



**LETÍCIA LOPES DE PAULA**

**MICROBIAL-BASED PRODUCTS AND PAPAYA-  
DERIVED COMPOUNDS AGAINST ROOT-KNOT  
NEMATODES**

**LAVRAS – MG  
2025**

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/ Fitopatologia, para a obtenção do título de Doutora.

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*Ao meu pai e minha mãe, por todo esforço e apoio durante esta longa jornada  
às minhas irmãs, avó e noivo Vinicius por todo  
apoio, carinho e amor.*

***Dedico***

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## RESUMO GERAL

Os fitonematoides são um dos fatores limitantes a produtividade agrícola. O seu manejo pode ser realizado utilizando formulados químicos, agentes biológicos e extratos vegetais. Os objetivos deste trabalho foram: i) investigar a eficácia da combinação de dois bionematicidas comerciais, *Purpureocillium lilacinum* cepa PL251 (PL) e *Bacillus amyloliquefaciens* cepa D747 (Ba), contra *Meloidogyne enterolobii*, *in vivo* em plantas de pepino e *in vitro* em diferentes fases de vida do nematoide e ii) avaliar o potencial nematicida do látex do mamão e papaína no controle de *Meloidogyne javanica* em diferentes fases de vida do nematoides e verificar a indução de resistência de ambos compostos em raízes de tomate. A aplicação combinada *P. lilacinum* cepa PL251 (PL) e *B. amyloliquefaciens* cepa D747 (Ba) em raízes de pepino, reduziu significativamente ( $p < 0,05$ ) o número de ovos por grama de raízes em 84% e o índice de galhas em comparação ao controle negativo, enquanto cada microrganismo isolado não mostrou efeito significativo. Ensaio *in vitro* demonstraram que os sobrenadantes de culturas livres de células combinadas de PL e Ba aumentaram a toxicidade dos juvenis de segundo estágio (J2) de *M. enterolobii* e inibiram a eclosão dos ovos. Além disso, os exsudatos das raízes de pepino inoculadas com ambos os microrganismos foram repelentes aos J2s, ao contrário dos exsudatos das raízes não inoculadas. Compostos orgânicos voláteis (VOCs) de Ba foram notavelmente tóxicos para J2s, no entanto, os voláteis de ambos os microrganismos combinados demonstraram uma maior toxicidade ( $P < 0,05$ ). Os ensaios utilizando látex de mamão e papaína demonstraram 100% de mortalidade de nematoides em concentração de 1% e reduziu a eclosão de J2s em uma média de 45% no primeiro experimento, com reduções de 39% e 61,9% em concentrações de 1,5% e 3%, respectivamente, no segundo experimento. A infectividade e a reprodução dos nematoides foram significativamente reduzidas em ambos os experimentos. O tratamento com papaína na concentração de 3,5% resultou em uma redução de 72% na mortalidade de J2s e reduziu significativamente a eclosão de juvenis e a reprodução dos nematoides. Tanto o látex de mamão quanto a papaína foram eficazes na ativação do sistema de defesa do tomate. Em resumo, ambos controles testados foram eficazes no controle no manejo sustentável dos fitonematoides, contribuindo para práticas agrícolas mais seguras.

**Palavras-Chaves:** Controle alternativo. Fitonematoides. Nematóide das galhas.

## ABSTRACT

Plant-parasitic nematodes are one of the limiting factors in agricultural productivity. Their management can be achieved using chemical formulations, biological agents, and plant extracts. The objectives of this study were: i) to investigate the efficacy of the combination of two commercial bionematicides, *Purpureocillium lilacinum* strain PL251 (PL) and *Bacillus amyloliquefaciens* strain D747 (Ba), against *Meloidogyne enterolobii*, in vivo in cucumber plants and in vitro in different life stages of the nematode and ii) to evaluate the nematocidal potential of papaya latex and papain in controlling *Meloidogyne javanica* at different nematode life stages and to verify the induction of resistance by both compounds in tomato roots. The combined application of *P. lilacinum* strain PL251 (PL) and *B. amyloliquefaciens* strain D747 (Ba) on cucumber roots significantly reduced ( $p < 0.05$ ) the number of eggs per gram of roots by 84% and the gall index compared to the negative control, while each microorganism alone did not show a significant effect. In vitro assays demonstrated that the combined cell-free culture supernatants of PL and Ba increased the toxicity of the second-stage juveniles (J2) of *M. enterolobii* and inhibited egg hatching. Additionally, root exudates from cucumber plants inoculated with both microorganisms were repellent to J2s, unlike the exudates from non-inoculated roots. Volatile organic compounds (VOCs) from Ba were notably toxic to J2s, however, volatiles from both combined microorganisms demonstrated greater toxicity ( $P < 0.05$ ). Assays using papaya latex and papain showed 100% nematode mortality at a 1% concentration and reduced J2 hatching by an average of 45% in the first experiment, with reductions of 39% and 61.9% at concentrations of 1.5% and 3%, respectively, in the second experiment. Nematode infectivity and reproduction were significantly reduced in both experiments. Treatment with 3.5% papain resulted in a 72% reduction in J2 mortality and significantly reduced juvenile hatching and nematode reproduction. Both papaya latex and papain were effective in activating the tomato's defense system. In summary, both tested controls were effective in the sustainable management of plant-parasitic nematodes, contributing to safer agricultural practices.

