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INSTITUTO DE BIOTECNOLOGIA**



PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

**TECNOLOGIAS ÔMICAS NA IDENTIFICAÇÃO E NO CONTROLE BIOLÓGICO
DE FITONEMATOIDES**

Aluno: Guilherme Nunes Moreira Costa

Orientador: Prof. Dr. Carlos Ueira Vieira

Coorientadora: Profa. Dra. Maria Amelia dos Santos

UBERLÂNDIA - MG

2024



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**Tese apresentada à Universidade
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Aos trinta e um dias do mês de julho de dois mil e vinte e quatro, às 14:00 horas, reuniu-se via web conferência pela Plataforma *Microsoft Teams*, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, Resolução de nº 06/2020 e Resolução nº 19/2022 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Dr^a. Luciana Nunes Gontijo, Dr^a. Marcela de Freitas Silva, Dr^a. Ana Carolina Silva Siquieroli, Dr^a. Nilvanira Donizete Tebaldi e Dr. Carlos Ueira Vieira, como presidente da mesa e demais convidados presentes conforme lista de presença. Iniciando os trabalhos o (a) presidente da mesa, Dr. Carlos Ueira Vieira apresentou a Comissão Examinadora e o (a) candidato (a), agradeceu a presença do público, e concedeu o (à) Discente a palavra para a exposição do seu trabalho. A duração da apresentação do (a) Discente e o tempo de arguição e resposta foram conforme as normas do Programa de Pós-graduação em Genética e Bioquímica. A seguir o (a) senhor (a) presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir o (a) candidato (a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais. Em face do resultado obtido, a Banca Examinadora considerou o candidato (a):

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Esta defesa de Tese de Doutorado é parte dos requisitos necessários à obtenção do título de Doutor. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.



Documento assinado eletronicamente por **Carlos Ueira Vieira, Professor(a) do Magistério Superior**, em 01/08/2024, às 08:40, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do [Decreto nº 8.539, de 8 de outubro de 2015](#).



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APRESENTAÇÃO

Os fitonematoides são uma ameaça para a produtividade agrícola e para a segurança alimentar da população mundial. As estratégias de manejo desses patógenos de plantas está passando por uma reformulação, onde ferramentas de controle biológico se mostram como um mercado promissor. Aliado a isso, o desenvolvimento de técnicas para detecção e identificação desses patógenos nas lavouras é uma necessidade constante. Por isso, estas pesquisas buscaram encontrar novos organismos para a utilização no controle biológico de fitonematoides, bem como novas técnicas para a identificação destes patógenos.

No Capítulo 1 desta Tese, discorro sobre a relevância dos fitonematoides na produção agrícola a nível mundial e sobre algumas das principais espécies destes patógenos. Apresento também o potencial da microbiota associada ao alimento larval das abelhas sem ferrão no controle biológico de patógenos e em outros processos biotecnológicos.

No Capítulo 2, apresento o artigo “Screening of microorganisms isolated from stingless bee’s larval food in the biocontrol of *Meloidogyne incognita*”, no formato de envio para publicação. Este trabalho apresenta uma triagem e identificação de micro-organismos associados ao alimento larval de abelhas sem ferrão que possuam efeito nematicida sobre a espécie *Meloidogyne incognita*.

No Capítulo 3, apresento o artigo “Differentiation of *Heterodera glycines* races by metabolomics” no formato de envio para publicação. Neste trabalho é abordado o uso de ferramentas de metabolômica na identificação de raças da espécie *Heterodera glycines*, com o intuito de facilitar a identificação deste patógeno nas lavouras bem como auxiliar na tomada de decisão nas estratégias de manejo.

CAPÍTULO 1
FUNDAMENTAÇÃO TEÓRICA

1. Fitonematóides

Os nematoides parasitos de plantas (fitonematoides) são animais aquáticos de formato vermiforme e tamanho geralmente microscópico que habitam o solo ou estruturas de plantas e se alimentam de tecidos vegetais (Howland & Quintanilla, 2023). A maioria dos nematoides encontrados em diversos ecossistemas se alimentam de bactérias, fungos e até mesmo de outros nematoides, sendo que os fitonematoides constituem a minoria dentro do filo Nematoda mesmo englobando mais de 4100 espécies (Decreamer & Hunt, 2006; Holterman *et al.*, 2017; Montarry *et al.*, 2021).

Devido ao fato de serem animais de pequeno tamanho e geralmente não visíveis a olho nu, muitas vezes os fitonematoides passam despercebidos mesmo sendo encontrados em altas densidades populacionais no ambiente. Contudo, os prejuízos causados pelo parasitismo destes organismos em plantas de interesse agrônômico são estimados em mais de 173 bilhões de dólares anuais a nível global (Elling, 2013; Holterman *et al.*, 2017).

Os fitonematoides possuem em sua cavidade oral, uma estrutura similar a uma agulha denominada estilete (Figura 1A), utilizada em sua alimentação para. Para sua alimentação, estes organismos utilizam seu estilete oral para perfurar a parede celular do tecido vegetal, injetar enzimas e hormônios e remover o conteúdo celular para sua obtenção de energia. Devido a este parasitismo os fitonematoides podem causar danos significativos em diversas culturas vegetais. As plantas infectadas podem apresentar sintomas como redução no tamanho, folhas cloróticas e deformidades do sistema radicular. Tais alterações podem levar a redução da produtividade das plantas infectadas bem como facilitar a infecção por outros organismos patogênicos oportunistas (Holterman *et al.*, 2017; Phani *et al.*, 2021).

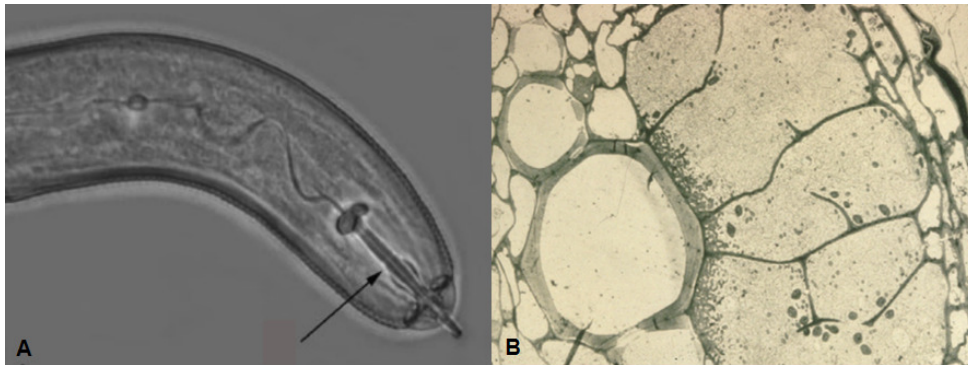


Figura 1: **A** - Região anterior do corpo de um fitonematóide com o estilete em evidência (Blok *et al.*, 2008). **B** - Sincício nas raízes de batata induzidas pela presença de fitonematoides sedentários (Jones *et al.*, 2013).

Dentre as diversas espécies de fitonematoides, todas apresentam o estilete na cavidade oral, porém diferentes hábitos alimentares são encontrados (Tabela 1). Alguns nematoides são denominados ectoparasitos migradores e parasitam as plantas sem adentrar o tecido vegetal, mas se deslocando pelo solo e se alimentando dos tecidos vegetais subterrâneos. Já os fitonematoides endoparasitos migradores, entram no tecido vegetal das plantas hospedeiras, causando danos significativos ao se deslocar e se alimentar de suas células. Contudo, a maioria dos nematoides de interesse econômico são endoparasitos sedentários, como os nematoides dos cistos (*Heterodera* sp.) e os nematoides das galhas (*Meloidogyne* spp.). Os fitonematoides endoparasitos sedentários adentram o tecido vegetal e induzem a formação de sítios especiais de alimentação (Figura 1B), onde as fêmeas adultas permanecem se alimentando até o fim do seu ciclo de vida (Taylor & Sasser, 1978; Jones, 2013).

Tabela 1. Classificação dos gêneros de fitonematoides de maior interesse econômico de acordo com seu gênero, hábito alimentar, sintomatologia e modo de ação (Pulavart *et al.*, 2021).

Gênero (Nome popular)	Hábito Alimentar	Sintomatologia	Modo de ação
<i>Meloidogyne</i> spp. (Nematoide das galhas)	Endoparasita Sedentário	Galhas nas raízes e caules subterrâneos	Induz a formação de células gigantes no sítio de alimentação.
<i>Heterodera</i> spp. (Nematoide dos cistos)	Endoparasita Sedentário	Cistos protegendo ovos viáveis	Dissolve a parede celular das plantas e funde os protoplasmas formando o sincício
<i>Pratylenchus</i> spp. (Nematoide das lesões)	Endoparasita Migrador	Lesões radiculares que podem levar ao apodrecimento da raiz	Migra pelos tecidos vegetais se alimentando de suas células

1.1. *Heterodera glycines*

Os nematoides dos cistos, nome popular das espécies do gênero *Heterodera* sp. e *Globodera* sp., são patógenos endoparasitos sedentários de culturas vegetais de grande importância econômica a nível mundial (Niblack *et al.* 2006; Jones, 2013). Dentre as espécies de nematoides dos cistos, a espécie *Heterodera glycines* Ichinohe (1952) se destaca por ser um patógeno de grande relevância para a cultura da soja (*Glycine max* [L.] Merr.) no mundo (Allen *et al.* 2017, Koenning & Wrather 2010). As perdas de produtividade da cultura da soja atribuídas a presença de áreas infestadas por *H. glycines* são estimadas em 5,24% na América do Sul e 9,31% na América do Norte (Savary *et al.*, 2019). Na China, foram estimadas perdas superiores a 120 milhões de dólares com o patógeno (Ou *et al.*, 2008). De maneira geral, as perdas econômicas mundiais atribuídas a *H. glycines* na cultura da soja ultrapassam 3 bilhões de dólares (Bandara *et al.*, 2020)

1.1.1. Ciclo de Vida

O nome popular da espécie *Heterodera glycines* (nematoide do cisto da soja) é devido a sua característica de formação da estrutura de sobrevivência denominada cisto (Figura 2). Entende-se por cisto o corpo da fêmea adulta morta que se torna resistente às condições ambientais adversas, enrijecendo e protegendo parte de seus ovos em seu interior.

Protegidos no interior do cisto, os ovos sofrem embriogênese resultando no juvenil de primeiro estágio (J1) que passa pela primeira ecdise ainda dentro do ovo e torna-se o juvenil de segundo estágio (J2). O J2 eclode do ovo e sendo ele a fase infectiva, migra no solo até encontrar e penetrar as raízes da planta hospedeira. Após a penetração, o J2 começa a induzir a formação de células especiais de alimentação, local que será o sítio de alimentação da fêmea adulta sedentária. O J2 passa por mais três ecdises, (J3, J4 e adulto) até atingir a fase adulta e ter a definição de macho ou fêmea, que apresentam dimorfismo sexual ao exibir morfologias bem distintas (Niblack *et al.* 2006).

Os indivíduos que se tornam machos apresentam corpo alongado de característica vermiforme, saem das raízes e migram para o solo e morrem após a fertilização de fêmeas. As fêmeas se tornam globosas, possuem entre 500 and 900 µm de comprimento, de coloração branca amarelada, e permanecem nas raízes se alimentando até a fase reprodutiva, onde elas expõem apenas uma pequena parte posterior do corpo para o lado de fora do tecido vegetal afim de possibilitar a cópula e a parte anterior permanece no interior dos tecidos radiculares. No processo de reprodução anfimítica de *H. glycines*, uma fêmea pode copular com diversos machos, promovendo uma grande variabilidade genética na espécie. As fêmeas de *H. glycines* depositam em torno de um terço de seus ovos envoltos por uma matriz gelatinosa, enquanto o restante permanece no interior do seu corpo. Assim, ao morrer a fêmea transforma-se no cisto de coloração marrom escura, capaz de

proteger os ovos em seu interior por cerca de oito a dez anos na ausência de plantas hospedeiras (Lauritis *et al.*, 1983; Triantaphyllou & Esbenshade, 1990; Niblack *et al.* 2006).



