present study paves the way for the development of an effective mycoinsecticide against *G. platensis* in Brazil that has minimal negative

impact on the environment and beneficial insects[33]. After examining a significant range of fungal strains, the highly virulent strains chosen here, *B. bassiana* (IBCB-240) and *M. anisopliae* (IBCB-364), appear to be excellent candidates for the development of new mycoinsecticides for use in a commercial biological control program specifically designed to manage this invasive pest in Brazilian eucalyptus plantations. Prospectively, additional research addressing the compatibility of these two virulent strains with the egg-parasitoid wasp *A. nitens* deserves special attention to establish a truly integrated strategy to deploy both biocontrol agents for ESB control in eucalyptus plantations.

4.5 Conclusion

In summary, these results constitute a major step in developing environmentally safe biocontrol entomopathogenic fungi to manage this invasive weevil pest. In addition, the two most lethal strains selected here could be used together with a novel *Gonipterus*-associated microsporidium pathogen recently identified by our group that naturally infects ESB populations in Brazil, or safely applied after the release of egg-parasitoid wasp (*A. nitens*) in field populations; both strategies could potentially contribute to mitigate the burden of this pest as well as to minimize any likelihood of host resistance to a high-virulence strain. Further, the widely variable natural virulence demonstrated among strains of *M. anisopliae* and *B. bassiana* offers the opportunity to better understand the molecular and genetic mechanisms underlying this heterogeneity and to support the potential development of improved biocontrol strains with desired properties.

4.6 Methods

4.6.1 Source and rearing of *Gonipterus platensis*

Insects were maintained at the Laboratory for Biological Control of Forest Pests (LCBPF), School of Agricultural Sciences, (FCA), São Paulo State University (UNESP), Campus of Botucatu, under controlled conditions (25 ± 1ºC, RH: 50 ± 10% and 12 h photoperiod). The colony was begun with *G. platensis* adults collected in outbreaks in São Paulo and Paraná states. The species identification was confirmed by taxonomic characters[34].

These adults were fed on fresh shoots of *Eucalyptus urophylla*, obtained from the same site where this study took place (UNESP, Botucatu, SP, Brazil), with their stems immersed in plastic containers containing water, changed once a week. Insect colonies were kept in rearing cages (40 cm long × 45 cm wide × 80 cm high), with glass roof and sides of voile fabric.

4.6.2 Source, culture, viability and deposition rate of fungi

We assayed 13 strains of *B. bassiana,* one strain of *Beauveria* sp., seven strains of *M. anisopliae*, and the active strains from two commercial products, Boveril WP (Koppert Ltda., Piracicaba, SP, Brazil) based on *B. bassiana* ESALQ-PL63 (used here as reference strain), which is registered to control *Gonipterus* spp.; and also the commercial product Metarril WP (Koppert Ltda., Piracicaba, SP, Brazil) based on *M. anisopliae* strain ESALQ-E9. All other fungal strains were originally obtained from the Collection of Entomopathogenic Microorganisms “Odemar Cardim Abreu” at the Experimental Center of the Instituto Biológico, Campinas, SP, or otherwise re-isolated and purified from commercial fungal formulations (Table 1) were preserved at –20 °C in 10% glycerol. Additional seed-cultures to serve as inoculum for bioassays were kept in test tubes containing Potato Dextrose Agar (PDA, 42 g L^{-1} [Sigma-Aldrich, St. Louis, MO, USA]) and stored at 6 °C. Such seed cultures of each strain were grown in Petri dishes (90 × 15 mm) containing PDA (15 mL per plate) in a climatic growth chamber at 25 ± 1 °C with 12 h photoperiod for 14 days until complete development and sporulation (conidiogenesis).

The collection, maintenance, and use in bioassays of these fungal isolates and the studied insect specimen were conducted in accordance with the Brazilian National System of Management of Genetic Heritage and Associated Traditional Knowledge – referred to as “SisGen” is approved under the protocol # AA51E70.

The viability of all test fungi was determined by spreading a 100-µL aliquot of a conidial suspension (10^6 conidia mL^{-1}), prepared with sterile surfactant solution (0.1% v/v) of Tween 80, on PDA medium (PDA 42 g L^{-1}) in Petri dishes (90 × 15 mm) and incubated in the dark at 25 ± 1 °C. Plates of *Beauveria* and *Metarhizium* were incubated for 16 and 18 h, respectively, prior to evaluation. Conidia were scored as viable if any germ tube was 2x longer than the diameter of the spore; a total of 100 conidia per sample were scored under 400× magnification in a phase-contrast microscope (Leica

Microsystems, DM 500, Heerbrugg, Switzerland)[35]. Fungal strains presenting ≥ 90% viability were used in the insect bioassays.

The inoculum deposition rate was assessed by a portable microspray tower. The concentration of 10^8 conidia mL^{-1} tested in the screening study was prepared in sterile Tween-80 solution (0.1%). The aqueous suspension was then sprayed onto 7 glass coverslips (20 × 20 mm) evenly placed on a plastic Petri dish (90 × 15 mm). The volume of spore suspension was chosen in accordance with the field application rate of 200 L ha^{-1} and sprayed on a 63.6 cm^2 Petri dish area. Then an aliquot of 127 µL of conidial suspension of each fungal strain was loaded in the reservoir of a professional dual-gravity airbrush DB134K (Fenghua Bida Machinery Manufacture Co., China), and applied to the coverslips from about 25 cm away from its nozzle and using 10 PSI working pressure (adapted from Mascarin et al.[36]) (Fig. 1A). The experiment was carried out in duplicate on different occasions, with three repetitions per treatment. After spraying, all 7 coverslips were transferred to 15-mL centrifuge tubes containing 5 mL of 0.1% Tween-80. This suspension was vortexed vigorously for 2 min to forcefully dislodge conidia from coverslips. Each suspension was quantified with a hemocytometer (Neubauer chamber, HGB, Precicolor, Germany) at 400× magnification.

4.6.3 Fungal screening bioassay (single concentration)

We assayed 14 strains of *B. bassiana,* one strain of *Beauveria* sp. and eight strains of *M. anisopliae* towards *G. platensis* adults. Conidia were produced in plastic Petri dishes containing PDA (42 g L^{-1}) at 25 ± 1 °C in total darkness. Bioassays were done with conidia harvested from 14-d-old cultures on PDA by superficial scraping with a Drigalski spatula; germination (viability) was assessed as noted above to be ≥ 90% prior to each bioassay.

Conidial suspensions were prepared with sterile Tween-80 solution (0.1% v/v) at 10^8 conidia mL^{-1}. The control group consisted of ESB adults sprayed with a sterile solution of Tween-80 (0.1%). In parallel, commercially-produced fungal strains as pure cultures and as their formulated products were used as standard treatments.

For each fungal strain, a conidial suspension of 127 µL containing 10^8 conidia mL^{-1} (equivalent to 143.0 – 287.5 conidia mm^{-2} within 18 – 21 seconds) was sprayed onto insects in four independent repetitions using the portable micro-sprayer, inside a

biological safety cabinet. Each biological repetition consisted of 5 ESB adults in a petri dish (90 × 15mm) lined with a sterile filter paper at the bottom. Each replicate was independently sprayed with each fungal suspension, and all fungal strains were assayed at the same time in each experiment.