**Keywords:** Plant-parasitic nematode. Root-Knot nematode. Management. Alternative Control.

## INDICADORES DE IMPACTOS

Os fitonematoides representam um dos principais desafios para a produtividade agrícola, causando danos significativos às culturas e comprometendo a segurança alimentar. O estudo apresentado demonstra o potencial de estratégias sustentáveis para o manejo desses patógenos, utilizando bionemáticas e compostos naturais. A aplicação combinada de *Purpureocillium lilacinum* (PL) e *Bacillus amyloliquefaciens* (Ba) mostrou uma redução expressiva na infestação de *Meloidogyne enterolobii*, tanto no desenvolvimento das galhas quanto na reprodução do nematoide. A toxicidade dos metabólitos liberados pelos microrganismos e sua capacidade de repelir juvenis reforçam sua viabilidade como uma alternativa biológica eficaz. Além disso, o uso do látex de mamão e da papaína demonstrou uma alta taxa de mortalidade dos nematoides *Meloidogyne javanica*, inibindo sua eclosão e reprodução. Esses compostos naturais também foram capazes de induzir a resistência em plantas de tomate, fortalecendo sua defesa contra futuras infestações. Os impactos desse estudo são relevantes para a agricultura sustentável, pois oferecem alternativas ao uso de nematicidas químicos, que podem ser prejudiciais ao meio ambiente e à saúde humana. O uso de bionemáticas e extratos vegetais reduz a dependência de agroquímicos sintéticos, promovendo práticas agrícolas mais seguras e ambientalmente responsáveis. Além disso, a eficácia comprovada dessas abordagens pode beneficiar produtores agrícolas, reduzindo perdas e aumentando a produtividade de culturas sensíveis a fitonematoides. A adoção dessas estratégias pode contribuir para um manejo mais sustentável do solo e para a produção de alimentos mais saudáveis, alinhando-se às demandas da agricultura moderna por soluções inovadoras e ecologicamente corretas.

## IMPACT INDICATORS

Phytoparasitic nematodes represent one of the main challenges to agricultural productivity, causing significant damage to crops and compromising food security. The presented study demonstrates the potential of sustainable strategies for managing these pathogens using bionematicides and natural compounds. The combined application of *Purpureocillium lilacinum* (PL) and *Bacillus amyloliquefaciens* (Ba) showed a significant reduction in *Meloidogyne enterolobii* infestation, both in gall development and nematode reproduction. The toxicity of the metabolites released by the microorganisms and their ability to repel juveniles reinforce their viability as an effective biological alternative. Additionally, the use of papaya latex and papain demonstrated a high mortality rate of *Meloidogyne javanica* nematodes, inhibiting their hatching and reproduction. These natural compounds were also able to induce resistance in tomato plants, strengthening their defense against future infestations. The impacts of this study are relevant to sustainable agriculture, as they offer alternatives to the use of chemical nematicides, which can be harmful to the environment and human health. The use of bionematicides and plant extracts reduces dependence on synthetic agrochemicals, promoting safer and more environmentally responsible agricultural practices. Furthermore, the proven effectiveness of these approaches can benefit farmers by reducing losses and increasing the productivity of crops susceptible to phytoparasitic nematodes. The adoption of these strategies can contribute to more sustainable soil management and the production of healthier food, aligning with the demands of modern agriculture for innovative and ecologically sound solutions.

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## PRIMEIRA PARTE

### 1 INTRODUÇÃO GERAL

As doenças estão entre os principais fatores limitantes da produção agrícola mundial. Entre os patógenos de plantas, os fitonematoides possuem elevada notoriedade por ocasionar perdas em diversas culturas de importância econômica (NICOL *et al.*, 2011). Parte dos danos são atribuídos ao nematoide de galhas (*Meloidogyne* spp.), devido a sua ampla distribuição geográfica, diversidade de gêneros e baixa especificidade por plantas hospedeiras (CASTAGNONE-SERENO, 2002; MOENS *et al.*, 2009; JONES *et al.*, 2013; HOOGEN *et al.*, 2019). Somado aos prejuízos diretos, o ferimento ocasionado durante a penetração do juvenil pode contribuir para a dinâmica de outros complexos de doenças (GOVERSE e SMANT, 2014; SARRI *et al.*, 2024).

Em geral, o controle de fitonematoides é realizado com a aplicação de nematicidas sintéticos ou uso de plantas resistentes. Moléculas como Aldicarbe (chumbinho), brometo de metila e carbofuran (Furadan) foram introduzidas no mercado durante os anos de 1960, 70 e 80 como nematicidas altamente eficazes no controle do fitopatógeno. No entanto, a alta toxicidade aos seres humanos e outros organismos fizeram com que tais moléculas fossem gradualmente retiradas do mercado (ANVISA, 2012; ANVISA, 2017; SOUSA, *et al.* 2015; DESAEGER *et al.*, 2020; SARRI *et al.*, 2024). Como forma de mitigar a redução de opções para o controle do patógeno, associado ao alto custo para a produção e comercialização de produtos químicos (250 milhões de dólares), empresas tem investido em formulados a base de compostos naturais e microrganismos (ARPINI *et al.*, 2018; DESAEGER *et al.*, 2020; ABD-ELGAWAD, 2021; SARRI *et al.*, 2024).

## 2 REFERÊNCIAL TEÓRICO

### 2.1 Os fitonematoides

O solo abriga uma ampla diversidade de microrganismos, entre eles, os nematoides constituem os grupos mais prevalentes. Tais microrganismos podem ser utilizados como indicadores da qualidade e atividade biológica do solo (HOOGEN *et al.*, 2019). Estima-se que em um hectare, nos primeiros 20 cm de solo, existam cerca de 7,5

bilhões de nematoides (DECRAEMER; HUNT, 2006). Dentre os diversos gêneros, aqueles que possuem estilete bucal e parasitam plantas são denominados fitonematoides, e totalizam, aproximadamente, 15% das espécies descritas no filo Nematoda (FERRAZ; BROWN, 2016).