Figura 2: Cistos de *Heterodera glycines*: estrutura de sobrevivência da espécie onde as fêmeas ao morrerem enrijecem a sua cutícula protegendo ovos no seu interior, podendo permanecer desta forma por diversos anos (Foto de acervo pessoal).

1.1.2. Sintomatologia

A presença de *H. glycines* em lavouras de soja induz a manifestação de sintomas como redução do tamanho das plantas, amarelecimento das folhas, formação de reboleiras e redução da produtividade. Contudo, vários estudos demonstram que a produtividade já é significativamente impactada mesmo antes do aparecimento dos demais sintomas (Young, 1996; Wang *et al.*, 2003). No entanto, esses sintomas recorrentes da infecção por *H. glycines* são bastante genéricos, se assemelhando a sintomas causados por outros fitonematoides, fitopatógenos e até mesmo fatores abióticos. Dessa forma, a melhor estratégia de diagnóstico é a observação de sinais do patógeno (cistos), que podem ser analisados por meio da amostragem de solo de áreas contaminadas, que devem ser encaminhadas para análise em laboratórios de nematologia, antes que as populações atinjam níveis elevados (Niblack *et al.*, 2006). Nestas análises são necessárias inúmeras etapas

de lavagem, peneiramento, trituração e centrifugação das amostras. Além disso, a contagem de ovos e cistos, bem como a interpretação do resultado dependem de profissionais capacitados para tanto (Arjoune *et al.*, 2022).

1.1.3. Estratégias de manejo

As principais estratégias adotadas no manejo de *H. glycines* são a rotação de cultura, manejo do solo e uso de cultivares resistentes. Dentre estas estratégias, a utilização de cultivares resistentes é a mais usada devido a sua praticidade e viabilidade econômica (Niblack *et al.* 2002; Niblack *et al.*, 2006). Contudo, a alta variabilidade genética oriunda da reprodução anfimítica de *H. glycines*, com uma fêmea copulando com diversos machos, dificulta o desenvolvimento de cultivares resistentes ao patógeno (Triantaphyllou & Esbenshade, 1990; Young, 1996; Li & Chen, 2005; Brucker *et al.*, 2005).

Por conta disto, o uso de cultivares resistentes ao nematoide do cisto da soja pode gerar uma pressão seletiva, onde os indivíduos que evoluírem a capacidade de parasitar essas cultivares, serão selecionados e aumentarão as suas populações na área. Devido a estes atributos, a identificação de novas fontes de resistência a *H. glycines* são constantes (Niblack *et al.* 2002; Niblack *et al.*, 2006). Para a seleção da cultivar resistente a ser utilizada em uma área de plantio é importante a correta identificação do fitonematoide presente na área a ser manejada. No caso de *H. glycines*, também é importante a caracterização da população presente na área a ser manejada para que se realize a escolha, tendo em vista que populações com padrões de parasitismo diferentes são encontradas (Riggs & Schmitt, 1988; Niblack *et al.* 2002).

O primeiro esquema de caracterização de populações de *H. glycines* a ser elaborado, consiste na utilização de linhagens de plantas diferenciadoras para a segregação dos diferentes perfis de parasitismo em raças fisiológicas. As linhagens 'Peking', 'Pickett', PI 88788 e PI 90763 foram escolhidas como cultivares de

resistência e a cultivar Lee como padrão de suscetibilidade. O teste foi capaz de evidenciar quatro padrões distintos de parasitismo e com isso separar em quatro raças (Golden *et al.*, 1970). Em uma expansão do modelo, Riggs & Schmitt (1988) encontraram 16 raças fisiológicas para *H. glycines* (Tabela 2). A determinação da raça é feita com base na média de fêmeas obtidas em cada diferenciadora, em relação à média de fêmeas encontradas na cultivar 'Lee'. Em cada cultivar diferenciadora é calculado o índice de fêmeas (IF), sendo $IF (\%) = (\text{média de fêmeas na cultivar diferenciadora} / \text{média de fêmeas na cultivar 'Lee'}) \times 100$. Caso a diferenciadora apresentar $IF < 10 \%$ é classificada como resistente (-). Ao contrário, se apresentar $IF \geq 10 \%$ é considerada suscetível (+).

Tabela 2 – Raças de *Heterodera glycines*, de acordo com o esquema de determinação proposto por Riggs & Schmitt, 1988.

Raça	'Pickett'	'Peking'	PI 88788	PI 90763
1	-	-	+	-
2	+	+	+	-
3	-	-	-	-
4	+	+	+	+
5	+	-	+	-
6	+	-	-	-
7	-	-	+	+
8	-	-	-	+
9	+	+	-	-
10	+	-	-	+
11	-	+	+	-
12	-	+	-	+
13	-	+	-	-
14	+	+	-	+
15	+	-	+	+
16	-	+	+	+

(+) Suscetível ($IF \geq 10 \%$); (-) Resistente ($IF < 10\%$).

A determinação de raças de *H. glycines* utilizando as cultivares diferenciadoras são processos que demandam tempo e envolvem cultivares de soja que não são adaptadas para as condições climáticas do território brasileiro (Riggs & Schmitt, 1988). Além disso, o esquema é criticado por não ter sido atualizado mesmo com o surgimento de novas cultivares resistentes que poderiam aumentar o número de raças conhecidos caso tivessem sido incorporadas no teste. Na tentativa de aperfeiçoar a classificação, um esquema alternativo para a caracterização de populações de *H. glycines* foi desenvolvido utilizando sete cultivares com resistência para a classificação de populações em uma específica região como um “HG Tipo” em detrimento de categorizar como uma raça (Niblack *et al.*, 2002). Contudo, o método ainda apresenta algumas das falhas do esquema de determinação de raças e ainda gera confusão entre pesquisadores e profissionais da área (Niblack *et al.*, 2006; Tylka, 2016).

Sendo assim, apesar do esquema de determinação de raças sofrer diversas críticas por diversos pesquisadores da área, o mesmo continua sendo o procedimento adotado por diversas empresas e produtores de soja para caracterização das populações de *H. glycines* presentes no Brasil. Com isso, existe atualmente a demanda para que novas técnicas de caracterização de populações de *H. glycines*, com maior grau de praticidade sejam desenvolvidas (Niblack *et al.*, 2006).

1.2. *Meloidogyne incognita*

Os nematoides das galhas são os fitonematoides de maior importância econômica a nível mundial, devido a sua distribuição geográfica e ampla gama de hospedeiros, que ultrapassam 3000 espécies de plantas (Abad *et al.*, 2003). Em torno de 100 espécies de *Meloidogyne* já foram descritas e identificadas em praticamente todas as espécies vegetais cultivadas pelo homem (Elling, 2013).

Embora a maior prevalência ocorra em climas tropicais, os danos econômicos causados por *Meloidogyne* spp. tem sido cada vez mais evidenciados em diversos locais no mundo, incluindo em climas temperados como o do continente europeu (Wesemael *et al.*, 2011). Dentre os *Meloidogyne* spp., a espécie *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, pode ser considerada a mais relevante ao se considerar a sua imensa gama de hospedeiros e alta taxa de reprodução (Vos *et al.*, 2013). São estimadas perdas de 100 bilhões de dólares todos os anos causadas por *Meloidogyne incognita* no mundo (Mukhtar *et al.*, 2014).

1.2.1. Ciclo de Vida

As fêmeas adultas de *Meloidogyne incognita* realizam a ovoposição em uma matriz gelatinosa que protege os ovos em seu interior. Essa estrutura denominada de massa de ovos, pode abrigar mais de 500 ovos em seu interior. No seu ciclo de vida (Figura 3), após o processo de embriogênese, o juvenil de primeiro estágio (J1) é formado e ainda no interior do ovo passa pela primeira ecdise, dando origem ao juvenil de segundo estágio (J2), o qual eclode do ovo e vai para o solo (Jones, 2013). O J2 em condições favoráveis, as quais dependem da umidade e temperatura, se desloca no solo até localizar as raízes de plantas hospedeiras (Goodell & Ferris, 1989).

Para a penetração do sistema radicular, o J2 utiliza uma combinação de seu estilete para perfurar e enzimas capazes de degradar a parede celular do tecido vegetal. Ainda no estágio J2, o nematoide induz alterações no tecido vegetal e inicia a formação de um sítio de alimentação, por meio da formação de células gigantes das quais obterá seus nutrientes durante o seu ciclo de vida. O mecanismo pelo qual ocorre a formação e manutenção das células gigantes não foi devidamente elucidado, mas efetores produzidos nas glândulas esofagianas dos nematoides desempenham papel fundamental no processo. Iniciando a sua alimentação, o J2 irá se desenvolver e passar por mais três ecdises até atingir a fase adulta. Os J3 e

J4 não possuem estilete funcional e, portanto, não se alimentam. Os machos adultos mantêm a morfologia vermiforme e se deslocam para o solo. As fêmeas adultas permanecem sedentárias, se alimentando no sítio de alimentação, aumentando de tamanho e adquirindo um formato de pêra. Os nematoides do gênero *Meloidogyne* spp. apresentam reprodução majoritariamente partenogênica, sendo que os machos são pouco numerosos em relação ao número de fêmeas formadas. As fêmeas adultas então iniciam o processo de ovoposição dentro da matriz gelatinosa iniciando um novo ciclo de vida (Moens *et al.*, 2009; Elling, 2013; Hewezi & Baum, 2013; Jones, 2013).

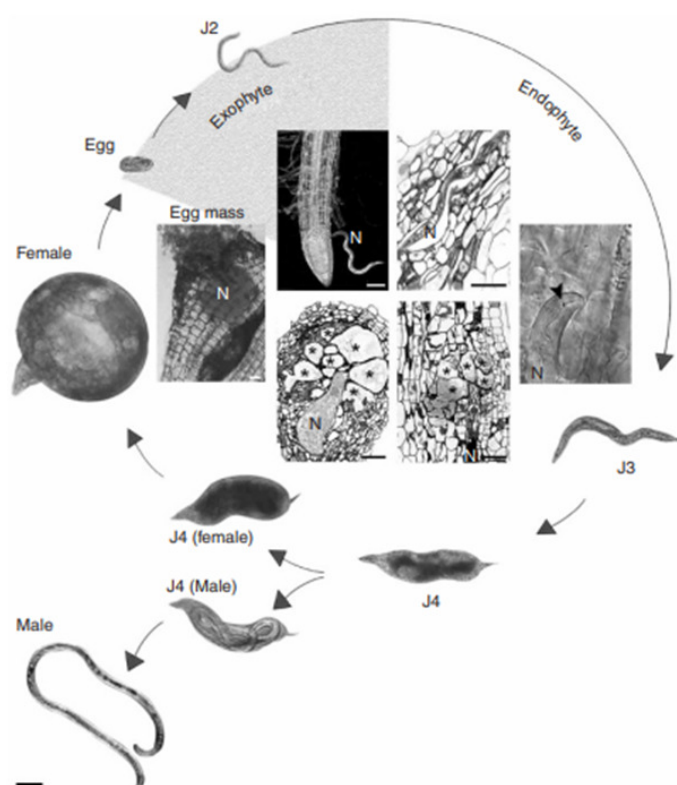


Figura 3: Ciclo de vida do fitonematoide *Meloidogyne incognita*. A fase infectiva J2 penetra a raiz e migra para o cilindro vascular. O estilete é utilizado para perfurar a parede celular, liberar secreções esofagianas e capturar nutrientes. O J2 induz a diferenciação das células das raízes para células de alimentação hipertrofiadas e multinucleadas, que servirão de alimento para o nematoide no restante de seu ciclo de vida. O nematoide se torna sedentário e passa por três ecdises (J3, J4 e adulto). Os machos se desenvolvem e migram para o exterior das raízes, não participando na reprodução da espécie. As fêmeas se tornam globosas se reproduzem por partenogênese e produzem massas de ovos que são liberadas para a superfície das raízes. Após o processo de embriogênese, o J1 passa pela primeira ecdise, tornando-se J2 e eclodindo do ovo, reiniciando o ciclo (Abad *et al.*, 2008).