After spraying fungal treatments, the insects remained for 24 h in Petri dishes in a growth chamber at 25 ± 1 °C, 83.0 ± 2.0% RH with 12 h photophase to ensure the residual contact of the fungus with insects. This methodology afforded two routes of contamination of this insect by fungi: *i)* direct contamination by topical spore deposition on the cuticle, and *ii)* indirect contamination through tarsal contact on a surface treated with the fungal conidia (Fig. 1B).

At 24 h after spraying, adults of *G. platensis* were transferred to plastic pots and fed with leaves of *E. urophylla* (clone 433) whose petioles were placed in hydrated phenolic foam to maintain leaf turgor. Leaves were replaced as needed. The incubation conditions were the same as described above. Adults were monitored on a daily basis during 20 days for assessing dead and living individuals. Dead insects were removed and superficially disinfected with sodium hypochlorite solution (1% v/v) for 30 s and subsequently rinsed twice in sterile distilled water for 30 s. Afterwards, dead insects were dried at room temperature and then individually transferred to wells of moist chambers made with 24-well culture plates. These cadavers were incubated at the same conditions above for more 15 days and assessed for fungus sporulation (i.e., emerging hyphae and spores) to verify mortalities caused by the applied fungus.

The experiment consisted of 26 treatments all 23 fungal strains, with 1 control, and two commercial formulated products as standards. Each treatment consisted of 4 independent repetitions, with 5 adults each, totalling 20 insects (sex ratio 1:1) per treatment, and the entire experiment was repeated twice on different occasions (blocks) using new fungal batches and insect cohorts to ensure data reproducibility. Virulence of fungal strains was evaluated according to the percentage of overall mortality as well as the median and 90% lethal times (LT_{50} and LT_{90}) for *G. platensis* obtained from survival analysis after exposure to each fungal strain.

4.6.4 Quantification of conidial production *in vivo*

After incubating dead individuals in humid chamber for 14 days, five adults covered with conidia (sporulated cadavers) were randomly removed from each sample of fungal treatment, surface sterilized with sodium hypochlorite (1% v/v) for 30 s, rinsed

twice in sterile distilled water for 30 s, and then cadavers were placed in a humid chamber maintained at 26 ºC with 14 h photophase for 14 days. Cadavers bearing conspicuous masses of aerial conidia were placed individually in 1.5-mL microcentrifuge (Eppendorf) tubes filled with 1 mL of sterile solution of 0.1% Tween-80 and vigorously vortexed for 1 min to dislodge conidia from insect's body. Subsequently, these same tubes were vortexed vigorously for 1 min and left in an ultrasonic bath for 2 min to enhance the suspension of conidia. Conidial density was calculated by haemocytometer counts at 40× magnification to determine the average of conidial production per insect. Two repeated assays were used for each fungal strain, totalling 10 cadavers per treatment. Altogether, the conidial production was measured in 230 cadavers (23 treatments x 2 experimental replicates x 5 cadavers).

4.6.5 Concentration-response assays with best strain

M. anisopliae IBCB-364 and *B. bassiana* IBCB-240 were previously selected for further evaluations of their virulence based on in terms of LC_{50} and LT_{50} and LT_{90} obtained from concentration-time-mortality bioassays using multiple conidial concentrations. Fungal suspensions containing conidia retrieved from PDA cultures were prepared and applied as described above. Experimental units consisted of 5 adults per plastic pot (repetition), with 4 repetitions per treatment (i.e., n = 20 adults with sex ratio 1:1). The strains were tested at concentrations of 5×10^6, 10^7, 5×10^7, 10^8, and 5×10^8 conidia mL^{-1}. After spraying fungal treatments likewise mentioned, beetles were kept in controlled conditions (25 ± 1 ºC, 83.0 ± 2.0% RH and 12 h photophase). Each repetition was monitored daily for up to 20 days; dead insects were recorded, removed and transferred to moist chambers to induce outgrowth and sporulation as a means to confirm mortality. Viability tests were performed on PDA as described previously, with both strains attaining viability > 90%. The experiment included a control group (as described above). Each treatment and 4 repetitions involved 20 insects. All fungal concentrations were assayed at once in each experiment, and repeated twice at different times (blocks) using new fungal batches and insect cohorts. ESB adults were fed with fresh leaves of *E. urophylla* (clone 433) and replaced as needed. The virulence of these two strains were compared according to their concentration-mortality curves and respective estimated lethal concentrations

(LC_{50}), and their estimated survival curves and respective estimated lethal times (LT_{50} and LT_{90}) for each fungal concentration.

4.6.6 Data analysis

All experimental designs followed a completely randomized arrangement, and experiments were always repeated at least twice on different dates to ensure data reproducibility. Cumulative mortality data for *G. platensis* for up to 20 days after application with fungal entomopathogens, as well as percent confirmed mortality data were separately fitted to a generalized linear model (GLM) with binomial distribution for errors and logit link function, in which fixed effects were attributed to fungal strains and experimental blocks in the linear predictor. The only exception is that values for mycosis in control group (no fungus) was "zero" for all replicates, and was dropped from the analysis. A total of 8 biological replications from two repeated experiments (blocks) were performed for each treatment (fungal strain). Once the treatment effect was significant, multiple pairwise comparisons of means were carried out using Tukey HSD method at 5% significance ($P < 0.05$) to separate fungal strains, using the package "emmeans"[37]. Regarding the mortalities recorded over time as well as for each conidial concentration tested in both screening and virulence bioassays, censored survival probability data on *G. platensis* adults were fitted to parametric Weibull and log-normal models, respectively, using the *survreg* function that enabled the estimation of median (50%) and 90% lethal times (LT_{50} and LT_{90}). In addition, survival curves constructed with the non-parametric Kaplan-Meier method were further pairwise comparisons were made between fungal strains within each conidial concentration based on the log-rank test at $P < 0.05$, using the function *pairwise_survdiff*. Differences between the control and the infected weevils were examined using a Cox proportional hazards model based on the risk of weevil death and respective hazard ratios (HR; the daily chance of death), implemented with the *coxph* function and visualized them using the *ggforest*. In addition, hazard ratios (HR) of insects exposed to different fungal strains in comparison with the commercial product Boveril were calculated. The survival analysis was performed with the "survival"[38] and "survminer"[39] packages.

Secondary inoculum production by sporulated cadavers was fitted to a generalized linear mixed model with Poisson distribution and log link function to account for overdispersion, using the package "lme4"[40]. Fixed effects were assigned

to fungal strains and experimental blocks, including random effects for the observational level in the linear predictor. Means were compared according to Tukey HSD test at $P < 0.05$, using the package "emmeans" [37]. As a complimentary analysis, the cluster analysis was performed to overall mortality, mycosis, LT_{50}/LT_{90} estimates and *in vivo* inoculum production by infected insects to facilitate visualization of similar and dissimilar fungal strains tested in the screening study. Predicted means derived from model fits were normalized prior to turning into distances based on the Euclidean method, a measure of similarity. Subsequently, Euclidean distances were subjected to a hierarchical clustering tree (in rows and columns) taking into account all variables and fungal strains using Ward's method. The heat map was computed and described using a function of heatmap.2 in the "gplots" package[41]. To determine the type of relationship between these response variables, average data on overall mortality rate was correlated with mycosis rate, LT_{50}, LT_{90} and *in vivo* inoculum production using Spearman's correlation method that allowed to compute *r* coefficient and *P*-value.