Comumente os sintomas associados a estes patógenos incluem redução no crescimento e desenvolvimento da planta, murcha e amarelecimento ou clorose (FERRAZ; BROWN, 2016). Além disso, ao penetrarem nas raízes, os fitonematoides ocasionam ferimentos nas raízes, que servirão como porta de entrada para outros agentes patogênicos (MOLINARI, 2009).

### **2.1.1 Gênero *Meloidogyne***

Popularmente conhecido por nematoide das galhas, o gênero *Meloidogyne* possui destaque dentre os diversos gêneros de fitonematoides (JONES *et al.*, 2013). Aproximadamente 70% dos danos ocasionados por fitonematoides são atribuídos a este grupo (SASSER E FRECKMAN, 1987), sendo tal fato associado ao seu comportamento polífago e de ampla distribuição geográfica, como por exemplo, o *M. incognita*. Estima-se que o grupo possa parasitar mais de três mil espécies vegetais, incluindo espécies agrícolas de importância econômica, florestais, daninhas e ornamentais (CASTAGNONE-SERENO, 2002).

O gênero *Meloidogyne* é classificado como endoparasita sedentário, ou seja, após a penetração do juvenil de segundo estágio e estabelecimento da relação parasítica, este completará o seu ciclo de vida no interior da raiz. Ao final do ciclo, ocorrerá a deposição de ovos, que serão aglutinados por substâncias excretadas pela fêmea, formando a massa de ovos (CARVALHO, 2017). Esta massa gelatinosa que agrega os ovos é rica em nutrientes e outras substâncias, que podem propiciar o crescimento, estabelecimento e até mesmo inibição de fungos e bactérias (ORION *et al.*, 2001; ESTUPIÑAN-LÓPEZ *et al.*, 2018).

Os sintomas característicos do gênero *Meloidogyne* spp. envolvem a formação de galhas nas raízes, redução e deformação do sistema radicular, decréscimo da absorção e translocação de água e nutrientes, clorose e nanismo da planta, gerando impactos diretos na produtividade (SIKORA; FERNÁNDEZ, 2005).

## 2.2 Controle biológico de fitonematoides

O objetivo do controle biológico de fitonematoides é manter a população do patógeno abaixo do nível de prejuízo econômico, reduzindo o inoculo inicial e os danos diretos ao hospedeiro. Existem evidências de que determinadas áreas apresentam supressividade natural ao patógeno, sendo tal fato correlacionado a composição microbiológica do solo (McSORLEY; WANG; CHURCH, 2008; DURÁN *et al.*, 2017). No entanto, grande parte das áreas de cultivo não apresentam este perfil, demandando recargas microbiológicas. Assim, uma das estratégias a serem integradas ao manejo do patógeno é a manipulação ambiental, por meio da introdução de um ou mais microrganismos (SOUZA *et al.*, 2015; SILVA *et al.*, 2018).

Ademais, a utilização de microrganismos antagonistas tem sido uma solução viável, eficaz e economicamente sustentável para o controle de pragas e doenças. Tal fato está relacionado aos múltiplos mecanismos de ação dos microrganismos antagonistas. Os mecanismos de ação podem ser diretos por meio de antibiose, competição, parasitismo e predação e, indiretos induzindo resistência, estimulando crescimento e desenvolvimento precoce das plantas hospedeiras (SOUZA *et al.*, 2015; RAIMUNDI, 2019).

O mercado de formulados biológicos voltados ao controle de pragas e doenças é crescente. No ano de 2018, o mercado global de biopesticidas foi avaliado em mais de 3.14 bilhões de dólares, com expectativas de aumento em mais de 14% entre os anos de 2019-2024 (ABD-ELGAWAD; ASKARY, 2020). Estima-se que em 2023, apenas o mercado de bionematicidas possa gerar 203 milhões de dólares em receita, e o crescimento estimado para o mercado é de 4.2% entre os anos de 2015-2023 (ANONYMUS, 2019).

Atualmente grande parte dos microrganismos isolados e prospectado são provenientes de áreas naturalmente supressivas aos fitonematoides (PIRES *et al.*, 2022). Para prospecção considera-se a efetividade do isolado no controle do patógeno alvo, facilidade na manipulação e reprodutibilidade em massa do microrganismo, tempo de conservação e custo de produção (MOTA *et al.*, 2003; FERRAZ *et al.*, 2010). Grande parte dos bio-produtos registrados possuem como ingrediente ativo diferentes cepas e isolados de bactérias rizosféricas e fungos nematófagos (FERRAZ; SANTOS, 1995).

### 2.2.1 Fungos no controle biológico de fitonematoides

Os fungos, denominados nematófagos, possuem mais de 700 espécies descritas, pertencentes aos filos Basidiomycota, Ascomycota, Chytridomycota, Zygomycota e Oomycota (LI *et al.*, 2015; SOARES; SUFIATE; QUEIROZ, 2018). Tais fungos podem ser classificados de acordo com o modo de parasitismo, em quatro grupos: produtores de toxinas, oportunistas ou ovicidas, endoparasitas e predadores (LI *et al.*, 2015; SOARES; SUFIATE; QUEIROZ, 2018).