1.2.2. Sintomatologia

Os nematoides do gênero *Meloidogyne* spp. são conhecidos pelo seu nome popular “nematoide-das-galhas” devido ao sintoma característico de sua infecção (Figura 4). As fêmeas adultas desta espécie possuem hábito sedentário e permanecem dentro do tecido vegetal infectado induzindo a formação de células gigantes das quais elas se alimentaram pelo restante de seu ciclo de vida. Este processo de hiperplasia e hipertrofia das células do tecido vegetal infectado acaba levando a formação de pontos de engrossamento das raízes conhecidas pelo nome “galhas” (Taylor & Sasser, 1978; Trudgill & Blok, 2001; Almeida-Engler & Gheysen 2013; Jones, 2013; Bartlem *et al.*, 2014; Cabrera *et al.*, 2015).



Figura 4: Raízes de soja com galhas. Sintoma típico da infecção do sistema radicular com nematoides do gênero *Meloidogyne*, onde ocorre a hiperplasia e hipertrofia dos tecidos vegetais gerando pontos de engrossamento nas raízes (Foto de acervo pessoal).

O aumento na demanda energética para manutenção das células gigantes presentes nos sítios de alimentação, faz com que a planta redirecione a energia produzida via fotossíntese da parte aérea para as raízes. Tal modificação metabólica pode acarretar em outros sintomas típicos da infecção por *M. incognita*, como redução no crescimento, descoloração das folhas e deformação do sistema radicular (Jones *et al.*, 2013)

1.2.3. Estratégias de Manejo

O manejo de *M. incognita* por muitos anos teve como principal estratégia o uso de nematicidas químicos e cultivares resistentes (Giannakou & Anastasiadis, 2005). A utilização de cultivares resistentes, apesar de ser uma estratégia viável, tem a limitação de que os processos de obtenção de novas cultivares são morosos e os recursos genéticos disponíveis para o desenvolvimento de resistência são limitados (Davies & Elling, 2015). Por diversos anos, cultivares portadoras do gene Mi foram utilizadas no controle de *Meloidogyne* spp. na cultura do tomate. Contudo, relatos de populações de *Meloidogyne javanica* e *Meloidogyne incognita* quebrando a resistência do gene tem levantado preocupações acerca da viabilidade da manutenção da estratégia de controle (Wesemael *et al.*, 2011).

Por outro lado, o uso de nematicidas químicos tem sido cada vez mais desencorajado ou até mesmo banido em vários países, devido aos potenciais danos ambientais e ao ser humano que essas formulações podem trazer (Aktar *et al.*, 2009; Coyne *et al.*, 2018; Abd-Elgawad, 2021).

Dentre os nematicidas químicos, a abamectina tem sido um dos princípios ativos mais utilizados no controle de *M. incognita* (Faske & Starr, 2007; Khalil *et al.*, 2012; Khalil, 2013; El-Marzoky *et al.*, 2022). A abamectina é uma molécula do grupo químico das avermectinas, que são lactonas macrocíclicas comumente utilizadas como ingrediente ativo de pesticidas contra insetos e de drogas no tratamento de

vermes parasitários (Batiha, 2020). A exposição a baixas concentrações de uso de abamectina normalmente não causa o aparecimento de sinais e sintomas adversos. Porém, o uso indiscriminado pode incorrer em sintomas como vômito, salivação excessiva, letargia, diarreia, entre outros (Nasr *et al.*, 2016).

Além disso, estudos recentes demonstraram o efeito tóxico da abamectina em espécies de abelhas, levando a redução da saúde das colmeias e maior taxa de mortalidade (Aljedani, 2017; Li *et al.*, 2022). A exposição à derivados de avermectina, em especial a abamectina, associada a mudanças no meio ambiente para práticas de agricultura e pecuária estão vinculadas com a maior mortalidade de espécie de abelhas sem ferrão (Obregon *et al.*, 2024). A perda da diversidade dessas abelhas sociais se torna um grave problema já que essas espécies são importantes polinizadores de diversas plantas e cultivares de interesse agrônomo, além de fornecerem matéria-prima para o desenvolvimento de produtos de uso biotecnológico (Heard, 1999; Slaa *et al.*, 2006; Santos *et al.*, 2022). Com isso em mente, alternativas mais viáveis e com preocupações ecológicas têm sido pesquisadas para o controle de *M. incognita*, dentre elas o controle biológico (Hu *et al.*, 2022; Tian *et al.*, 2022).

Nos últimos anos, diversos estudos com micro-organismos, como fungos e bactérias, vêm identificando potenciais agentes para o uso no controle biológico de fitonematoides (Tian *et al.*, 2010; Silva *et al.*, 2017; Jin *et al.*, 2019; Migunova & Sasanelli, 2021). Tais métodos de controle biológico têm se mostrado como estratégias ambientalmente seguras e eficazes de combate a pragas e patógenos (Mhatre *et al.*, 2019; Lahlali *et al.*, 2022). Alguns destes estudos têm descritos micro-organismos com ação específica contra *Meloidogyne incognita* (Kim *et al.*, 2018; Park *et al.*, 2020; Pocurull *et al.*, 2020; Sharma *et al.*, 2020; Wang *et al.*, 2022).

O mecanismo de ação descrito no controle biológico varia de acordo com o micro-organismo utilizado. Alguns dos mecanismos mais comuns envolvem fungos e bactérias capazes de parasitar ovos e juvenis de fitonematoides ou micro-organismos capazes de inibir a eclosão de ovos (Davies & Curtis, 2011; Jin *et al.*, 2017; Topalović *et al.*, 2019; Cheng *et al.*, 2017; Park *et al.*, 2020; Khoja *et al.*, 2021;

Sun *et al.*, 2021). Algumas espécies dos gêneros *Pasteuria* spp. e *Pochonia* spp. apresentam outras formas de controle, podendo parasitar desde ovos até as fêmeas de *Meloidogyne* spp., mas também podem secretar metabólitos com efeito nematicida ou que possam ativar defesas das plantas contra os nematoides (Giné *et al.*, 2016; Ghahremani *et al.*, 2019).

1.2.4. Identificação

O adequado manejo de fitonematoides exige a correta identificação da espécie presente na área a ser tratada. Contudo, a identificação de espécies de *Meloidogyne* sp. apresenta um histórico de dificuldades, com espécies novas sendo identificadas erroneamente como outras já descritas. Tradicionalmente, a identificação de *Meloidogyne* spp. é baseada em caracteres morfológicos, em especial o padrão perineal das fêmeas adultas (Figura 5), sendo que todas as espécies descritas possuem o registro do padrão. Com isso, a técnica segue sendo crucial para pesquisadores que trabalham com *Meloidogyne* spp., porém, essas análises demandam profissionais em taxonomia com experiência e habilitados para a correta identificação (Wesemael *et al.*, 2011; Elling, 2013).



Figura 5: Padrão perineal de *Meloidogyne chitwoodi*. Os diferentes padrões perineais são importantes ferramentas para a identificação de espécies de *Meloidogyne* (Elling, 2013).

Como alternativa, diversos laboratórios de pesquisa utilizam a eletroforese de isoenzimas, em especial de esterases e malato desidrogenase (Esbenshade & Triantaphyllou, 1985). A técnica não exige profissionais com alto grau de conhecimento, oferece custos baixos e tem a cobertura de vasta gama de espécies de *Meloidogyne* sp. descritas. Contudo, a identificação por técnicas morfológicas e por eletroforese de isoenzimas podem apenas ser realizadas em fêmeas, não sendo possível utilizar as demais fases do ciclo de vida, criando dificuldade na hora da coleta do animal (Wesemael *et al.*, 2011; Elling, 2013).

Para a identificação de espécies de *Meloidogyne* spp. em qualquer fase do ciclo de vida, a reação em cadeia polimerase (PCR), se mostra como uma técnica valiosa, uma vez que o DNA não depende da fase de desenvolvimento do animal (Wesemael *et al.*, 2011). Diversos primers e métodos de PCR estão disponíveis na literatura e a técnica inclusive permite a correta identificação com alto grau de sensibilidade mesmo em amostras que apresentam mistura de várias espécies (Wishart *et al.*, 2002; Adam *et al.*, 2007).

Sendo assim, as técnicas de PCR são um recurso valioso na identificação de fitonematoides, mas a utilização dessas técnicas é mais cara que a análise morfológica e eletroforese de isoenzimas e sem a presença de profissionais taxonomistas podem aumentar os riscos de resultados falsos (Elling, 2013). Estudos prévios mostram primers específicos para *M. fallax* indevidamente reagindo com primers de *M. minor* e gerando uma fita amplificada de mesmo tamanho (Nischwitz *et al.*, 2013). Com isso em mente, pode-se afirmar que apesar de várias técnicas estarem disponíveis para a identificação de espécies de *Meloidogyne* spp., ainda existe a necessidade de maior integração entre os métodos ou o surgimento de métodos mais simples e de menor custo (Wesemael *et al.*, 2011; Elling, 2013).

2. Abelhas sem ferrão (Hymenoptera, Apidae)

2.1. Classificação e importância

As abelhas sem ferrão, (Meliponini), se caracterizam por ser um grupo de espécies de abelhas que apresentam uma atrofia do ferrão, que perde a sua funcionalidade como uma ferramenta de defesa. Com cerca de 605 espécies, são o grupo de abelhas mais abundantes em regiões tropicais e subtropicais (Slaa, 2006; Engel *et al.*, 2023). Estas abelhas sociais estão diretamente vinculadas a processos de polinização de plantas nativas e cultiváveis, sendo consideradas extremamente relevantes no processo produtivo de diversos itens que compõem a alimentação humana. Algumas estimativas indicam que 30% da polinização de plantas cultiváveis para a alimentação humana dependem de abelhas (Kerr *et al.*, 1996; Heard, 1999; Slaa *et al.*, 2006; Atmowidi *et al.*, 2022).

Apesar da relevância de inúmeras espécies de abelhas ser amplamente evidenciada em trabalhos científicos, muitas espécies do grupo estão em risco de extinção devido à perda de habitat natural e práticas agrícolas (Obregon *et al.*, 2024). Alguns dos efeitos do desmatamento de áreas naturais e de práticas agrícolas às abelhas sem ferrão incluem a exposição a doses letais de defensivos agrícolas, mudanças na composição da dieta, mudanças no tamanho corporal, redução na performance produtiva da colônia e conseqüentemente a redução da densidade populacional (David *et al.*, 2016; Gutiérrez-Chacón *et al.*, 2018; Buchori *et al.*, 2019; Mayes *et al.*, 2019; Machado *et al.*, 2020).

2.2. O Alimento Larval de Abelhas sem ferrão

O alimento larval das abelhas sem ferrão é constituído por uma mistura de pólen maturado, mel e secreções glandulares. Após a ovoposição da rainha, este alimento é depositado nas células de cria, que permanecem fechadas até a eclosão

dos adultos (Kerr *et al.*, 1996; Nogueira-Neto, 1997). O alimento larval é composto por 40 a 60% de água, 5 a 12 % de açúcares, 1,1 a 19,4 % de proteínas e 0,2 a 1,3% de aminoácidos (Hartfelder & Engels, 1989).

As abelhas sem ferrão apresentam uma diversidade de micro-organismos associados às colônias, como bactérias, leveduras e fungos filamentosos que parecem estar associados à fermentação do néctar e maturação do pólen, produção de moléculas antimicrobianas e nutritivas para as abelhas, além de protegerem a colmeia contra patógenos (Dillon, 2004; Hamdi *et al.*, 2011; Vásquez *et al.*, 2012; Voulgari-Kokota *et al.*, 2019). O papel efetivo da microbiota associada ao alimento larval de abelhas sem ferrão na manutenção das colônias ainda exige estudos mais aprofundados (Ngalimat *et al.*, 2020; de Paula *et al.*, 2021).