Proportion data for a specific time interval after spraying from multiple concentration-mortality response bioassays involving two fungal strains were fitted with a two-parameter log-logistic model with binomial distribution using the package "drc"[42]. The following model fitted to the mortality-concentration data can be written as:

$$y = c + \frac{d - c}{1 + exp(b(\log(x) - \log(e)))}$$

Where: *y* is the mortality probability, *b* is the slope (steepness of the curve), *e* is the inflection point, while lower limit *c* is zero (i.e., natural mortality in control was null) and upper limit *d* is equal to 1. We chose to analyse data on 15-day mortality for *G. platensis* exposed to these fungi, because most fungal concentrations had reached maximum mortality levels while control group had no natural mortality. Estimated effective median concentration (LC_{50}) for each fungal strain were extracted from these dose-response models along with their corresponding robust standard errors (using function *sandwich*) and the delta method for 95% confidence intervals adjusted for over-dispersion (i.e., multiplication by a scaling factor). Model slopes (*b*, curve steepness) and estimated LC_{50} values were compared using Student t-test to identify significant contrasts at $P < 0.05$, while difference between dose-response curves was assessed with the log-likelihood ratio test.

All graphs were plotted with package “ggplot2”[43]. Goodness-of-fit of models was assessed using half-normal plots with simulation envelopes[44] or Akaike’s Information Criterion (AIC) along with residual plot examination. All analyses were performed in the statistical environment R[45].

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Table and Figures

Table 1. Two-parameter log-logistic model fitted to concentration-mortality data for describing the virulence of two fungal strains to *G. platensis* adults in terms of their estimated median lethal concentrations (LC50) with corresponding 95% confidence intervals. Concentration is expressed as conidia mL-1. *Total number of insects in the experiment. † Slope for mortality represents regression of proportion of weevils versus log (concentration) of conidia. Different letters indicate significant contrast between slopes according to Student t-test ($P < 0.05$). **§** Student t-test and *P*-values represent the probability of slope ≠ 0. **¥** Student t-test contrasts for estimated lethal concentrations indicate significant differences when followed by distinct letters at $P < 0.05$.

		Model parameters			**Median lethal concentrations (conidia mL^{-1}) ¥**
Fungal isolate	**n***	**Inflection point (*e*) ± SE**	**Slope (*b*) ± SE†**	***t* (*P*-value)§**	**LC_{50} (95% ci)**
B. bassiana ICBC-240	240	7.37 ± 0.16	-7.79 ± 1.73 a	4.50 (<0.0001)	2.33 (1.12 – 4.86) × 10^7 a
M. anisopliae ICBC-364	240	7.82 ± 0.13	-13.35 ± 2.18 b	6.13 (<0.0001)	6.62 (3.75 – 11.69) × 10^7 b

Table 2. Origin, date of collection, host and species of fungal strains native to Brazil used in this study. All fungal strains used in the current study are registered at the National System for the Management of Genetic Heritage and Associated Traditional Knowledge—SisGen under the code AA51E70. *All fungal strains were deposited in the microbial germplasm named Collection of Entomopathogenic Microorganisms “Odemar Cardim Abreu” at the Experimental Center of the Instituto Biologico, Campinas, SP, Brazil, with a prefix code ‘IBCB’. Fungal strains ESALQ-PL63 and ESALQ-E9 are proprietary of Koppert do Brasil Holding Ltd. (Piracicaba, SP, Brazil) and were re-isolated and purified in PDA from serial dilutions of their respective commercial formulated products named Boveril WP and Metarril WP, respectively.

Species	**Strain**	**Host**	**Date of collection**	**Geographical origin**	**Geographical coordinates**
Beauveria bassiana	**IBCB-6**	*Cosmopolites sordidus*	02/01/1986	Miracatu – SP	24°17'13.2"S 47°27'28.1"W
	IBCB-18	*Hypothenemus hampei*	11/11/1987	Tapiratiba – SP	21°29'03.8"S 46°46'10.2"W
	IBCB-31	*Nezara viridula*	03/01/1986	Piracicaba – SP	22°43'25.0"S 47°38'35.5"W
	IBCB-35	*Cosmopolites sordidus*	03/01/1986	Cruz das Almas – BA	12°39'06.1"S 39°07'17.8"W

	IBCB-66	*Hypothenemus hampei*	09/01/1986	São José do Rio Pardo – SP	21°35'46.0"S 46°53'17.5"W
	IBCB-74	*Aphidoidea*	03/01/1988	Campinas – SP	22°54'10.8"S 47°03'52.9"W
	IBCB-80	*Hypothenemus hampei*	12/01/1988	Caconde – SP	21°31'14.5"S 46°38'40.2"W
	IBCB-87	*Cosmopolites sordidus*	04/01/1989	Goiânia – GO	16°40'08.4"S 49°16'00.5"W
	IBCB-240	*Hypothenemus hampei*	06/01/1999	Campinas – SP	22°54'05.0"S 47°04'05.2"W
	IBCB-246	*Hypothenemus hampei*	06/01/1999	Taubaté – SP	23°01'40.4"S 45°33'22.0"W
	IBCB-259	*Hypothenemus hampei*	06/01/1999	Taubaté – SP	23°01'40.4"S 45°33'22.0"W
	IBCB-329	Native forest	06/01/1999	Guaraniaçu – PR	25°05'34.8"S 52°52'18.1"W
	IBCB-620	*Hypothenemus hampei*	08/08/2008	São Paulo – SP	23°32'24.0"S 46°38'22.9"W
	ESALQ PL63	*Atta* sp.	01/01/1992	Piracicaba – SP	22°42'45.27"S 47°37'38.5"W
***Beauveria* sp.**	**IBCB- 634**	*Epacroplon cruciatum*	05/23/2009	Cel. Macedo – SP	23°38'15.7"S 49°18'49.0"W
Metarhizium anisopliae	**IBCB-196**	Soil	05/01/1999	Piracicaba – SP	22°43'56.6"S 47°38'56.4"W
	IBCB-333	*Mahanarva fimbriolata*	07/01/1999	Valparaíso – SP	21°13'42.2"S 50°52'01.9"W
	IBCB-348	*Mahanarva fimbriolata*	06/02/1999	Sertãozinho – SP	21°08'38.4"S 48°00'41.8"W
	IBCB-364	*Anthonomus grandis*	07/14/1999	Araras - SP	22°21'32.0"S 47°22'47.6"W
	IBCB-383	*Mahanarva fimbriolata*	06/01/1999	Araras - SP	22°21'32.0"S 47°22'47.6"W
	IBCB-391	*Mahanarva fimbriolata*	08/02/1999	Tabapuã - SP	20°57'27.0"S 49°01'37.6"W
	IBCB-425	Soil	01/05/2000	Iporanga - SP	24°35'11.4"S 48°35'41.3"W
	ESALQ-E9	*Mahanarva posticata*	05/05/1981	Boca da Mata - MT	9°40'1.73"S 36°07'23.04"W