Majoritariamente, os fungos nematófagos são considerados parasitas facultativos, sobrevivendo com a presença ou não do hospedeiro. Tal fato, está relacionado a capacidade adaptativa do fungo ao ambiente, podendo este sobreviver como saprófitas no solo (HALLMANN *et al.*, 2009; SOARES; GÔLO; FERNANDES, 2020). No entanto, fungos endoparasitas são obrigatórios, apresentando alta especificidade ao hospedeiro e baixa capacidade adaptativa ao ambiente (FERRAZ; SANTOS, 1995; BRAGA; ARAÚJO, 2014).

Os fungos nematófagos classificados como oportunistas ou parasitas de ovos são altamente promissores para o desenvolvimento de formulados biológicos. Tais fungos são extremamente competitivos, estabelecendo-se principalmente em solos com matéria orgânica (SHARON *et al.*, 2001). O seu principal mecanismo de controle é o parasitismo de ovos, fêmeas sedentárias e cistos. Após o contato com o hospedeiro o fungo produzirá hifas apressórias e enzimas hidrolíticas, tais como quitinases e proteases, que auxiliaram a infecção em virtude da degradação da camada quitinosa do ovo (SHARON *et al.*, 2001; DEVI *et al.*, 2018; SOARES *et al.*, 2018). Ademais, alguns fungos do grupo são capazes de metabolizar moléculas que irão impactar no reconhecimento do patógeno ao hospedeiro, mortalidade, eclosão e quimiotaxia (WANG *et al.* 2010; SOARES *et al.*, 2016; PACHECO *et al.*, 2022). Os fungos parasitas de ovos mais estudados e frequentemente utilizados como ingredientes ativos na formulação de produtos biológicos são *Pochonia chlamydosporia* e *Purpureocillium lilacinum*.

*Purpureocillium lilacinum*, anteriormente nomeado *Paecilomyces lilacinus*, é um ascomiceto caracterizado por apresentar colônias com coloração arroxeadas. Por se tratar de um fungo saprófita, este pode ser isolado em diferente ambiente, tais como solo, insetos, nematoides, restos vegetais e etc (LUANGSA-ARD *et al.*, 2011). A sua

virulência a fitonematoides está relacionada a capacidade do isolado em produzir enzimas hidrolíticas (quitinases e proteases) e metabolitos (ácido acético e leucinostatina) (KHAN et al., 2004; YANG et al., 2015; WANG et al., 2016). Tais enzimas e metabolitos irão auxiliar na degradação da membrana constituinte do ovo, facilitando o estabelecimento fúngico (WANG et al., 2010; MUKHTAR et al., 2013; WANG et al., 2016).

Em 2001, a Bayer CropScience Biologics foi a primeira empresa a obter os direitos a produção e comercialização de bionematicidas utilizando *P. lilacinum* como ingrediente ativo. Posteriormente, a variante 251 (PL251) passou a ser utilizada para a formulação de biológicos, como por exemplo Melocon® e BioAct (WILSON AND JACKSON, 2013; DAS et al., 2022). Os produtos foram registrados para diversos gêneros de fitonematoides tais como, *Meloidogyne* spp, *Pratylenchus* spp., *Heterodera* spp., *Globodera* spp., *Rotylenchulus reniformis*, etc. Além disso, os bionematicidas a base de *P. lilacinum* podem ser utilizados no manejo integrado de diversas culturas de importância (WILSON AND JACKSON, 2013; ABD-ELGAWAD e ASKARY, 2018; DAS et al., 2022).

## 2.2.2 Bactérias no controle biológico de fitonematoides

A principal fonte utilizada para a prospecção de bactérias, com potencial bionematicida, é a rizosfera (ZHOU et al., 2016; MIGUNOVA; SASANELLI, 2021). As substâncias produzidas por bactérias rizosféricas, podem inibir o crescimento, desenvolvimento e estabelecimento de patógenos radiculares, atuando por antibiose e/ou traduzindo sinais para o sistema imunológico da planta (TIAN et al., 2007; MACHADO et al., 2012; ABALLAY et al., 2013; RAIMUNDI, 2019). Adicionalmente, as bactérias rizosféricas podem estimular o crescimento do hospedeiro, por meio da síntese de hormônios e colonização radicular (STRZELCZYK et al., 1994; KHABBAZ et al., 2019; GLICK, 2020). Alguns gêneros de bactérias promotoras de crescimento, isoladas em diversos tipos de solo e com atividade nematicida conhecida são: *Bacillus*, *Pseudomonas*, *Bradyrhizobium*, *Rhizobium*, *Herbaspirillum*, *Azospirillum*, *Gluconacetobacter* e *Burkholderia* (PROBANZA et al., 1996; KHABBAZ et al., 2019; MIGUNOVA; SASANELLI, 2021).

Os *Bacillus* spp. são endofíticos, epífitas, formadores de endósporos (estruturas de resistência) e constituem o gênero mais amplo, diverso e estudado (ONGENA et al., 2005). Os seus isolados apresentam alta diversidade antagonista, sendo frequentemente utilizados na prospecção e formulação de bionematicidas (ONGENA et al., 2005). Os modos de ação atribuídos ao gênero podem ser diretos, produzindo substâncias tóxicas, e indiretos, induzindo resistência de plantas e/ou competindo pelo sítio ativo de penetração do fitonematídeo (STIRLING, 1991; LEELASUPHAKUL et al., 2008). Dentre as espécies do gênero, as espécies *Bacillus subtilis* e *Bacillus amyloliquefaciens* lideram o mercado de bionematicidas (LIANG et al., 2019; MIGUNOVA; SASANELLI, 2021).