O uso de bactérias associadas ao alimento larval de abelhas sem ferrão tem se mostrado promissor na produção de probióticos, enzimas e agentes de controle biológico de patógenos. Em associação com o alimento larval de abelhas dos gêneros *Melipona* sp. e *Tetragonisca* sp., foram encontradas bactérias dos gêneros *Enterococcus* sp., *Providencia* sp., *Serratia* sp. e *Vagococcus* sp. capazes de produzir substâncias antimicrobianas contra patógenos Gram-positivos e Gram-negativos (Santos *et al.*, 2022).

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CAPÍTULO 2

Artigo científico a ser submetido para a revista BMC Microbiology

**Screening of microorganisms isolated from stingless bee's larval food in the
biocontrol of *Meloidogyne incognita***

Screening of microorganisms isolated from stingless bee's larval food in the biocontrol of *Meloidogyne incognita*

Guilherme Nunes Moreira Costa, Ana Carolina Costa Santos, Tamires dos Santos Paschoal, Anna Paula Martins Garcia, Anderson Rodrigues dos Santos, Carlos Ueira-Vieira.

Abstract

Background: Plant-parasitic nematodes can infect several plant species and cause numerous financial losses for agricultural producers. The species *Meloidogyne incognita* occupies a prominent position due to its large range of hosts. The use of several microorganisms for the biological control of nematodes has been in high demand, given their effectiveness and greater safety over chemical nematicides. With this in mind, this work evaluated the potential for using microorganisms present in stingless bee's larval food in the biological control of *M. incognita*.

Results: The screening of microorganisms present in stingless bee's larval food showed the presence of a bacteria with nematicidal effect on *M. incognita*. Tests in pots with soybean plants demonstrated that the bacteria Mq-MCK-07 reduced the population of *M. incognita*, while in vitro tests demonstrated the nematicidal capacity against second-stage juveniles (J2) as well as the inhibitory effect on egg hatching. By sequencing the bacteria's DNA, we were able to identify the bacteria as *Enterococcus faecalis mandacaium* and identify possible genes of relevance in the control of multiple pathogens.

Conclusion: The *E. faecalis mandacaium* strain isolated from stingless bee's larval food, exhibited nematicidal activity against *M. incognita*. The genome analysis of this bacteria indicated several potential genes implicated in biocontrol effects of several pathogens. Thus, this work provided a theoretical background for *E. faecalis mandacaium* as a potential candidate for an eco-friendly nematicide.

Keywords: Plant-parasitic nematodes, Root-knot nematode, Biological Control, Nematicidal activity, Phytopathology, Bacteria.

Background

Plant-parasitic nematodes (PPNs) infect several plant crops to obtain nutrients, harming the normal growth of their hosts [1]. Estimates indicate that the damage caused to crops by these organisms can lead to enormous financial losses that could exceed US\$100 billion annually worldwide [2-5]. Among PPNs species, Root-Knot nematodes (*Meloidogyne* spp.) have one of the widest host ranges and can infect several plant species, from vegetables and grasses to even fruit trees [6-8].

Adult females of *Meloidogyne* sp. exhibit sedentary behavior and during their parasitism, these nematodes induce hyperplasia and hypertrophy of cells in their feeding site, forming thickening points in the root, where several giant cells are used for their feeding and interrupt the normal growth of plant tissue [9-12]. These points of root thickening are called galls (root-knots), the main symptom of *Meloidogyne* spp. infection and gives the vernacular name to the genus [13]. The root-knot formation causes destruction of the vascular system and affects the ability of infected plants to absorb water and nutrients [11].

The species *Meloidogyne incognita* is one of the main species of the genus due to its frequent occurrence, short life cycle, high reproduction capacity and high economic damage. Furthermore, these nematodes can facilitate infection by secondary pathogens such as fungi and bacteria, leading to secondary damage and significant reductions in crop productivity [13,14].

Currently, several control strategies for *M. incognita* populations are used, the most common being the use of chemical nematicides and resistant cultivars [15,16]. However, the excessive use of chemical nematicides can be harmful to the environment and humans, in addition to generating high costs in cases of wide geographic distribution of the nematode in the field [4,17,18]. The use of resistant cultivars has its limitation in the difficulty of obtaining new cultivars, as they require long processes and have limited genetic resources for the development of resistance to PPNs [19]. Crop rotation can help control this nematode, but it is not always a viable strategy due to the equipment needed to maintain different crops

[20]. As a result, more viable alternatives with ecological concerns have been researched, including biocontrol [5,21].

Biocontrol methods have proven environmentally safe and effective strategies to control pests and pathogens, including PPNs [22,23]. In recent studies, some microorganisms, such as fungi and bacteria, have been identified as potential agents for use in the biocontrol of PPNs [24-27]. Some studies have described microorganisms with specific action against *M. incognita* [28-32]. Bacteria from the genera *Bacillus* and *Pseudomonas*, microorganisms present in the rhizosphere of plants, play a prominent role in the biological control of PPNs [22].

The microorganisms used in biocontrol have different forms of interaction with PPNs. Some microorganisms parasitize eggs and juveniles, while others are capable of inhibiting egg hatching or repelling the second-stage juveniles (J2s) of root-knot nematodes [29,33-38]. Species of the genera *Pasteuria* and *Pochonia* present other forms of control, being able to parasitize from eggs to females of *Meloidogyne* spp., but they can also secrete metabolites with a nematocidal effect or that can activate plant defenses against nematodes [39,40].

Several microorganisms associated with stingless bee's colonies appear to be associated with nectar fermentation and pollen maturation, production of antimicrobial and nutritional molecules for bees, in addition to protecting the hive against pathogens [41-44]. In recent studies, bacteria associated with stingless bee's larval food have shown promise in the production of probiotics, enzymes and biocontrol agents for pathogens. Our group recently showed that microorganisms association with larval food from bees of the genera *Melipona* and *Tetragonisca*, were capable of producing antimicrobial substances against Gram-positive and Gram-negative pathogens [45]. Therefore, the objective of this study was to evaluate the potential use of microorganisms from stingless bee's larval food in the biocontrol of *Meloidogyne incognita*.

Methods

Microorganisms obtention

The fungi and bacteria used in this work were obtained from the collection of microorganisms isolated from stingless bee's larval food (CoMisBee) at the Laboratory of Genetics from the Federal University of Uberlândia [46]. Bacteria were reactivated on BHI agar and incubated for 24 h at 37 °C. One loop was transferred to 50 mL of Luria Berthani (LB) medium and incubated at 37 °C $\pm$ 1 for 48 h at 200 rpm shaking. To obtain the supernatant, the medium was centrifuged at 10,000 g for 4 minutes to sediment the bacterial cells and the supernatant was separated from the precipitate and filtered at 22 μ m. The fungi were reactivated in Potato-Dextrose Agar medium and incubated for 120 h at 30 °C, and after the formation of the spore mat, they were washed with 1 mL of 0.9% saline and collected.

Nematode inoculum

The initial inoculum of *Meloidogyne incognita* was provided by the Laboratory of Nematology from the Federal University of Uberlândia and multiplied in pots with tomato and okra plants kept for 60 days in a greenhouse. After this period, the roots were processed using the described nematode extraction technique with modifications. The roots were placed in a blender for 20 seconds in a 0.5% sodium hypochlorite solution and poured into superimposed 100 and 500 mesh sieves [47]. The contents of the 500 mesh sieve were collected and the suspension with *M. incognita* eggs was calibrated to the desired concentration.

Screening for microorganisms with nematicidal potential

To search for possible biocontrol agents and evaluate the nematicidal effect, 10 microorganisms from CoMisBee were tested, five bacteria (Mq-MCK-07; Mq-

NUT-16; Ms-MSA-9; Ms-MH-21; Ms-BDA2-41C) and five fungi (Fv-SAB-01; Mq-OAT-03; Ms-BDA2-35; Ms-ISP-46C; Ta-BDA2-26). The experiment was conducted in a completely randomized design in a greenhouse and for each treatment, 5 pots with 1.5 L capacity were prepared with a mix of sand and clay substrate (2:1 ratio), previously analyzed to ensure the absence of nematodes. For the bacteria treatments, 10 mL of medium containing bacteria grown for 48 h were used whereas the fungi treatments were prepared with 10 mL of suspension with 3×10^7 fungal spores. Each treatment was applied to the planting furrow at the time of sowing the soybean cultivar Brasmax Desafio RR (8473 RSF). For the negative control, pots were prepared with only soybean seeds and for the positive control, pots were prepared with the commercial product Nemat® (Ballagro product based on *Paecilomyces lilacinus*), used for biocontrol of *M. incognita*, using the recommended dose suggested by the manufacturer. The 60 pots were inoculated with 10 mL of *M. incognita* suspension containing 500 eggs/mL (5000 eggs). After 60 days of inoculation, the root mass was measured and the nematodes were extracted from the roots using the previously described nematode extraction technique and counted in a Peters chamber with the aid of an optical microscope.

Validation of nematicidal potential

Based on screening assay, the bacterial isolate Mq-MCK-07 was chosen and its nematicidal effect was validated in this second experiment. For this, 4 different treatments were compared, being: untreated (soybean plants without nematodes), negative control (soybean plants inoculated with *M. incognita*), Mq-MCK-07 (soybean plants inoculated with *M. incognita* and Mq-MCK-07) and positive control (soybean plants inoculated with *M. incognita* and treated with Nemat®). In each treatment, 10 pots with 1.5 L capacity were prepared with the same mix of the previous assay and seeds of the soybean cultivar 8473 RSF were sown. For the test subject, 10 mL of Mq-MCK-07 medium grown for 48 h were applied to the planting furrow at the time of the sown. The pots with the presence of *M. incognita* were inoculated with 10 mL of a suspension containing 500 eggs/mL (5000 eggs).

After 60 days of inoculation, nematodes were extracted from the roots and counted in a Peters chamber with the aid of an optical microscope. The nematicidal effect was evaluated by comparing the initial and the final nematode population.

Egg hatching inhibition test

To evaluate the ability of the isolate Mq-MCK-07 to inhibit *M. incognita* egg-hatching, a test was performed in a 24-well plate containing 4 treatments: distilled water as negative control, Luria Bertani medium, Mq-MCK-07 medium grown for 48 h and the supernatant of Mq-MCK-07 medium grown for 48 hours. For each treatment, 5 replications were performed, where each well contained a known number of 50 eggs. The plate was kept for 72 hours at 28 °C and the number of hatched J2s and eggs were counted using an optical microscope [21,48]. The hatching rate was calculated with the formula: $\text{Hatching rate} = [(\text{Number of J2s hatched} / \text{Total eggs}) \times 100]$.

***In vitro* nematicidal effect test**

The nematicidal effect of the isolate Mq-MCK-07 on *M. incognita* J2s was tested in a 24-well plate containing 4 treatments: distilled water as negative control, Luria Bertani medium, Mq-MCK-07 medium grown for 48 h and the supernatant of Mq-MCK-07 medium grown for 48 h. For each treatment, 5 replications were performed, where each well contained a known number of 100 J2s of *M. incognita*. The plate was kept for 24 hours at 28 °C and then evaluated under optical microscopy. Nematodes were considered dead when their bodies were straight and did not move when stimulated with 0.5 M NaOH [21,48]. The mortality rate was calculated by the formula: $\text{Mortality rate} = [(\text{Number of dead J2s} / \text{Total J2s}) \times 100]$.

DNA extraction

The isolate Mq-MCK-07 were cultured in Brain Heart Infusion (BHI) medium and incubated for 48 hours at 37°C under 200 rpm agitation. Once the incubation ended, the bacterial culture was centrifuged at 14,000 rpm for 10 minutes at 25°C, and the supernatant was discarded. Next, 1 mL of extraction buffer (0.1 M Tris-HCl or 100 mM Tris-HCl, 500 mM NaCl, 50 mM EDTA, and 0.4% SDS) was added to the bacterial pellet and vortexed for 15 seconds. Subsequently, 50 µL of proteinase K was added, and the samples were incubated at 56°C for 30 minutes. After incubation, the samples were centrifuged at 14,000 rpm for 10 minutes at room temperature.