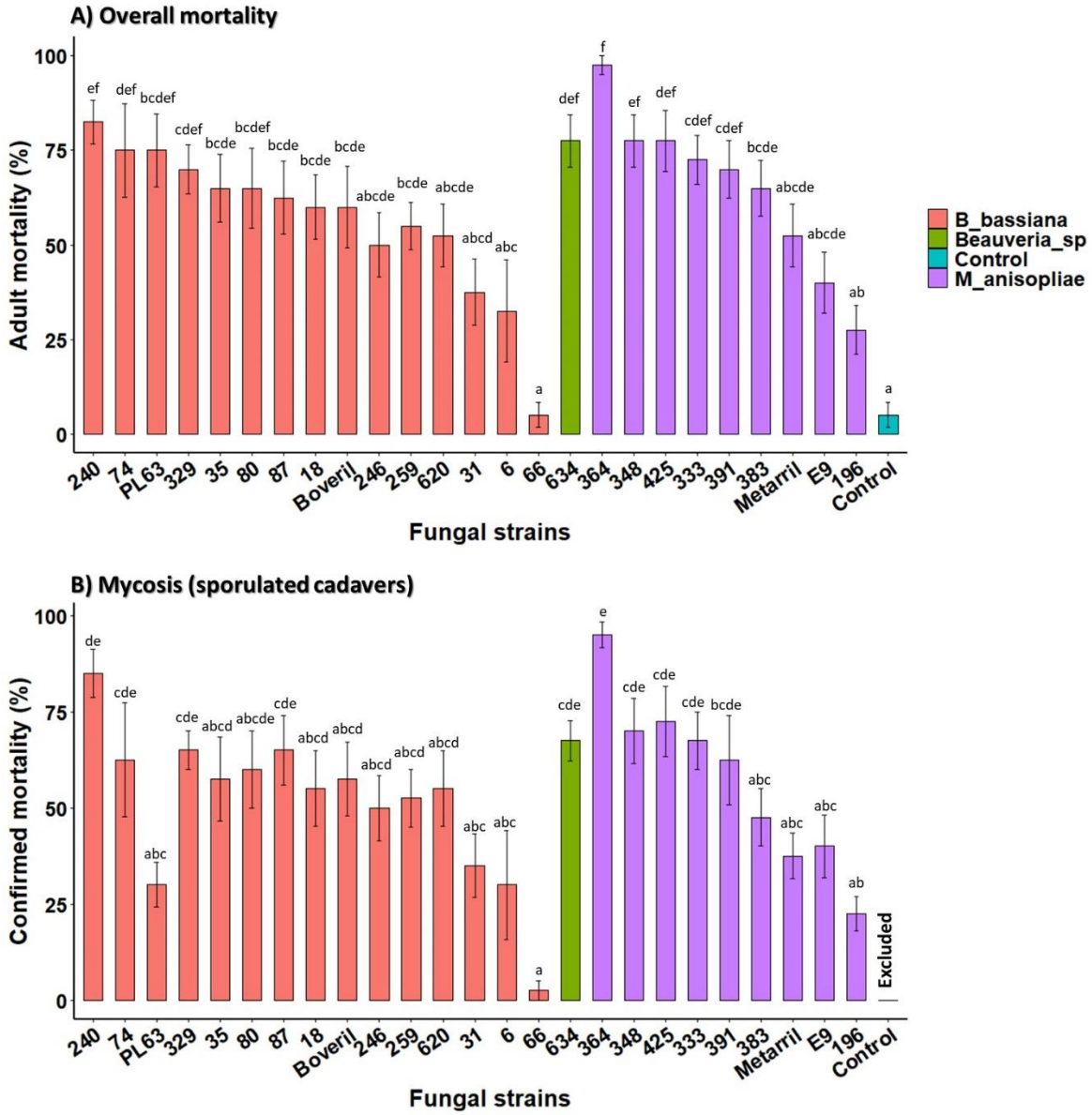

Figure 1. Cumulative mortality rate by day 20 post-spraying (A) and confirmed mortality (i.e., sporulated cadavers or mycosis rate, B) of *G. platensis* adults after inoculation with 1 × 10^8 conidia mL^{-1} of several strains of *M. anisopliae*, *Beauveria* sp. and *B. bassiana*. Untreated weevils from control group were sprayed only with Tween-80 solution (0.1%). Vertical bars represent means (± SE, n = 8 replicates with a total of 40 weevils per treatment), while different letters denote significant contrasts between treatments by Tukey HSD method (P < 0.05). There are four commercial isolates explored in Brazil and used here as positive controls for the sake of comparison: *B. bassiana* IBCB-66 (only unformulated) and ESALQ-PL63 (unformulated and formulated as Boveril); *M. anisopliae* IBCB425 (only unformulated) and ESALQ-E9 (unformulated and formulated as Metarril).