O Grupo Operacional *B. amyloliquefaciens* (GOBa) constitui das espécies *B. amyloliquefaciens*, *B. velezensis* e *B. siamensis*. A denominação grupo operacional foi criada após a reclassificação de diversas cepas de *B. amyloliquefaciens*, evidenciando que as espécies do grupo compartilhavam alto índice de similaridades genômicas (FAN et al., 2018; NGALIMAT et al., 2021). As cepas do GOBa são usualmente prospectadas para a produção de biofertilizantes, bionematicidas e biofungicidas. Tal fato, está relacionado com a capacidade do grupo em produzir sideróforos, sintetizar hormônios vegetais (Ácido indolacético - IAA), produzir enzimas e auxiliar na fixação de nitrogênio (CHOWDHURY et al., 2015; SIBPONKRUNG et al., 2020; NGALIMAT et al., 2021). Além disso, o grupo é capaz de sintetizar elicitores associados ao sistema de defesa da planta, como por exemplo, surfactina (CHOWDHURY et al., 2015). As propriedades bionematicidas atribuídas ao grupo, estão relacionados a produção de compostos orgânicos voláteis (COVs) (álcoois, ésteres, cetonas, terpenoides e sulfurados) e enzimas (proteases, quitinases, colagenases), além dos outros fatores ligados a resistência de plantas, mencionados anteriormente (MIGUNOVA; SASANELLI, 2021).

### **2.2.3 Interação entre *Bacillus* spp e *Purpureocillium lilacinum* no controle biológico de fitonematídeos**

Diversos estudos sobre compatibilidade entre diferentes espécies do gênero *Bacillus* e *Purpureocillium lilacinum* foram realizados, e estes demonstraram que ambos interagem positivamente, não possuindo interação antagônica (CRUZ-MAGALHÃES et al., 2021; SEDHUPATHI et al., 2022). Chen et al. (2023) demonstraram que o uso

combinado de *B. subtilis* e *P. lilacinum*, em áreas de cultivos sucessivos de melancia, reduziu a abundância de microrganismos que estão negativamente relacionados a qualidade do fruto. Conseqüentemente, os frutos coletados das áreas tratadas com ambos os microrganismos apresentaram maior teor de açúcar e tamanho, quando comparado com áreas não tratadas

Como bionemáticas, estudos tem demonstrado que a interação entre bactérias do gênero *Bacillus* e *P. lilacinum* podem potencializar o controle de fitonematoides, bem como auxiliar na promoção de crescimento e desenvolvimento das plantas (LEONG et al., 2021; ENGELBRECHT et al., 2022; SEDHUPATHI et al., 2022). Em casa de vegetação, o uso combinado de *P. lilacinum* e *B. subtilis* resultou em redução de 78.48% na população de *M. incognita*, quando comparado com a testemunha (SEDHUPATHI et al., 2022).

No entanto, diversos aspectos sobre a interação entre microrganismos no controle de fitonematoides permanecem obscuros, necessitando de estudos complementares acerca de como dois ou mais microrganismos atuam quando aplicados simultaneamente nas diferentes fases do ciclo dos fitonematoides e nas relações nematoide/planta. Soma-se a isto a falta de informações acerca do uso simultâneo de produtos à base do grupo *GOBa* e *P. lilacinum* no incremento do controle de fitonematoides.

### **2.3 Controle de Fitonematoides com látex de diferentes culturas**

O principal método de controle de fitonematoides é a utilização de produtos químicos. Este método apesar de eficiente possui diversas desvantagens, como a alta toxicidade ao meio ambiente, animais e ao homem, resultando assim, na proibição de diversas formulações, amplamente utilizadas no controle desses fitopatógenos. Por consequência, as opções de formulados químicos, disponíveis no mercado, foram reduzindo ao longo do tempo (NOLING, 2002; ANVISA 2012; ANVISA 2017).

Atualmente a busca por qualidade de vida e conseqüentemente sustentabilidade, fez com que a sociedade passasse a buscar novas alternativas de controle, que reduzissem o impacto ambiental. Diante do novo cenário, os pesquisadores começaram a buscar moléculas, agentes biológicos e métodos alternativos de controle, com menores impactos e efeitos residuais. Neste contexto, métodos alternativos foram priorizados como: incorporação de plantas, extratos ou resíduos vegetais, envolvendo as práticas de

biofumigação, fertilização verde, além do uso exsudatos e metabólitos extraídos de plantas, que possuem ação comprovada na redução da densidade populacional (CARBONI E NTALLI, 2014).

O látex é produzido por vasos laticíferos presentes nos frutos verdes, folhas e caule. Este composto é produzido como mecanismo de defesa da planta estresses bióticos e abióticos. Diversos estudos comprovaram a toxicidade de látices extraídos de diferentes espécies de plantas a fitonematoides. A alta toxicidade de látices pode ser explicada pela diversidade na composição e alta concentração de enzimas (MACALOOD, 2014).

Siddiqui e Alam (1988) foram um dos primeiros pesquisadores a comprovarem o efeito de látices extraídos de *Calotropis gigantea* L., *Calotropis procera*, *Euphorbia milii* e *Euphorbia tirucalli* L. no controle de *Rotylenchulus reniformis*. O tratamento realizado com o látices de *Cajanus cajan* e *Cicer arietinum* também demonstraram efeito no controle dos fitonematoides *M. incognita* e *Rotylenchulus reniformis* (ANVER e ALAM, 1992). Lopes *et al.* (2004), visando o controle de *M. incognita*, trataram sementes de feijão com os látices de *C. procera*, *Cryptostegia madagascariensis* e *E. milii*, e constataram redução significativa no número de galhas e ovos das plantas previamente tradas. Todos os autores citados sugerem que os látices contribuem para a aquisição de resistência ou tolerância, a nematoides, pela planta, além de auxiliar na promoção do crescimento.