The supernatant was transferred to a new microtube containing 330 µL of isopropanol. The tubes were gently inverted 30 times and then centrifuged at 14,000 rpm for 10 minutes at room temperature. The supernatant was discarded, and 500 µL of 70% ethanol was added to the pellet. After 30 seconds of centrifugation, the supernatant was discarded, and this step was repeated twice to ensure DNA purification. The supernatant was discarded, and the DNA pellet was air-dried for 15 minutes. Finally, the pellet was resuspended in 50 µL of ultrapure water, and the quantity and quality of the extracted DNA was assessed using a Nanodrop 2000 Spectrophotometer and agarose gel electrophoresis.

Genome sequencing and data analysis

The genome sequencing was performed by the BGISEQ-500 sequencing system using DNBSEQ™ Sequencing Technology at BGI Genomics (Hong Kong, China). For DNBSEQ™ Short-read library preparation, the genomic DNA was fragmented and underwent size selection. For the sequencing library, insert size was 300 bp with a pair-end sequencing length of 150 bp. The selected fragments were 3'-adenylated through end-repair and adapter-ligation. A single-strand circular

DNA was form as the final library and quality control was performed using a clean FastQ phred quality score encoding a threshold of Phred +33.

After the sequencing, all the raw data were trimmed and low-quality sequences was filtered using SOAPnuke v.2.1.9 [49]. Assembled contigs with lengths less than 150 bp or with the N content in the sequencing read equal 0.1% or more were discarded in the subsequent analysis. The output read quality value system was set to Phred+33.

The whole genome was assembled using the SPAdes genome assembler v3.15.4 contrasting with the NCBI data base using BLASTN tool. The species identification was made using an algorithm developed in house (unpublished data).

The prediction of regions for antimicrobial resistance or virulence genes was performed using ABRicate [50], the secondary metabolites analysis was performed using antiSMASH 7.0 software [51] and Artemis for genome visualization and annotation [52].

Statistical analysis

The data analysis was performed using Graphpad Prism software version 10.1.1. using One-Way analysis of variance (ANOVA) and Tukey multiple comparisons test ($p < 0.05$). All data are presented as mean $\pm$ standard deviation.

Results

Screening for microorganisms with nematicidal potential

The evaluation of the nematicidal potential of 10 microorganisms tested from CoMisBee demonstrated that only the bacterial isolate Mq-MCK-07 exhibits a probable nematicidal effect, as it was able to reduce the number of nematodes per

gram of soybean root when compared to the control treatment and other treatments (Figure 1).

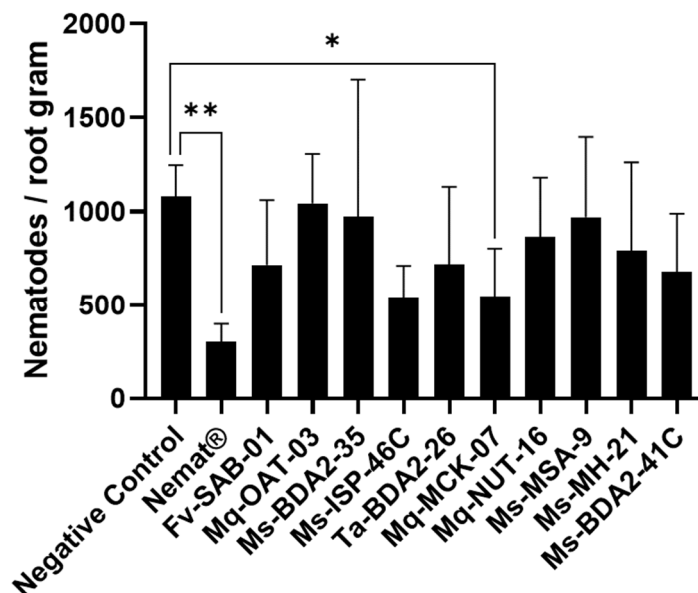


Figure 1: Nematicidal activity of microorganisms isolated from stingless bee's larval food against *M. incognita* inoculated in pots with soybean. The negative control was water and the positive control was the commercial biocontrol product Nemat® (recommended dosage). Data analysis was performed using One-Way analysis of variance (ANOVA) and Tukey multiple comparisons test, in the Graphpad Prism software version 10.1.1.. *p < 0.05; **p < 0.01

Validation of the nematicidal effect of Mq-MCK-07 on *M. incognita*

In the validation assay, the bacterial isolate Mq-MCK-07 was efficient in reducing the total number of nematodes in soybean roots and in reducing the number of nematodes per gram of root when compared to the control treatment. This result validates its potential effect against *M. incognita*. When compared with the commercial product Nemat®, there was no significant difference between treatments (Figure 2).

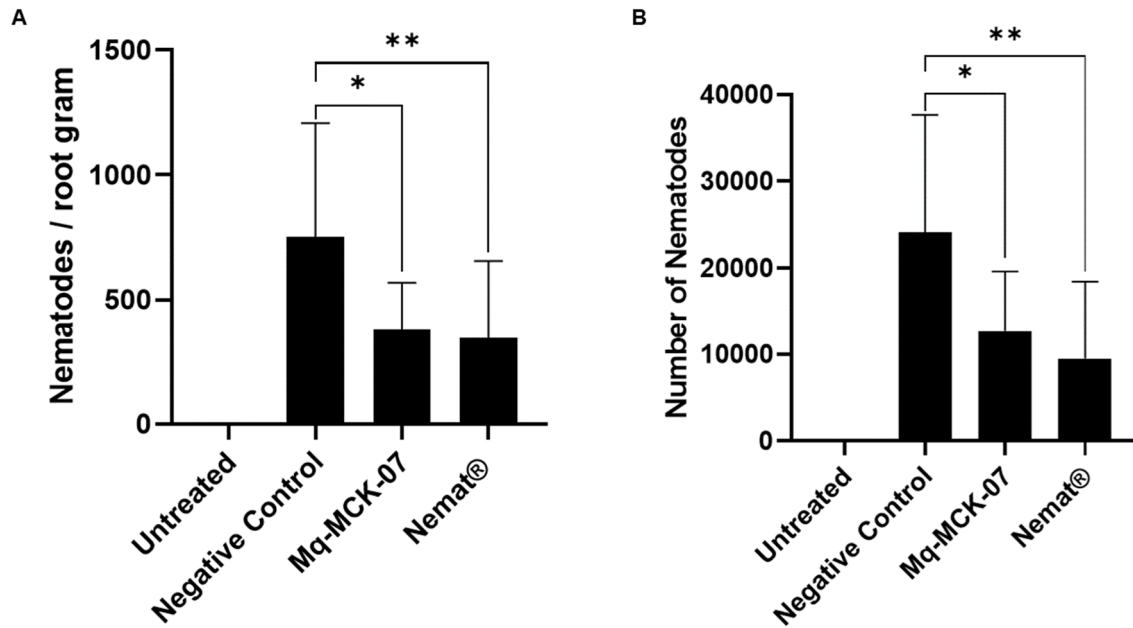


Figure 2: The isolate Mq-MCK-07 effect on *M. incognita* inoculated in pots with soybean. **(A)** Number of nematodes per root gram 60 days after inoculation. **(B)** Number of nematodes in the soybean roots 60 days after inoculation. Untreated pots with no nematode inoculation were set to check possible contaminations among treatments. The negative control was water and the positive control was the commercial biocontrol product Nemat® (recommended dosage). Data analysis was performed using One-Way analysis of variance (ANOVA) and Tukey multiple comparisons test, in the Graphpad Prism software version 10.1.1. *p < 0.05; **p < 0.01

Inhibition of *Meloidogyne incognita* egg hatching

The *M. incognita* egg-hatching rate after 72 h of incubation with different treatments were 26.8% in water (100% v/v, pH 6.8); 23.6% in Luria Bertani medium (50% v/v, pH 7.2); 22.8% in Mq-MCK-07 supernatant (50% v/v, pH 8.6) and 8% in culture medium with Mq-MCK-07 (50% v/v, pH 8.6), indicating egg hatching inhibition caused by the presence of Mq-MCK-07 (Figure 3).

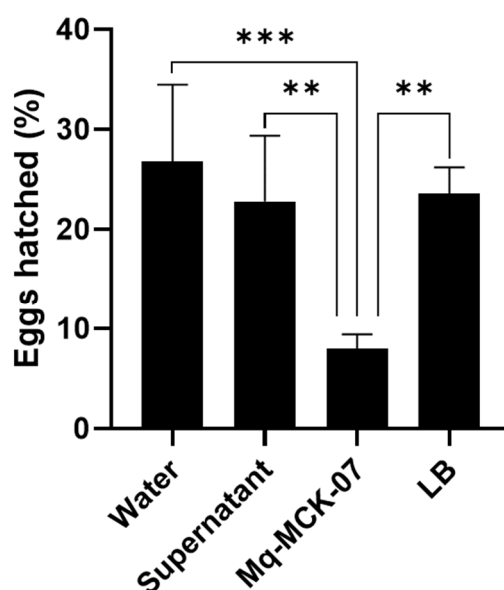


Figure 3: The bacteria Mq-MCK-07 inhibited *M. incognita* egg-hatching *in vitro*. Egg hatching rates were evaluated with 50 *M. incognita* eggs incubated at 28°C for 72 h with different treatments. The negative control was distilled water. Data analysis was performed using One-Way analysis of variance (ANOVA) and Tukey multiple comparisons test, in the Graphpad Prism software version 10.1.1. **p < 0.01; ***p < 0.001

Nematicidal effect on juveniles (J2s) of *Meloidogyne incognita*

The mortality rates of J2s of *M. incognita* after incubation with different treatments were 10% in water (100% v/v, pH 6.8); 21.6% in Luria Bertani medium (50% v/v, pH 7.2); 28.8% in Mq-MCK-07 supernatant (50% v/v, pH 8.6) and 65.2% in culture medium with Mq-MCK-07 (50% v/v, pH 8.6), indicating nematicidal effect of isolate Mq-MCK-07 against J2s of *M. incognita* (Figure 4).

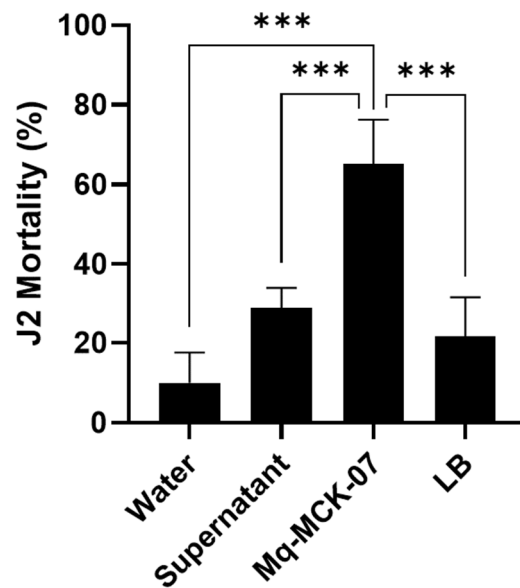


Figure 4: The isolate Mq-MCK-07 showed nematicidal effect on J2s of *M. incognita* in the *in vitro* test. Mortality rates were evaluated with 100 J2s of *M. incognita* incubated at 28°C for 24 h with different treatments. The negative control was distilled water. Data analysis was performed using One-Way analysis of variance (ANOVA) and Tukey multiple comparisons test, in the Graphpad Prism software version 10.1.1. ***p < 0.001

Species identification and genomic features

The genome analysis with "Species Matching " indicated that the bacterial isolate Mq-MCK-07 matched with *Enterococcus faecalis* sequences deposited on NCBI database. Due to its origin, we decided to name this isolate of *Enterococcus faecalis* strain mandacaium (*E. faecalis mandacaium*). Further analysis of genome characteristics revealed that the *E. faecalis mandacaium* genome comprised a circular chromosome of 2.6 Mb (Figure 5), and was deposited in NCBI with the accession number CP159511. The *E. faecalis mandacaium* chromosome included 2,612,373 bp, with an average GC-content of 37.91%. There were found 2,481 protein-coding genes, being that 2,155 of those presented 95% or more of identity with the protein of the reference genome of the alignment. There were also found 46 tRNAs and 8 rRNAs comprising a total of 2,535 genes. Two plasmid sequences were predicted in the analysis, named plasmid 1 with 20,406 bp (Figure 6) and plasmid 2 with 74,988 bp (Figure 7).