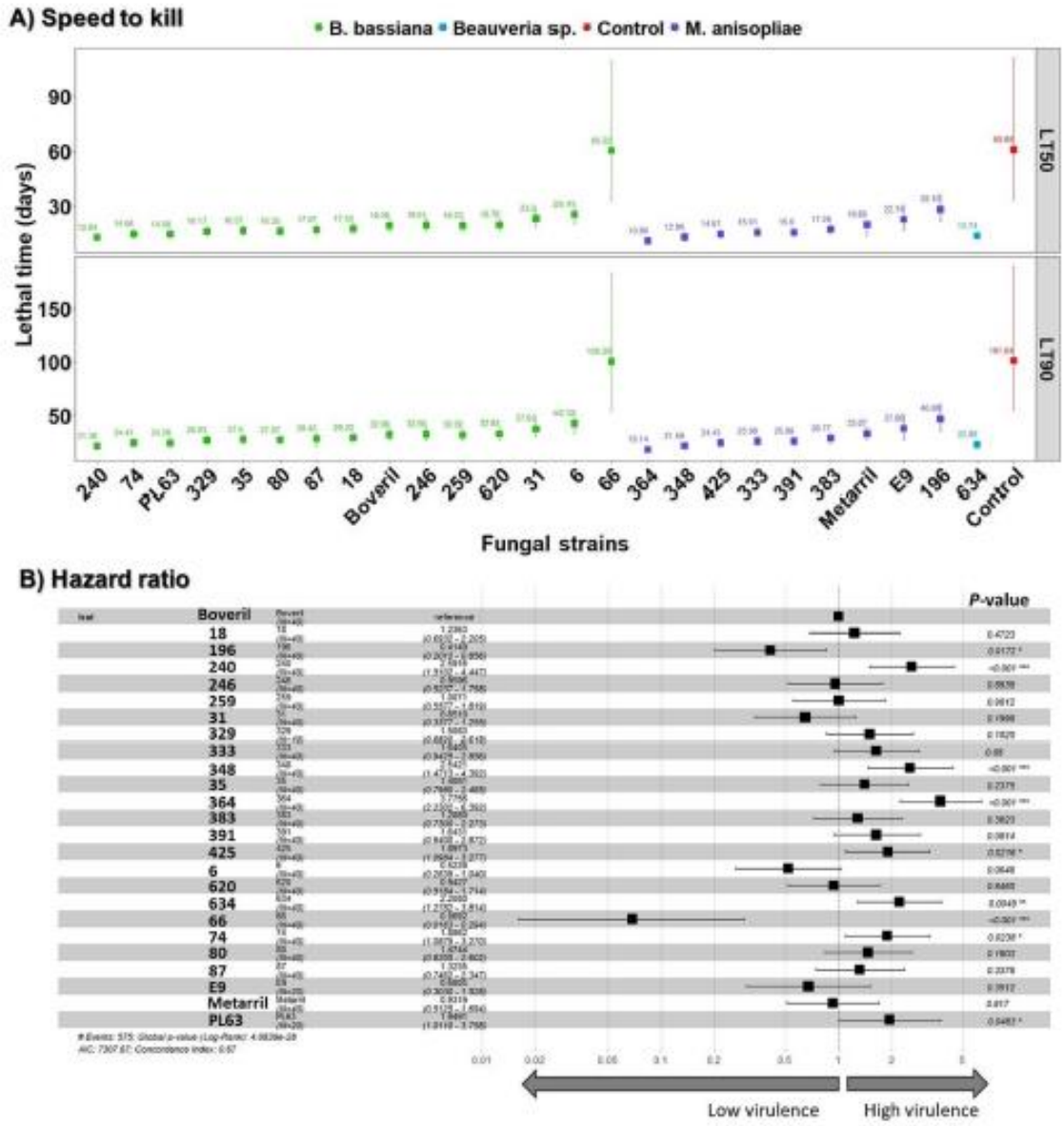

Figure 2. Susceptibility of *G. platensis* adults after exposure to several strains of *M. anisopliae* and *B. bassiana* applied with a single concentration of 1×10^8 conidia m^{-1}. (A) Speed to kill represented by 50% and 90% lethal times (LT_{50}/LT_{90}, in days) estimated with Weibull models fitted to survival censored data of several fungal strains. Lethal times are significantly different when their 95% confidence intervals (whiskers) do not overlap. (B) Hazard ratios (square symbols) for fungal infection compared to the commercial mycoinsecticide Boveril, which is assigned as the reference group and currently registered to control Gonipterus spp. in Brazil. Significant P-values are indicated by asterisks (*< 0.05, **< 0.01 or ***< 0.001) and represent the strains that were significantly different (more/less virulent) from the reference Boveril. Whiskers represent the 95% CIs.

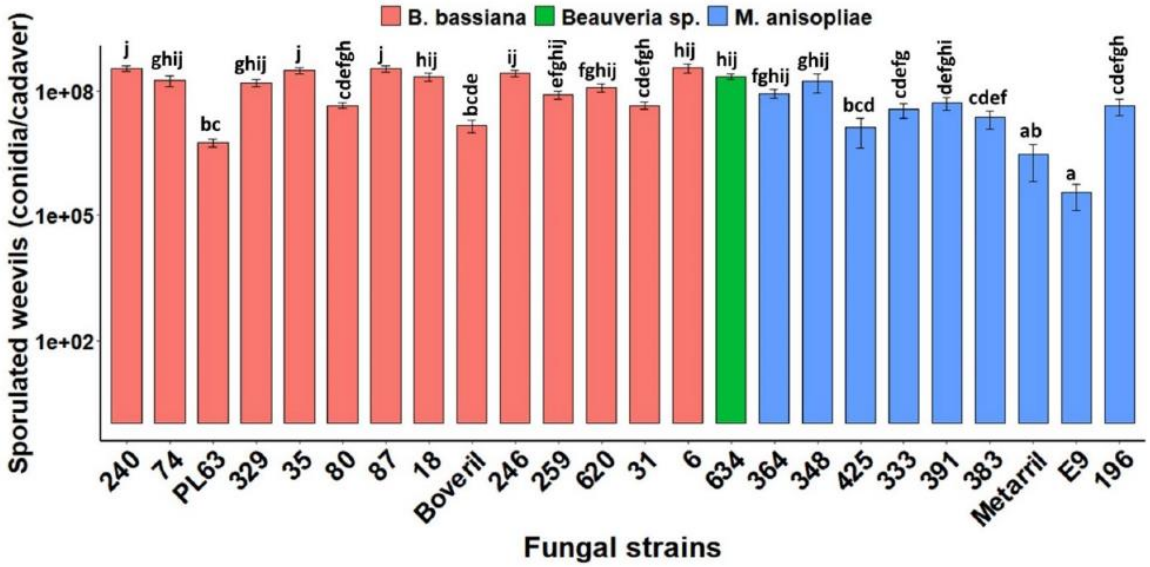

Figure 3. Secondary inoculum production by infected weevils' cadavers of various strains of *M. anisopliae* and *B. bassiana* after incubation in saturated humid chambers. Data were fitted to a generalized linear model with negative binomial distribution, and estimated means (± SE, n = 10 replicates) were statistically separated by Tukey HSD test at $P < 0.05$. Note that *B. bassiana* IBCB-66 was not included in this analysis, as it had one weevil that sporulated.