Recentemente os autores Kalaiselvi *et al.* (2019), utilizaram o látex extraído de *E. tirucalli*, em diferentes concentrações, para a síntese de nanopartícula objetivando o controle de *M. incognita*, e os resultados demonstraram que independente da concentração, as nanopartículas possuem efeito no controle do patógeno.

Os látices são fontes para isolamento e obtenção de enzimas e compostos biocidas. Kumar *et al.* (2018) isolaram compostos biocidas do látex de *C. procera* e testaram *in vitro* e *in vivo* contra *M. javanica*. Os compostos extraídos foram tóxicos ao fitonematóide, ocasionando alta mortalidade em baixas concentrações e foram capazes de controlar a formação de galhas e ovos. O látex de *Synadenium grantii* possuiu efeito nematicida *in vitro* contra *M. incognita* e *Panagrellus redivivus*. Como forma de explicar os resultados, os autores isolaram as enzimas presentes no látex de *S. grantii* constatando alto teor de proteases com atividades biológicas, sendo o efeito nematicida atribuído a elas (GOMES *et al.*, 2019).

## **2.4 Látex e papaína no controle de *M. javanica***

### **2.4.1 *Carica papaya* L.**

O cultivo do mamão (*Carica papaya* L.) é realizado principalmente nas regiões tropicais e subtropicais do mundo, sendo consumido *in natura* ou industrializado (sucos, geleias, doces, etc) (EMBRAPA, 2013). O Brasil é o segundo maior produtor da fruta, sendo os estados de Espírito Santos, Bahia, Ceará e Rio Grande do Norte os maiores produtores. Em 2022, a produção total do fruto no país foi de mais de um milhão de toneladas (EMBRAPA, 2023).

O mamão possui composição variável de acordo com o grau de maturação. Como uma forma de proteção, o fruto verde, quando lesionado, libera látex rico em enzimas, sendo a principal a papaína. Quando maduro se torna rico em açúcares, vitaminas e compostos voláteis (EMBRAPA, 2013). Os frutos, as folhas e sementes também produzem proteínas e alcaloides de alta relevância para a indústria farmacêutica (VENTURINI *et al.*, 2012). Além disso, as sementes são utilizadas por várias décadas como vermífugo (GOMES *et al.*, 2020).

### **2.4.2 Propriedades do Látex e atividade nematicida**

O látex proveniente do mamão é produzido por vasos laticíferos presentes nos frutos verdes, folhas e caule. Este composto é produzido como mecanismo de defesa da planta contra insetos, animais e estresses ambientais (MACALOOD, 2014). O látex extraído do mamão verde contém enzimas proteolíticas como papaína e quimopapaína A, B e C, endopeptidases de cisteína, glutamina, ciclotranferase, quitinases, peptidases A e B, lisozimas e inibidores de serina (ARVIND *et al.*, 2013).

A produção de látex varia com as estações do ano, fator sexual (feminina ou hermafrodita) e alterações do ambiente (BORELLA E STEVANATO, 2015). Na medicina popular o látex é utilizado em diversos tratamentos de enfermidades, como infertilidade, doenças do fígado (hepatoprotetor), anti-histamínicos, antifúngicos, antimalárico e antihelmíntico (KARISHNA *et al.*, 2008; PINNAMANENI, 2017).

Diversas pesquisas no âmbito veterinário comprovaram que o látex do mamão possui ação nematicida, contra nematoides do trato digestivo de ratos, suínos e equídeos (SATRIJA *et al.*, 1995; SATRIJA *et al.*, 1994; PEACHEY *et al.*, 2016). O uso do látex do mamão resultou em completa destruição de ovos e larvas de *Strongyloides venezuelensis*, na maior concentração, quando testado *in vitro* (MORAES *et al.*, 2017). Chandrasekaran *et al.* (2018) testaram a eficácia do látex, utilizando nanopartículas de prata, contra larvas de mosquitos de *Aedes aegypti* e *Culex quinquefasciatus*, e constataram que o metabólito possui alta toxicidade a ambos os mosquitos, e que possivelmente poderá ser utilizado como uma nova estratégia para o manejo destes mosquitos.

Os estudos da toxicidade do látex do mamão a fitonematoides têm sido conduzidos prioritariamente *in vitro*. Em ovos de *M. incognita*, Saravanapriya *et al.*, (2004) testaram diferentes produtos extraídos de plantas, inclusive o látex do mamão, e obtiveram que mesmo em baixas concentrações o metabólito foi capaz de inibir 98% da eclosão quando comparada a testemunha. Em juvenis de segundo estágio de *M. incognita*, quando aplicada a concentração de 5% de látex, houve 100% de mortalidade após 72 horas de exposição (SARAVANAPRIYA e SIVAKUMAR, 2004).

No entanto, apesar das pesquisas realizadas, não foram demonstrados o efeito do látex do mamão na infectividade e reprodução dos fitonematoides, bem como não foram realizados testes que comprovem a aquisição de resistência pela planta. Sendo assim, faz-se necessário pesquisas adicionais acerca do assunto como uma ferramenta no controle de fitonematoides.