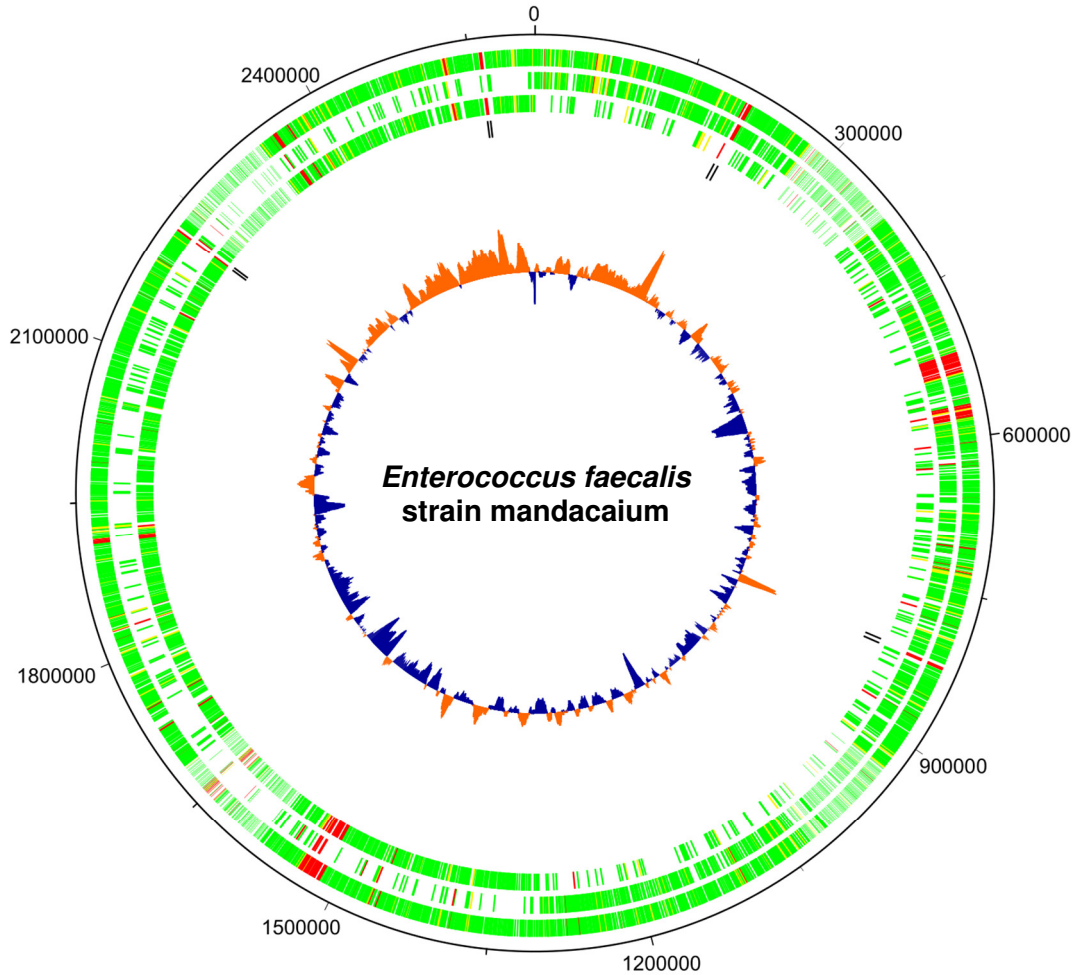


Figure 5: Graphical circular map of *Enterococcus faecalis* strain mandacaium chromosome (2.61 Mb). From outer circle to the center: All CDS on forward and reverse strands, all CDS on forward strand, all CDS on reverse strand, all rRNAs on forward and reverse strands. **Green:** > 95% of identity with reference genome, **Yellow:** 70% to 95% of identity with reference genome, **Red:** < 70% of identity with reference genome, **Black:** rRNAs sequences. Inner circle show GC content (**Blue:** Bellow average / **Orange:** above average). The map was generated using DNAPlotter from Artemis [52].

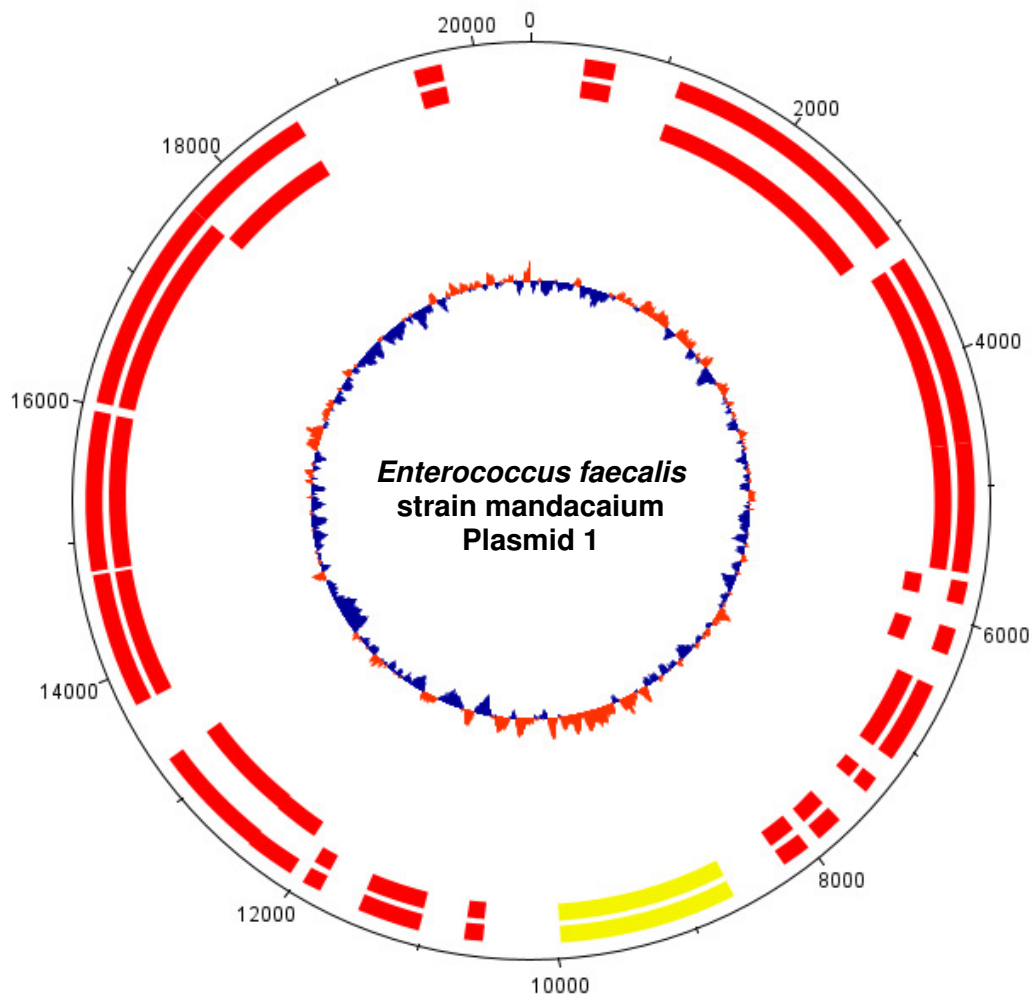


Figure 6: Graphical circular map of *Enterococcus faecalis* strain mandacaium plasmid 1 (20,406 bp). From outer circle to the center: All CDS on forward and reverse strands, all CDS on forward strand, all CDS on reverse strand. **Green:** > 95% of identity with reference genome, **Yellow:** 70% to 95% of identity with reference genome, **Red:** < 70% of identity with reference genome. Inner circle show GC content (**Blue:** Bellow average / **Orange:** above average). The map was generated using DNAPlotter from Artemis [52].

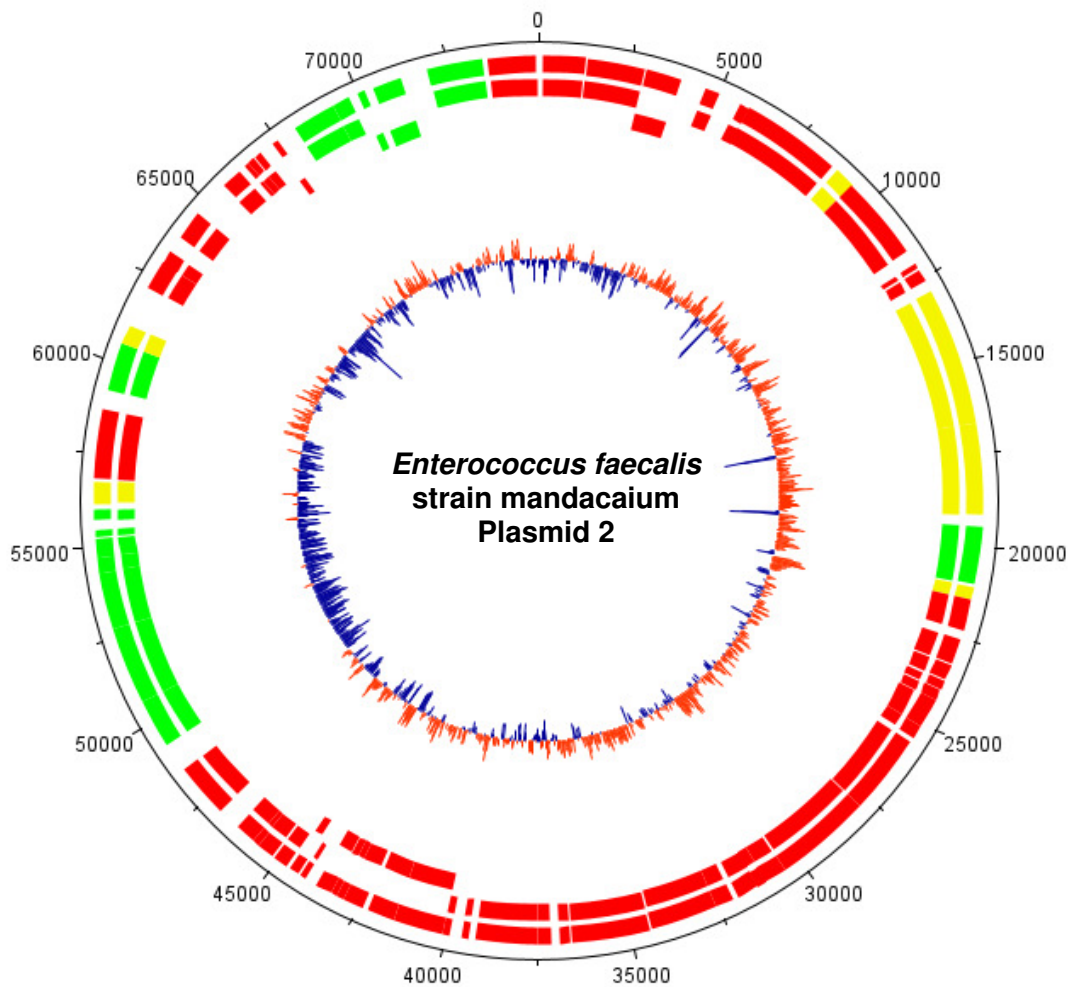


Figure 7: Graphical circular map of *Enterococcus faecalis* strain mandacaium plasmid 2 (74,988 bp). From outer circle to the center: All CDS on forward and reverse strands, all CDS on forward strand, all CDS on reverse strand. **Green:** > 95% of identity with reference genome, **Yellow:** 70% to 95% of identity with reference genome, **Red:** < 70% of identity with reference genome. Inner circle show GC content (**Blue:** Bellow average / **Orange:** above average). The map was generated using DNAPlotter from Artemis [52].