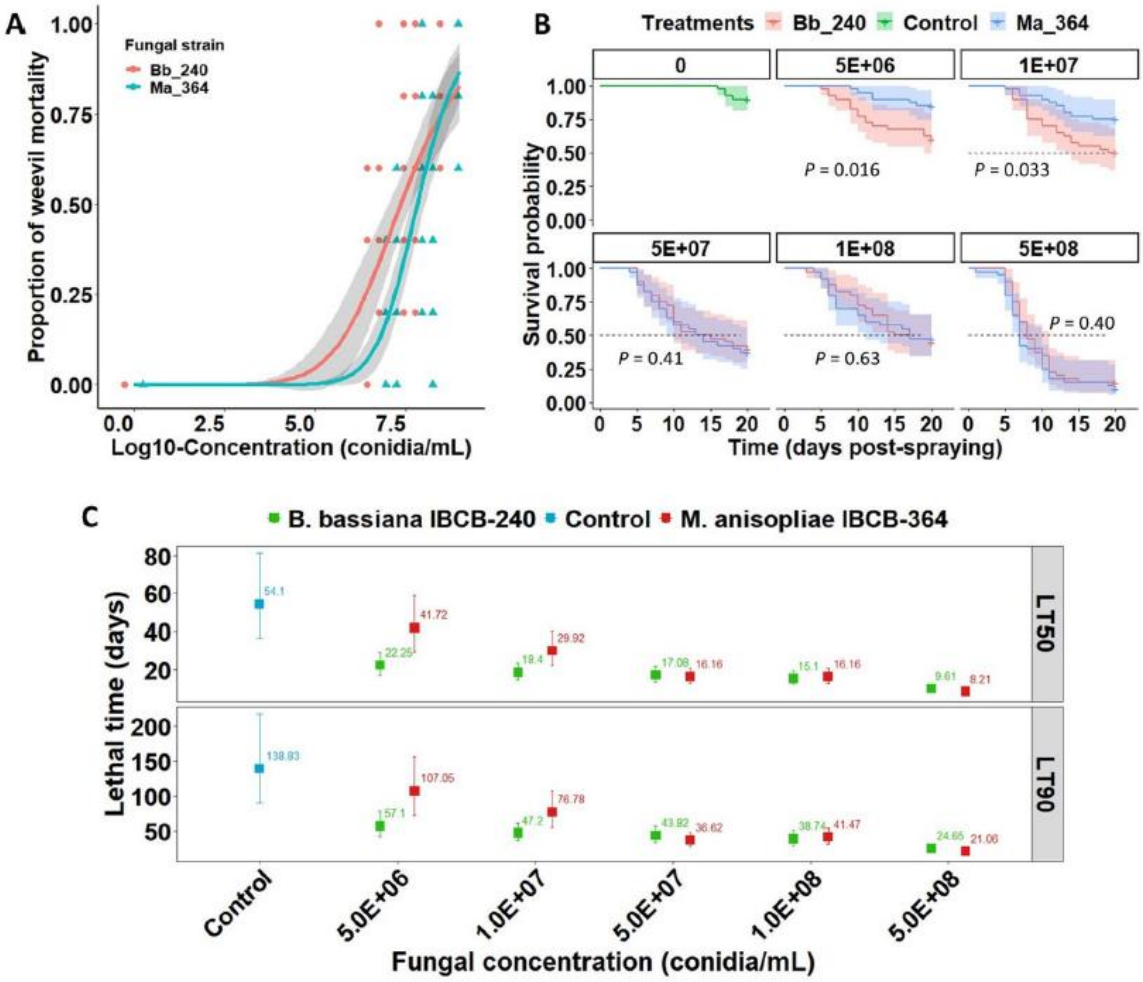

Figure 4. Comparative virulence between strains IBCB-240 (*B. bassiana*) and IBCB 364 (*M. anisopliae*) against *G. platensis* adults assessed with time-concentration-mortality bioassays. (A) Concentration-mortality response curves at 15 days post spraying fitted with a two-parameter log-logistic models (solid lines are fitted models, grey bands are 95% confidence intervals and points with different shapes are observational data). (B) Kaplan–Meier survival curves of *G. platensis* adults exposed to two fungal strains and compared with log-rank test ($P < 0.05$) within each conidial concentration tested. Log-rank P-values for all the pairwise comparisons between survival curves are presented in Table S2. (C) Estimated median (LT50) and 90% (LT90) lethal times with their corresponding 95% confidence intervals (whiskers) for *G. platensis* adults after being sprayed with five conidial concentrations of each fungal strain. Statistical differences between LT50 or LT90 values are indicated when their 95% confidence intervals do not overlap.

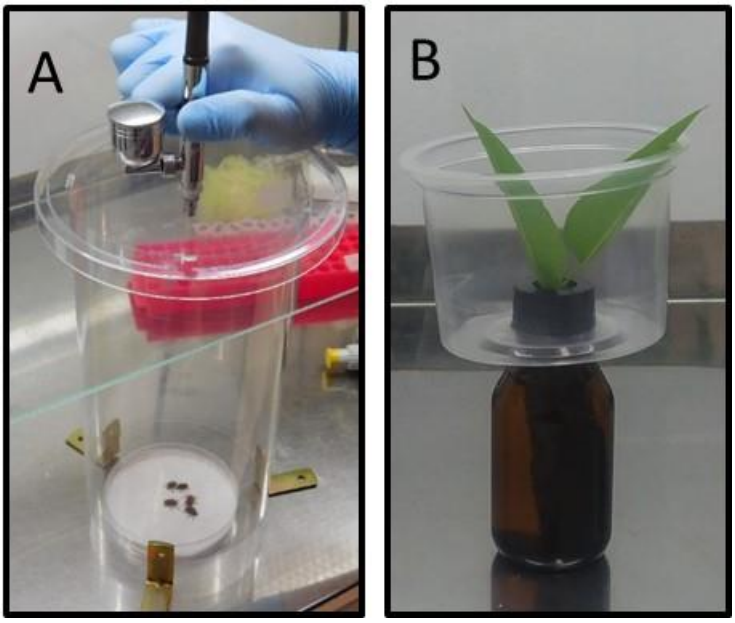

Figure 5. Detail of the experiment set up for screening fungal strains against adults of *G. platensis* under laboratory conditions. (A) Portable mini-sprayer assembled with a dual-gravity airbrush, modified from Mascarin et al. (2013), operated with 10 PSI and loaded with 127 µL of fungal suspension for application on to *G. platensis* beetles. Acrylic cylinder measured 11.5 cm in diameter and 23 cm in height. The triplet created a free space of 2 cm to allow depressurization during spraying. (B) Experimental unit consisting of eucalyptus leaf with its petiole immersed in a glass vial filled with water to keep its turgor and serving to feed the beetles confined in a plastic container.

Supplementary Material

Table S1. *P*-values adjusted with Bonferroni-Holmes were obtained from pairwise comparisons of survival curves of *G. plantesis* adults exposed to different fungal strains using log-rank test at $P < 0.05$ (significant *P*-values are highlighted in bold). Data refer to screening bioassays using single concentration of 1×10^8 conidia mL^{-1}.

	18	**196**	**240**	**246**	**259**	**31**	**329**	**333**	**348**	**35**	**364**	**383**	**391**	**425**	**6**	**620**	**634**	**66**	**74**	**80**	**87**	**Boveril**	**Control**	**E9**	**Metarril**
196	**0.00507**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
240	**0.01799**	3.3e-07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
246	0.47166	**0.05079**	**0.00244**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
259	0.57402	**0.02334**	**0.00198**	0.92298	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	0.10738	0.36433	**8.8e-05**	0.38733	0.28388	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
329	0.55034	**0.00055**	0.08586	0.19998	0.22003	**0.02455**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
333	0.40659	**9.4e-05**	0.14162	0.11573	0.12146	**0.00907**	0.81990	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
348	**0.02200**	**3.5e-06**	0.96753	**0.00501**	**0.00501**	**0.00032**	0.11747	0.21625	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	0.72256	**0.00117**	**0.03220**	0.30451	0.31989	**0.04521**	0.80644	0.65376	**0.03875**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
364	**9.1e-05**	**5.6e-11**	0.21419	**6.5e-06**	**2.2e-06**	**9.0e-08**	**0.00122**	**0.00271**	0.23085	**0.00032**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
383	0.94784	**0.00169**	**0.02219**	0.39331	0.45047	0.05988	0.64794	0.47436	**0.03781**	0.75843	**7.9e-05**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
391	0.42950	**0.00018**	0.16525	0.12601	0.13045	**0.01272**	0.81351	0.99529	0.23009	0.63318	**0.00501**	0.49335	-	-	-	-	-	-	-	-	-	-	-	-	-
425	0.16884	**9.8e-06**	0.33132	**0.03921**	**0.03786**	**0.00211**	0.46899	0.66283	0.43643	0.35163	**0.01401**	0.21843	0.67004	-	-	-	-	-	-	-	-	-	-	-	-