### **2.5.3 Papaína e atividade nematicida**

Enzimas são moléculas que possuem função de digestão ou quebra de ligações, auxiliando no incremento da velocidade da reação e redução da energia requerida para a formação de subprodutos. As enzimas podem ser classificadas como: hidrolases, ligases, oxidoreduases, transferases, liases e isomerases (PEREIRA, 2015). Proteases ou enzimas proteolíticas, são enzimas pertencentes a classe das hidrolases, ou seja, necessitam de água para que a reação ocorra. Estas, podem ser classificadas de acordo com o seu sítio de

atuação e formação peptídica, como: cisteínas, glutâmicas, aspárticas, asparaginas, metálicas, serinas e treoninas (MOTA *et al.*, 2017).

A papaína é uma protease classificada como cisteína, extraída principalmente do látex do mamão. Constituída de 212 aminoácidos, possui massa molecular de 23,406 g/mol e alta estabilidade. Apresenta boa solubilidade em água e baixa em álcool, éter e clorofórmio, atuando em pH entre 3,5 a 9 e temperatura entre 65° a 80°C (MOTA *et al.*, 2017; BORELLA *et al.*, 2015; GHOSH, 2005). A papaína possui baixo custo e variabilidade funcional, sendo amplamente utilizada por indústrias alimentícias, cosméticas e farmacêuticas. Além de possuir ação anti-inflamatória, antifúngicas, bacteriostática, proteolítica, bactericida, agente cicatrizante e esfoliantes, pode ainda ser utilizada como clarificante na produção cervejeira e amaciante de carne (MOTA *et al.* 2017; BORELLA, *et al.*, 2015).

Nutricionalmente, em aves, a papaína tem sido estudada como aditivo em ração para avaliação de diversos aspectos como peso, produção de ovos, qualidade da casca e avaliação de carcaça. Em galinhas poedeiras observou-se aumento de peso corpóreo e do ovo produzido. Em frangos de corte, apesar de não ser observada diferença na qualidade da carcaça, os animais que receberam a ração com papaína tiveram incremento da resposta imunológica e sobrevivência (HARUANA E ODUNSI, 2018).

A papaína possui efeito comprovado contra nematoides parasitas do trato intestinal de diferentes animais e humanos. A alta toxicidade da protease aos nematoides de animais está relacionada com a atividade catalítica das ligações dissulfeto, digerindo a cutícula dos nematoides, afetando diretamente a mobilidade, trocas gasosas, evoluindo para morte (STEPEK *et al.*, 2004; SOARES *et al.*, 2019). Stepek *et al.* (2007) demonstraram que tal fato também ocorre em fitonematoides dos gêneros *Meloidogyne* spp. e *Globodera* sob o tratamento *in vitro* de diferentes cisteínas, incluindo a papaína.

Bioensaios realizados com diferentes concentrações de papaína, demonstraram diversos efeitos da papaína sobre juvenis de *M. incognita*. A quantidade necessária para imobilizar 50%, após 24 horas de exposição, foi de 100 µM. A recuperação da mobilidade foi avaliada após 24 horas da primeira leitura e, poucos nematoides recuperaram a mobilidade. A atração e penetração de *M. incognita* em raízes de *Arabidopsis thaliana* e *Solanum lycopersicum*, foram reduzidas desde a menor concentração utilizada (5 µM). Após a exposição do J<sub>2</sub> a 100 µM de papaína foram constatados danos na cutícula do

nematoide. Para explicar como os danos ocorriam, análises de SDS-PAGE, MALDI-TOF e ESI-MS demonstraram que a papaína é capaz de hidrolisar diversas proteínas presentes na cutícula, inclusive a camada de colágeno (GORNY, 2013).

Os estudos com papaína relacionados a toxicidade a fitonematoides ainda são insuficientes. Portanto, são necessárias pesquisas que demonstrem e expliquem o efeito da papaína nas diferentes fases de vida dos nematoides, além de testes *in vitro* para avaliar o efeito na infectividade e reprodução do nematoide expostos a ela e inoculados em plantas. Também se faz necessário explicar os efeitos indiretos da aplicação da papaína em plantas, por exemplo, na aquisição de resistência ao fitonematoides.

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**SEGUNDA PARTE ARTIGOS**

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**ARTIGO 1 The combination of *Bacillus amyloliquefaciens* and *Purpureocillium lilacinum* in the control of *Meloidogyne enterolobii*.**

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**Abstract**

424

425

426 Nowadays, numerous microorganisms have been identified as effective agents against  
427 plant-parasitic nematodes (PPNs) when applied individually. However, under field  
428 conditions, the combination of biocontrol agents is a desirable strategy to improve control  
429 efficacy against soil-borne pathogens. Since, as different biogents species possess  
430 different mechanisms, the combination of strains is an alternative to improve the  
431 biocontrol effects. The present study aimed to investigate *in planta* the efficacy of the  
432 association of two commercial bionematicides against *M. enterolobii*. Here, the  
433 combination of *Purpureocillium lilacinum* strain PL251 (PL) and *Bacillus*  
434 *amyloliquefaciens* strains D747 (Ba) simultaneously inoculated on cucumber roots, in  
435 Florida (USA) soil, significantly reduced ( $p<0.05$ ) the number of eggs per gram of roots  
436 (84%) and gall index compared to negative control. Contrasting with the mixture, the  
437 application of each microorganism alone didn't show any difference to the negative  
438 control. In *in vitro* assays, the combined cell-free supernatants cultures of these  
439 microorganisms were also effectiveness on increasing *Meloidogyne enterolobii* second-  
440 stage juvenile (J2) toxicity and inhibit the eggs hatching. Also in *in vitro* assays, the  
441 chemotaxis of *M. enterolobii* J2 showed to be repellent to both microorganisms. *M.*  
442 *enterolobii* (J2) repellence was also obtained when root exudates of cucumber were  
443 simultaneously inoculated with both microorganisms. On the other side, the exudate of  
444 cucumber roots free of those microorganisms was highly attractive to the J2. Despite the  
445 notable toxicity of Ba's volatile organic compounds (VOCs) to *M. enterolobii* J2  
446 compared to the control, the impact of VOCs from Ba alone was significantly more  
447 pronounced ( $P<0.05$ ) when the combined microorganisms were examined. Hence, the  
448 enhanced nematode control observed in *in vivo* experiments is substantiated by the  
449 combined impact of the mixture.