Using the ABRicate tool, possible regions encoding proteins for resistance to antimicrobials agents were found in the genome (Table 1), but none were found in the plasmid sequence. An antibiotic sensitivity test was performed and confirmed resistance to clindamycin, but vancomycin showed an antibiotic effect.

Table 1: Predictions of genes conferring antibiotic resistance found in *E. faecalis mandacaium* genome using the ABRicate tool.

Gene	Resistance Mechanism	Antibiotic Resistance
VanHBX_2	(vanHAX resistance cassette) Enzymes replacing the terminal D-Ala-D-Ala motif of the stem peptide with a D-Ala-D-Lac or D-Ala-D-Ser motif, reducing the affinity of vancomycin	Vancomycin
Lsa(A)_3	ABC-F subfamily protein (ATP binding cassette transporter)	Lincomycin Clindamycin Dalfopristin Pristinamycin_IIA Virginiamycin_M

The antiSMASH genome analysis tool, detected 3 regions of secondary metabolite biosynthesis gene clusters of *E. faecalis mandacaium* (Figure 8), encoding the bacteriocins Enterocin NKR-5-3B (100% of similarity), Linocin M18 (94% of similarity), Microcin C7 (100% of similarity) and Linocin M18 (94% of similarity).



Figure 8: (A) RRE-containing region with a core biosynthetic gene encoding a sequence similar to the molecule Microcin 07 from *Escherichia coli*. (B) RiPP-like region with a core biosynthetic gene encoding a sequence similar to the Linocin M18. (C) Cyclic-lactone-autoinducer region with a core biosynthetic gene encoding a sequence similar to the molecule Enterocin NKR-5-3B from *Enterococcus faecium*.

Discussion

The use of chemical nematicides has been increasingly discouraged or even banned in several countries, due to the potential environmental and human harm that these formulations can cause [4,53]. One of the most effective chemical nematicides in controlling *M. incognita*, the abamectin, can pose risks to humans and the environment if used in high concentrations [54,55].

The toxic effect of products used in the chemical control of pathogens on stingless bee's species has been demonstrated in several recent studies [56-59]. The loss of biodiversity of this bees presents not only an ecological problem, but also an economic problem considering the importance of these species in pollination processes of crops of agronomic interest [45,60-62]. Furthermore, the microbiota associated with stingless bee's larval food has been shown to be an important source of organisms with numerous biotechnological applications, including being capable of pathogens biocontrol [45].

Considering this, research has focused on more ecologically viable alternatives for controlling *M. incognita*, including biological control methods [5,21]. In recent years, some studies have described microorganisms with specific action against *M. incognita* [28-32]. For this purpose, this work evaluated the applicability of microorganisms from stingless bee's larval food in the biocontrol of *M. incognita*.

According to the results found our test on soybean plants, among the 10 microorganisms evaluated, the bacteria Mq-MCK-07 isolated from *Melipona quadrifasciata* stingless bee which is commonly known as mandaçaia, showed a nematicidal effect and was capable of reducing the number of nematodes in soybean roots, as well as the number of nematodes per root gram. After whole genome sequenced this bacteria was identified as *Enterococcus faecalis mandacaium*. To investigate *E. faecalis mandacaium* biocontrol mechanism, incubations of the microorganism and its supernatant with nematode eggs and juveniles were carried out according to protocols used in previous studies [21,48]. The tests demonstrate that *E. faecalis mandacaium* was capable of inhibiting egg hatching and presenting a nematicidal effect against J2s of *M. incognita*. The

bacteria supernatant did not exhibit effects neither on the egg hatching or the J2s mortality. To date, there have been no published reports on the use of *Enterococcus* spp. for the biological control of plant-parasitic nematodes (PPNs) or other pathogens of agronomic significance.

The species *Enterococcus faecalis* is a Gram-positive bacteria that commonly lives in the guts of humans and animals, and under certain circumstances or depending of the strain can become mildly pathogenic to its host [63,64]. Like other species of *Enterococcus* sp., they are notable for being a common source of hospital infections, being able to infect surgery sites, the bloodstream and urinary tract [65]. The relevance of the species in hospital environments becomes even more accentuated due to its recognized intrinsic and acquired resistance to antibiotics [66,67].

In the analysis of *E. faecalis mandacaium* genome, it was possible to infer the presence of antibiotic resistance genes. The VanHBX gene identified in the sequence is responsible for conferring resistance to the antibiotic Vancomycin. This finding is in line with the resistance of *Enterococcus* spp. to this antibiotic that is already widely reported [66-72]. The Lsa(A) gene, also found in *E. faecalis mandacaium*, is known to encode an ATP binding cassette transporter and has been identified as intrinsic resistance gene in *E. faecalis*, responsible for conferring resistance to antibiotics Clindamycin and Quinupristin-Dalfopristin [73-75]. However, the analysis of the phenotypic profile carried out in an antibiotic sensitivity test indicated that *E. faecalis mandacaium* has resistance to clindamycin, conferred by the Lsa(A) gene, but did not present resistance to the antibiotic vancomycin, as would be expected in the expression of the VanHBX gene.

Interactions between *E. faecalis* and nematodes has been described usually in model organisms [64]. Several reports indicate that *E. faecalis* frequently occur in animal and human microbiomes both as a commensal or pathogenic agent [63,76,77]. The bacteria can colonize the gut of *Caenorhabditis elegans* and act as a pathogenic agent reducing its population [63,78]. Several strains of *E. faecalis* and *E. faecium* have nematicidal effect in *C. elegans* eggs and juveniles, but strains of *E. faecalis* could also kill *C. elegans* adults [79]. The antinematodic effect of *E.*

faecalis was also demonstrated against mammalian nematode parasite *Trichinella spiralis* [80].

Interestingly, *E. faecalis* can also protect its *C. elegans* host against infection of more virulent pathogens such as *Staphylococcus aureus*, crossing the parasitism–mutualism continuum [64,81]. Much of this protective trait can be attributed to the production of bacteriocins, antimicrobial peptides often encoded on pheromone responsive plasmids on enterococcal colonization dynamics [77]. Bacteriocins are ribosomally synthesized and post-translational modified peptides (RiPPs) with described antimicrobial activity that usually manifest antagonistic activity towards microbial strains that are closely related to the producer strain. The bacteriocins can be used as natural food preservatives and therapeutic antibiotics [82,83]. Using the antiSMASH genome analysis tool, regions of secondary metabolites biosynthesis gene clusters of *E. faecalis mandacaium* was identified encoding sequences with great similarity with previous described bacteriocins genes.

The bacteriocin Enterocin NKR-5-3B (Ent53B), with a sequence of 100% of similarity found in the analysis, is a circular bacteriocin originally found in *Enterococcus faecium* NKR-5-3 strain, with described antimicrobial activity against Gram-positive bacteria such as *Bacillus* spp., *Lactococcus* spp. and *Listeria innocua* [83,85]. Many bacteriocins has been described in *E. faecalis* [77,86].

Bacteriocins from *Bacillus subtilis* and *Bacillus amyloliquefaciens* has been described in biocontrol of *M. incognita* [27]. The borosins, other group of RiPPs widespread in bacteria, has also been described as nematicidal agents. The omphalotins and the lentinulins are cyclic peptides from the borosin group with potent nematicidal activity *M. incognita* [87,88]. However, in the initial analysis of *E. faecalis mandacaium* genome, none of the peptides found has been described with nematicidal activity. Due to this background, the mechanism of action in *E. faecalis mandacaium* in *M. incognita* biocontrol need to be investigate in further analysis.

Conclusion

In summary, the *Enterococcus faecalis mandacaium* strain, isolated from stingless bee's larval food, exhibited nematicidal activity against *Meloidogyne incognita* both *in vitro* as well in the soybean pots test. The genome analysis indicated that it encodes several potential genes implicated in biocontrol effects of several pathogens. Thus, this study provided a theoretical background for *Enterococcus faecalis mandacaium* as a potential candidate for an eco-friendly nematicide.

Declarations

Acknowledgments

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Authors' contributions

GNMC, CUV and ACCS conceived and supervised the project. GNMC, TSP and APMG performed the soybean pots test. GNMC performed the *in vitro* tests and statistical analysis. ARS performed the genome analysis and species identification. GNMC wrote this manuscript and all authors approved its final version.

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Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All the biological material was obtained in accordance with Brazilian laws. The species does not fall under the IUCN Red List categories as a threatened species.

Competing interests

The authors declare no conflict of interest related to the results reported in this study

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CAPÍTULO 3

Artigo científico a ser submetido para a revista Metabolites

Differentiation of *Heterodera glycines* races by metabolomics

Differentiation of *Heterodera glycines* races by metabolomics.

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† In Memoriam

Abstract: The nematode *Heterodera glycines* is one of the main phytosanitary problems of soybean and the main management strategy is the use of pathogen resistant cultivars. For this, it is necessary to characterize the populations of *H. glycines*. Race determination schemes present flaws and there is a demand for more practical methods. This study objective was to identify possible target metabolites for the characterization of populations of *H. glycines*. For this, the metabolic profile of the *H. glycines* races present in the Brazilian territory was analyzed by High-Performance Gas Chromatography linked to Mass Spectrometry and contrasted with a database. The results showed different metabolites profiles with the potential to be used as indicators of the races determined by the differentiating cultivars method. The concentrations patterns of metabolites found were different among the races analyzed and allow the individualization of each race within a single profile. These findings indicate the possibility of race discrimination based on metabolomics tests. Further investigations may provide fundamental information in the development of new rapid tests for the determination of *H. glycines* races.

Keywords: nematode; phytosanitary; soybean; mass spectrometry

1. Introduction

The nematode species *Heterodera glycines* Ichinohe (1952) is one of the main phytosanitary problems of soybean (*Glycine max* [L.] Merr.) in the world [1,2]. The symptoms of *H. glycines* in soybean crops are initially manifested by patches of yellow and stunted plants and low productivity. However, if the pathogen reaches high population rates, it can lead to an increase in premature plant mortality. In situations where there are very high populations of the nematode in the soil, productivity losses related to *H. glycines* can reach 100% [3,4].

The management strategies to this nematode consists on crop rotation, soil management and the use of resistant cultivars, which is the most used *H. glycines* control measure due to its practicality and economic viability [3,4]. However, the use of resistant cultivars is difficult due to the high genetic variability of *H. glycines* [5-7]. The reproduction of *H. glycines* is amphimictic and the female can copulate with several males, promoting a great genetic variability within the species [8]. Because of those features, the management strategies of *H. glycines* by the continuous use of a resistant cultivar can promote a selective pressure where the individuals capable of parasitizing the root system of these cultivars will be selected and increase its population. This characteristic makes it necessary to constantly identify new sources of resistance [3,4].

The selection of resistant cultivars was usually made through the identification of *H. glycines* races following the methodology of differentials soybean lines [9,10]. Studies carried out in Brazil detected the presence of 11 races (1, 2, 3, 4, 4+, 5, 6, 9, 10, 14 and 14+) distributed across the producing states [11].

The determination of *H. glycines* races using the differential soybean lines demand time and involves soybean cultivars that are not adapted to the soil and climatic conditions of the Brazilian territory [10]. Furthermore, the identification scheme is criticized for not having been updated even with the discovery of new resistant cultivars that could lead to an increase in the number of known races. Such modifications could lead to the conclusion that populations of a certain race should be separated into distinct races. Despite criticism by several field researchers, the

procedure continues to be adopted by companies, diagnostics laboratories and soybean farmers to characterization of *H. glycines* populations in Brazil. An alternative scheme was proposed in which 7 cultivars with resistance are used for the identification of 'HG types' of *H. glycines* for the characterization of populations in a specific region and not for the determination of races [3]. However, the method still presents some of the flaws of the race determination scheme and still generates some confusion amongst researchers and agribusiness professionals [4,12].