6	**0.02219**	0.63562	**3.9e-06**	0.15432	0.10239	0.65441	**0.00364**	**0.00135**	**1.6e-05**	**0.00740**	**1.7e-09**	0.01318	**0.00181**	**0.00020**	-	-	-	-	-	-	-	-	-	-
620	0.44873	**0.03701**	**0.00092**	0.94628	0.87822	0.38204	0.15436	0.08139	**0.00252**	0.22003	**8.7e-07**	0.35163	0.08742	**0.02409**	0.15479	-	-	-	-	-	-	-	-	-
634	0.07024	**4.9e-06**	0.68224	**0.01401**	**0.01409**	**0.00074**	0.23624	0.36108	0.72256	0.12916	0.08139	0.08742	0.36909	0.63562	**4.6e-05**	**0.00696**	-	-	-	-	-	-	-	-
66	**1.4e-06**	**0.01997**	**2.7e-11**	**4.5e-05**	**9.8e-06**	**0.00163**	**6.3e-08**	**6.5e-09**	**2.6e-10**	**2.0e-07**	**1.3e-15**	**2.3e-07**	**1.9e-08**	**5.4e-10**	**0.00612**	**2.2e-05**	**3.1e-10**	-	-	-	-	-	-	-
74	0.19998	**2.2e-05**	0.33206	**0.04247**	**0.04789**	**0.00288**	0.50223	0.66283	0.35684	0.34815	**0.01434**	0.21691	0.66917	0.96212	**0.00022**	**0.02822**	0.62915	**1.7e-09**	-	-	-	-	-	-
80	0.62915	**0.00106**	0.06287	0.22003	0.25670	**0.03450**	0.94429	0.77789	0.07024	0.87260	**0.00104**	0.65376	0.77111	0.43674	**0.00501**	0.17556	0.19998	**1.8e-07**	0.45047	-	-	-	-	-
87	0.85631	**0.00293**	**0.02219**	0.39319	0.46353	0.07851	0.65441	0.47815	**0.02782**	0.89264	**0.00011**	0.94311	0.49135	0.21742	**0.01397**	0.36108	0.08139	**4.1e-07**	0.26330	0.79561	-	-	-	-
Boveril	0.53966	**0.02367**	**0.00177**	0.94216	0.96292	0.29689	0.20492	0.11608	**0.00335**	0.29595	**1.6e-06**	0.44483	0.12916	**0.03322**	0.10184	0.92972	**0.01143**	**9.8e-06**	**0.04026**	0.24148	0.44382	-	-	-
Control	**7.9e-07**	**0.01702**	**1.5e-11**	**3.0e-05**	**5.9e-06**	**0.00135**	**3.3e-08**	**3.3e-09**	**2.0e-10**	**1.0e-07**	**4.2e-16**	**1.2e-07**	**9.3e-09**	**2.6e-10**	**0.00501**	**1.3e-05**	**2.0e-10**	0.98771	**8.0e-10**	**1.0e-07**	**2.5e-07**	**6.0e-06**	-	-	-
E9	0.20366	0.36130	**0.00239**	0.50545	0.43674	0.95867	0.08291	**0.04789**	0.00571	0.11573	**2.5e-05**	0.15063	0.05301	**0.01997**	0.66599	0.52605	0.00884	**0.00210**	**0.01997**	0.09214	0.16966	0.43084	**0.00156**	-	-
Metarril	0.41469	**0.03875**	**0.00081**	0.94628	0.85631	0.38737	0.14471	0.07507	0.00211	0.20702	**6.8e-07**	0.33307	0.08139	**0.02031**	0.15806	0.96753	0.00596	**2.2e-05**	**0.02409**	0.16670	0.32119	0.87233	**1.3e-05**	0.52605	-
PL63	0.22003	**0.00024**	0.53966	0.08673	0.08857	**0.01072**	0.50995	0.69852	0.65376	0.32929	0.07508	0.32929	0.72256	0.96753	**0.00179**	0.06131	0.80824	**6.5e-09**	0.93017	0.41469	0.21843	0.07507	**3.3e-09**	**0.04163**	0.05718

Table S2. *P*-values adjusted with Bonferroni-Holmes were obtained from pairwise comparisons of survival curves of *G. plantesis* adults exposed to different concentrations of *B. bassiana* IBCB-240 and *M. anisopliae* IBCB-364 using log-rank test at $P < 0.05$ (significant *P*-values are highlighted in bold).

	B_bassiana_ 1E+07	**B_bassiana_ 1E+08**	**B_bassiana_ 5E+06**	**B_bassiana_ 5E+07**	**B_bassiana_ 5E+08**	**M_anisopliae_ 1E+07**	**M_anisopliae_ 1E+08**	**M_anisopliae_ 5E+06**	**M_anisopliae_ 5E+07**	**M_anisopliae_ 5E+08**
B_bassiana_ 1E+08	0.07757	-	-	-	-	-	-	-	-	-
B_bassiana_ 5E+06	0.39935	**0.00758**	-	-	-	-	-	-	-	-
B_bassiana_ 5E+07	0.37971	0.41994	0.07806	-	-	-	-	-	-	-
B_bassiana_ 5E+08	**0.00020**	**0.02940**	**8.7e-06**	**0.00759**	-	-	-	-	-	-
M_anisopliae_1E+07	**0.03254**	**6.5e-05**	0.19333	**0.00213**	**1.3e-08**	-	-	-	-	-
M_anisopliae_1E+08	0.65795	0.18272	0.24223	0.60316	**0.00261**	**0.01363**	-	-	-	-
M_anisopliae_5E+06	**0.00125**	**4.3e-07**	**0.01615**	**4.7e-05**	**3.2e-11**	0.30045	**0.00049**	-	-	-
M_anisopliae_5E+07	0.24738	0.59369	**0.04171**	0.76138	**0.01542**	**0.00109**	0.45688	**1.8e-05**	-	-
M_anisopliae_5E+08	**1.1e-05**	**0.00382**	**3.6e-07**	**0.00077**	0.38516	**2.8e-10**	**0.00023**	**7.8e-13**	**0.00200**	-
Control	**0.00012**	**9.9e-09**	**0.00261**	**3.4e-06**	**7.8e-13**	0.07981	**4.8e-05**	0.50178	**1.0e-06**	**5.5e-14**

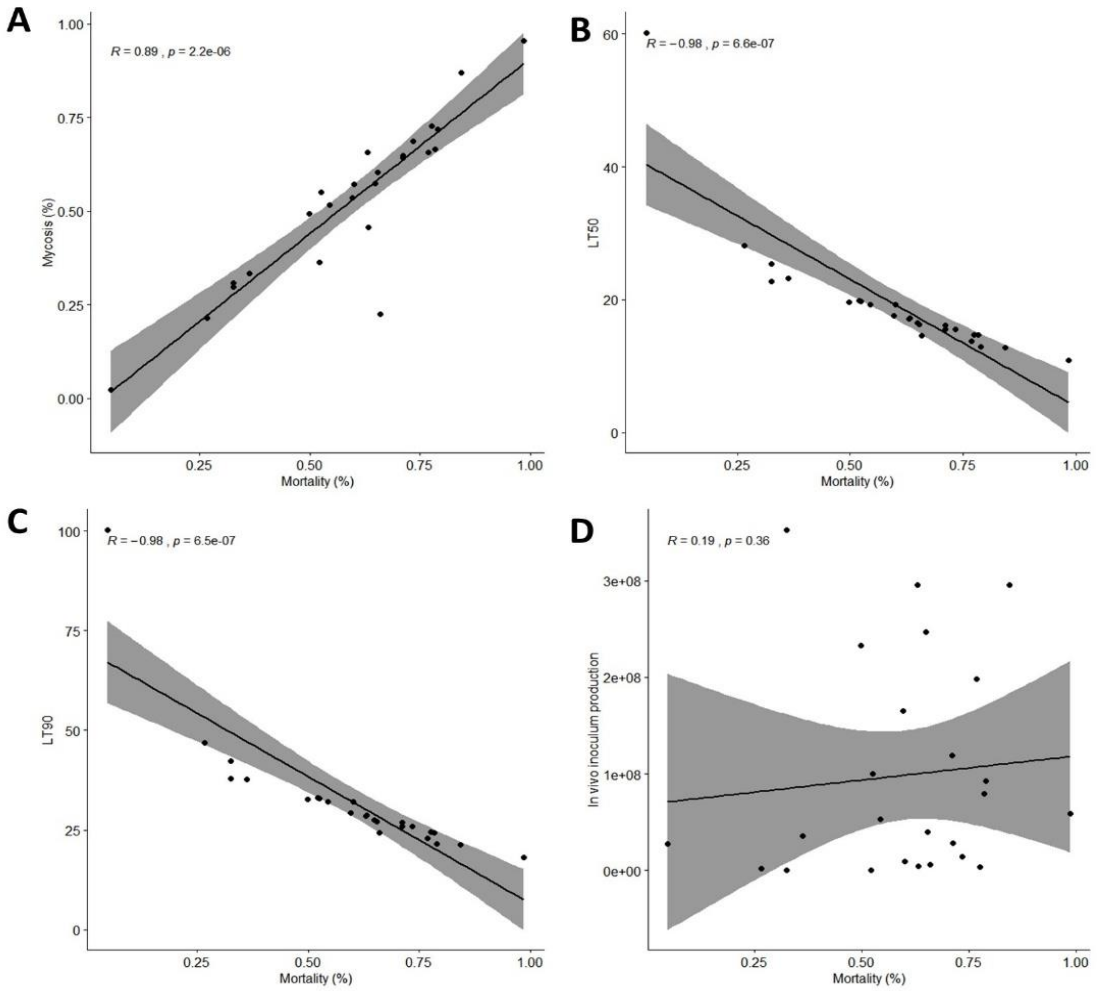

Figure S1. Spearman's correlation (*R* coefficient and *P*-value) carried out to determine the relation of overall adult mortality with mycosis (confirmed mortality) (A), LT_{50} (B), LT_{90} (C) and *in vivo* inoculum production (conidia produced by weevil's cadaver) (D). Significant correlation coefficient was attribute to $P < 0.05$, while 95% confidence bands are represented by the grey shade and black circles refer to observational points.

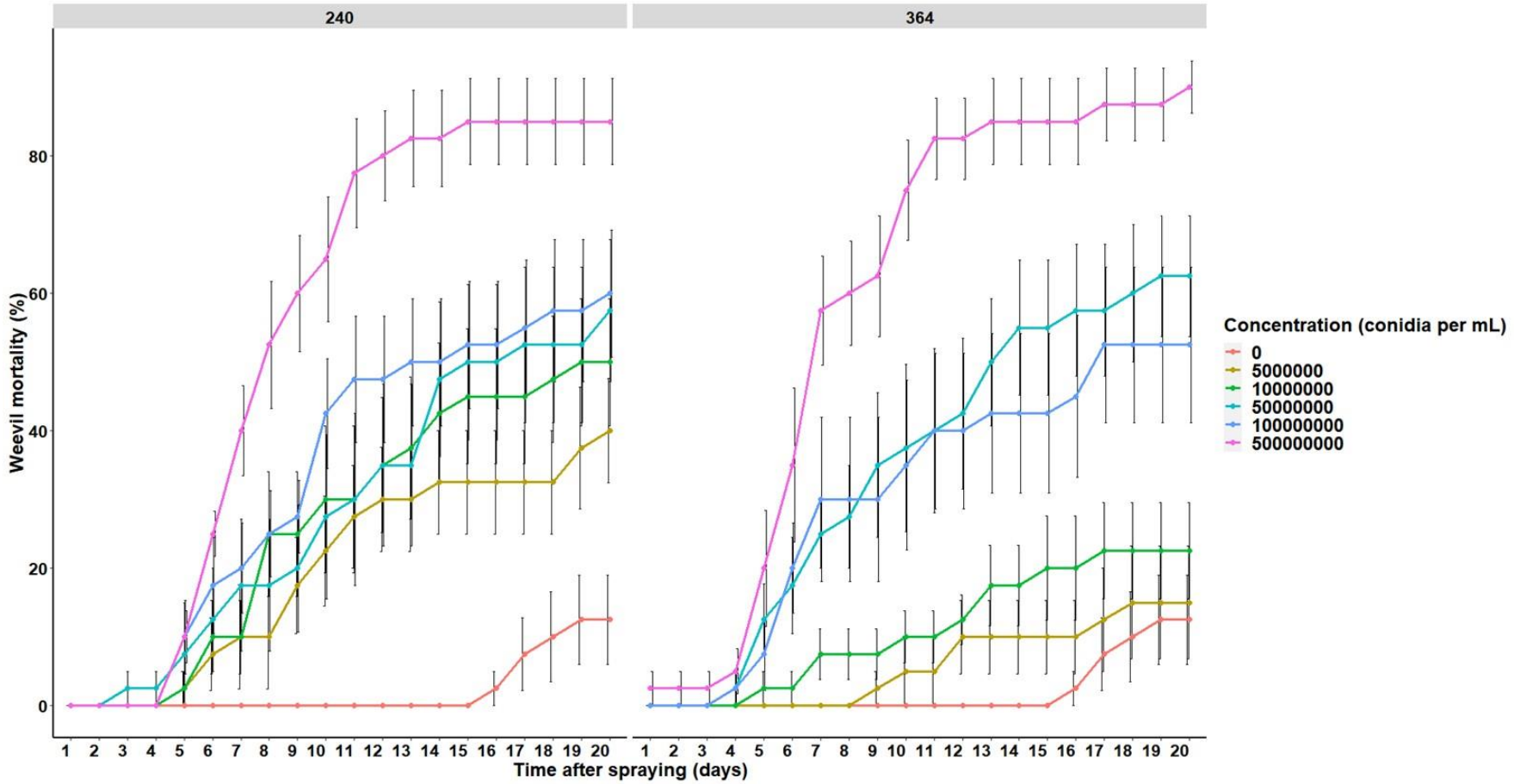

Figure S2. Cumulative daily proportional mortality of adult *G. platensis* weevils exposed to six spore concentrations of two fungal strains, *B. bassiana* IBCB-240 and *M. anisopliae* IBCB-364. Control (red line, concentration "0") were exposed to only Tween 80 at 0.1%. Solid lines with different colors represent mean cumulative daily mortality rates accompanied by standard errors for each concentration with 8 replicates each and a total of 40 insects.

CONSIDERAÇÕES FINAIS

A busca por novas estratégias de controle biológico para *G. platensis* proporcionou nesse trabalho resultados pioneiros e inovadores para o controle microbiano, respondendo os quatro objetivos inicialmente propostos.

Tivemos como principais resultados: a detecção da ocorrência de microsporidiose em adultos de *G. platensis,* indicando uma provável nova espécie deste patógeno, a identificação de dois novos vírus em adultos de *G. platensis*, denominados Gonipterus platensis macula-like vírus (GPMLV) e Gonipterus platensis bunya-like vírus (GPBLV); a expressão de toxicidade em larvas neonatas para *B. thuringiensis* variando conforme a expressão gênica; a ampla variação na virulência entre os isolados de *M. anisopliae* e *B. bassiana* oferecendo a oportunidade de abordar o desenvolvimento potencial de isolados em biocontrole aprimorado com propriedades personalizadas.

Os resultados encontrados nos sugerem novos passos para a pesquisa no manejo integrado do *Gonipterus platensis,* iniciando-se por testes de patogenicidade com os isolados de *B. thuringiensis* selecionados em larvas de outros instares e no inseto adulto, como também testar os produtos a base de *Bacillus thuringiensis* específicos para coleóptera disponíveis em outros paises. Iniciar testes em campo com os fungos selecionados e assim iniciar o desenvolvimento de um novo produto, como também identificar a presença dos novos vírus e microsporideos em outros países e a sua interação com o inseto praga e seu parasitoide.

Em resumo, esses resultados constituem um passo importante no desenvolvimento de alternativas de biocontrole do gorgulho do eucalipto ambientalmente seguras e potencialmente eficientes. Além disso a descoberta de novos entomopatógenos para essa praga, mesmo que ocorrendo de forma endêmica, auxilia no controle biológico complementar de *G. platensis*. As possíveis interações entre os novos entomopatógenos e o parasitoide *A. nitens* e predadores ainda precisam ser devidamente estudadas para se conhecer a abrangência das relações de controle biológico no complexo eucalipto-gorgulho-inimigos naturais.

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