Thus, there is currently a demand for the development of new techniques for *H. glycines* identification with a greater degree of practicality and applicability in management decision-making process [4]. Therefore, the objective of this study was to identify, through metabolomics techniques, different metabolites profiles that can increase the practicality in the characterization of *H. glycines* populations and generate knowledge for the elaboration of management techniques that can contribute to the increase in crop productivity.

2. Materials and Methods

2.1. Nematode inoculum.

The initial inoculum (eggs and second stage juveniles suspension) of *H. glycines* races was obtained by multiplication in the soybean cultivar Brasmax Potência RR for 60 days in a greenhouse. After this period, the roots were processed to extract the females, which were smashed in 60 mesh sieves superimposed on a 500 mesh sieve to obtain the eggs and the suspension was calibrated to appropriate concentration.

2.2. Experimental strategy.

The 11 races of *H. glycines* present in the Brazilian territory were inoculated in the susceptible cultivar Brasmax Potência RR for the multiplication and collection

maintenance. The metabolic profile of females of each race was analyzed 60 days after inoculation. The data obtained from the analysis of females in the susceptible cultivar were interpreted for the identification of functional biomarkers for the differentiation of *H. glycines* races and metabolic characterization of the infection process.

2.3. Metabolites extraction.

The *H. glycines* whole females were collected from the roots in triplicates of 10 individuals for each race in a 2 mL microtube. The extraction of metabolites was performed by adding 1 mL of spectroscopic grade methanol in the 2 mL microtube and incubating in an ultrasonic bath for 15 minutes. The material was then incubated for 4 hours in an ultrafreezer (-80°C). In the sequence, centrifuged for 15 min at 13000 g and the supernatant transferred to another 2 mL microtube, which was concentrated in a vacuum concentrator for 30 min. The material was stored in an ultrafreezer (-80°C) until the time of analysis.

In the mass spectrometry analyses, the samples were resuspended in 700 µL of spectroscopic grade methanol and filtered through a syringe tip filter with a pore size of 0.22 micrometer.

2.4. GC-MS.

Samples were analyzed in triplicate using a gas chromatography/mass spectrometry (GC/MS) system (Agilent Technologies model 7890B GC System/5977B GC/MSD). Capillary chromatography column DB-5HT (30 m x 250 µm x 0.25 µm). The chromatographic parameters were: injector temperature at 290 °C, high purity helium as a carrier gas at a constant flow rate of 1,2 mL/min with 6 µL injections. The MS Scan was set on 50–550 m/z, with a 150 count threshold and 4 min solvent delay. The temperature was set to 150° C for the quadrupole MS and 280° C for the ion source.

2.5. Statistical analysis.

The acquired mass spectra were extracted using the software MassHunter Qualitative v. 10.0 and converted to “.CEF” extension using the “Molecular feature extraction tool. The software Agilent Mass Profiler Professional v. B.13.1.1 was used to filter and align the extracted metabolites. The filters used were: minimum absolute abundance = 5000 counts; minimum number of allowed ions = 2; all charges were permitted. Alignment parameters were: retention time tolerance of 0.15 min; mass window 15 ppm + 2mDa. Metabolites present in 75% of at least one group with a coefficient of variation below 25% were selected for further data analysis. Statistical analyzes were performed with log2 transformed values. Principal component analysis (PCA) was used to verify the analytical performance. Metabolites were identified using the METLIN database (included in the MPP).

3. Results

3.1. Metabolites profiling.

The analysis of data acquired from mass spectrometry of material extracted from females of *H. glycines* indicated the presence of 245 metabolites. Considering the 75% frequency filter within each race and applying the Benjamini-Hochberg multiple testing correction, the statistical significance analysis (ANOVA) test suggested the presence of 8 metabolites that present a significant difference ($p < 0.05$ and fold change > 2) among the different races of *H. glycines* (Table 1).

Table 1. Metabolites profiling of *Heterodera glycines* races.

Races	R2	R9	R3	R5	R6	R4	R14	R4+	R10	R1	R14+
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R2	8	2	2	2	2	1	2	1	2	2	2
R9	6	8	1	0	0	1	0	1	0	4	0
R3	6	7	8	1	1	0	1	0	1	3	1
R5	6	8	7	8	0	1	0	1	0	4	0
R6	6	8	7	8	8	1	0	1	0	4	0
R4	7	7	8	7	7	8	1	0	1	3	1
R14	6	8	7	8	8	7	8	1	0	4	0
R4+	7	7	8	7	7	8	7	8	1	3	0
R10	6	8	7	8	8	7	8	7	8	4	0
R1	6	4	5	4	4	5	4	5	4	8	4
R14+	6	8	7	8	8	7	8	8	8	4	8

Table 1. Statistical analysis: ANOVA, Tukey, $p < 0.05$, / Multiple Testing Correction: Benjamini-Hochberg, *Fold change* > 2.00. Blue: metabolites that showed a significant difference. Pink: metabolites that showed no significant difference. Gray: All metabolites analyzed.

3.2. Hierarchical clustering.

The *H. glycines* races were organized into hierarchical clusters according to metabolite similarity and the normalized intensity of the 8 metabolites that showed a significant difference between races was used (Figure 1).

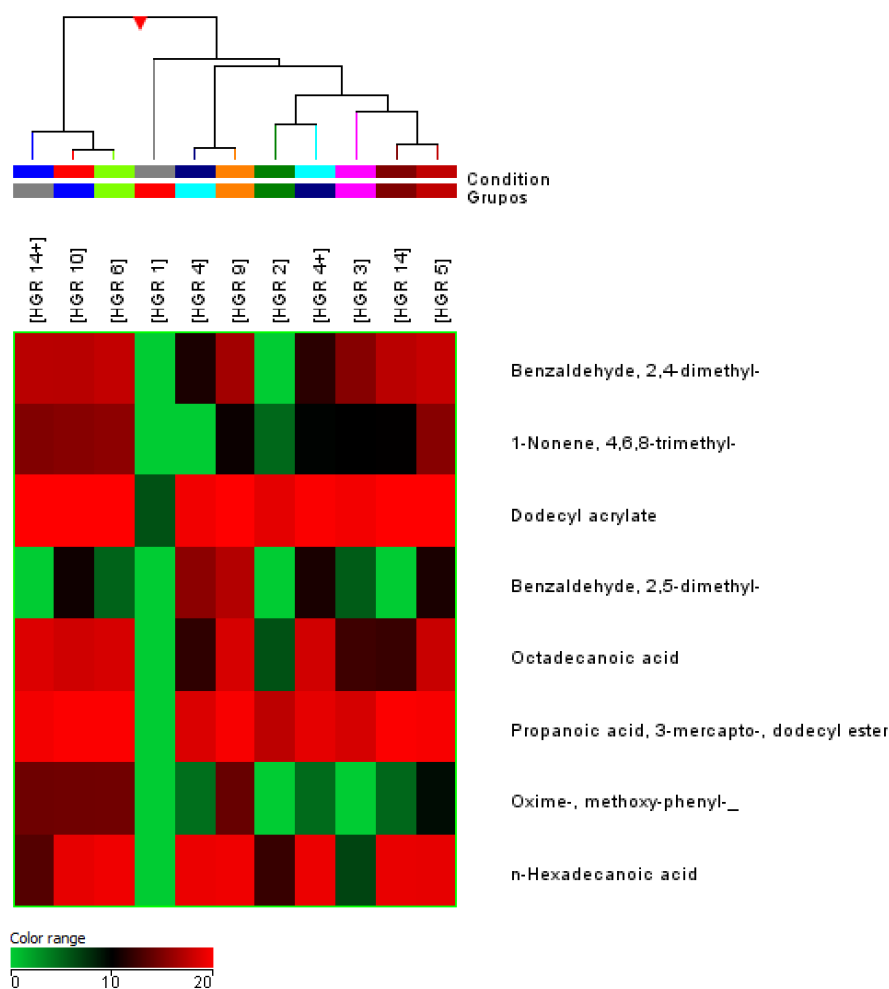


Figure 1. Hierarchical clustering of *Heterodera glycines* races according to its metabolites similarity.

The 8 metabolites found were compared with the database and searched on the literature and organized in Table 2.

Table 2. The 8 metabolites with significant differences between races of *Heterodera glycines*.

Metabolite	Mass (Da)	PubChem CID
2,4-Dimethylbenzaldehyde	134.17	61814
1-Nonene, 4,6,8-trimethyl-	168.32	41077
Dodecyl acrylate	240.38	75084
2,5-Dimethylbenzaldehyde	134.17	22015
Stearic Acid	284.47	5281
Propanoic acid, 3-mercapto-, dodecyl ester	274.46	80796
Oxime-, methoxy-phenyl-	151.16	9602988
Palmitic Acid	256.42	985

4. Discussion

The nematode *H. glycines* is one of the majors phytosanitary problems for the soybean crop. Productivity losses with the nematode in the largest producing regions are estimated at 5.24% in South America and 9.31% in North America [13]. The main management strategy for *H. glycines* is the development of pathogen resistant cultivars, which can result in higher productivity and lower reproduction rates [12]. For this purpose, a race identification scheme was used for many years to avoid population increases in the selected resistant cultivars [3,8].

The race determination scheme has been criticized by nematologists, but it continues to be adopted by several companies and soybean breeders to characterize the populations of *H. glycines* in Brazil. Alternatives presented for the characterization of *H. glycines* populations continue to present some of the flaws of the previous scheme and have created confusion among researchers and agribusiness professionals. Considering this, the demand for new techniques for characterizing *H. glycines* populations that facilitate management decisions remains high [4,12].

In several studies, mass spectrometry has been used as a tool to identify pathogenic fungi, bacteria and parasites [14-19]. In addition, proteomic and metabolomic analyzes of nematodes and other parasites were also performed with various mass spectrometry techniques in several studies [20,21]. The potential of the methodology has already been described in the identification of several parasites species, presenting the advantage of usually being an easy and fast method of identification. However, it is worth noting that the cost of acquiring the equipment is one of the biggest disadvantages of the methodology [22].

Our results showed significantly different metabolites with the potential to be used as indicators of the races determined by the differentiating cultivars method. The concentrations patterns of these metabolites found were different among the 11 races analyzed and allow the individualization of each one of the races within a single profile. Additional investigations may allow the corroboration of the results and the elucidation of the profile of all races present in the Brazilian territory. Complementary analyzes with the proper identification of these metabolites can

generate knowledge of possible new compounds or targets for the development of tests for specific detection of the race.

Among races found on Brazilian territory, races 4+ and 14+ differ in the determination scheme of races 4 and 14 only by their ability to parasitize the Hartwig cultivar [11]. However, according to our findings races 4 and 4+, as well as races 14 and 14+, did not show similarities in the presence of metabolites that would allow the clustering of these groups. These findings may indicate that despite the similarity in the race determination scheme, these races do not have a metabolic profile that allows segregation from other nematode races. This result is in line with criticism that the increase in differential cultivars in the race determination test could lead to segregation of new races [3].

New tests for the detection and characterization of *H. glycines* are needed to increase the practicality of field management. Techniques such as multiplex sensors have been widely used for detection and identification of diseases in the field [23]. However, such techniques use phenotypic aspects of the plants and much of the productivity loss caused by *H. glycines* can occur without the manifestation of apparent symptoms in the crop [1,24]. Despite not been a tool capable of on-site detection, the presence of functional biomarkers makes identification in the laboratory faster and simpler [21]. Therefore, laboratories with structure for mass spectrometry could perform the characterization of *H. glycines* populations based on the metabolic profile with a high level of reliability and eliminate the long process of race determination. Beyond that, new analyzes and the proper identification of these metabolites may provide sufficient information to search for ligands and create biosensors for faster identifications [25].

Our study suggests that the *H. glycines* races present in the Brazilian territory have different metabolic profiles. These findings indicate the possibility of race discrimination based on metabolomics tests. Further investigations may provide fundamental information in the development of new rapid tests for the determination of *H. glycines* races.

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