450

451 **Keywords:** Biocontrol, root-knot nematodes, metabolites, volatiles compounds,  
452 chemotaxis.

## 453 1. Introduction

454 Plant-parasitic nematodes (PPNs) represent one of the most significant limitations  
455 to global food production. The parasitism of these plant pathogens on cultivated plants  
456 results in billions of US dollars in losses every year (Sikora et al. 2022). Among the PPNs,  
457 the *Meloidogyne* genus, commonly known as root-knot nematodes, stands out as the most  
458 important. About 80% of the damage caused by PPNs is attributed to this group, which is  
459 associated with their polyphagous behavior and wide geographical distribution (Jones et  
460 al. 2013).

461 The application of bioproducts based on antagonistic microorganisms has proven  
462 to be a practical, efficient, and financially feasible method for managing PPNs (Singh et  
463 al., 2017; Mingunova & Sasanelli, 2021). Research has shown that most isolated and  
464 prospecting microorganisms are currently sourced from naturally suppressive areas  
465 (Kremer et al., 2006; Bonatelli et al., 2021; Cruz-Magalhães et al., 2021). Nowadays, the  
466 registered bio-nematicide involve different strains of rhizosphere bacteria or  
467 nematophagous fungi as their active ingredients (Ferraz et al., 2010).

468 The nematophagous fungus, *Purpureocillium lilacinum* is one of the most studied  
469 nematode antagonistic bioagents. *P. lilacinum*, is an ascomycete characterized by  
470 colonies with a purple coloration. As a saprophytic fungus, it can be isolated from various  
471 environments such as soil, insects, nematodes and plant debris (Luangsa-Ard et al., 2011).  
472 Its virulence to PPNs is related to the ability to produce hydrolytic enzymes (chitinases  
473 and proteases), metabolites (acetic acid and leucinostatin) (Yang et al., 2015; Wang et al.,  
474 2016). These enzymes and metabolites aid in the degradation of the constituent membrane  
475 of the nematode eggshell, facilitating the establishment of the fungus (Mukhtar et al.,  
476 2013; Wang et al., 2016). Moreover, *P. lilacinum* has been characterized as an endophytic  
477 fungus. Endophytic fungi can form associations with plants, assisting in the activation of  
478 their defense mechanisms and responses to abiotic stress (Lopez et al., 2014).

479 In the bacteria group of biological agents for the control of PPNs, *Bacillus* is the  
480 most widely studied and diverse genus, featuring a vast range of strains with potent  
481 antagonistic properties which are frequently employed in the screening and development  
482 of bionematicides (Khabbaz et al., 2019; Migunova & Sasanelli, 2021). The Operational  
483 Group *B. amyloliquefaciens* (OGBa) comprises the species *B. amyloliquefaciens*, *B.*  
484 *velezensis*, *B. siamensis* and *B. nakamurai*. OGBa strains are usually screened to produce

485 biofertilizers, bionematicides, and biofungicides (Fan et al., 2018; Ngalimat et al., 2021).  
486 This is related to the group's ability to produce siderophores, synthesize plant hormones  
487 (Indole-3-Acetic Acid - IAA), enzymes, as well as to assist in nitrogen fixation. The  
488 bionematicidal properties attributed to the group are related to the production of volatile  
489 organic compounds (VOCs) (alcohols, esters, ketones, terpenoids, and sulfured  
490 compounds) and enzymes (proteases, chitinases, collagenases) (Sibponkrung et al., 2020;  
491 Ngalimat et al., 2021).

492 Studies have shown that the interaction between *Bacillus* spp. and *P. lilacinum*  
493 can enhance the PPNs control as well as promote plant growth and development (Leong  
494 et al., 2021; Engelbrecht et al., 2022; Sedhupathi et al., 2022). Based on this data, our  
495 hypothesis is that the combined application of the commercial products MeloCon® WG  
496 (*P. lilacinum* strain 251) plus Double Nickle LC® (*B. amyloliquefaciens* strain D747)  
497 control *M. enterolobii* more efficiently than applying either product alone. Here, in in  
498 planta assays, *B. amyloliquefaciens* strain D747 and *P. lilacinum* strain 251 were used to  
499 assess their biocontrol activity against *M. enterolobii* either alone or in combination. To  
500 verify their possible biocontrol mechanisms, we analyzed the effects of 1) VOCs and 2)  
501 cell-free supernatant molecules released by these microorganisms against *M. enterolobii*  
502 eggs and J2. Finally, through in vitro tests, the effect of these microorganisms on the *M.*  
503 *enterolobii* J2 chemotaxis was measured.

504

## 505 **2. Materials and methods**

506

### 507 **2.1. Obtaining isolates of *P. lilacinum* and *B. amyloliquefaciens***

508 The microorganisms used in the experiments were isolated from the commercial  
509 products MeloCon® WG (*P. lilacinum* strain 251 - Certis Biological, Columbia USA) and  
510 Double Nickle LC® (*B. amyloliquefaciens* strain D747 - Certis Biological, Columbia  
511 USA). Isolation was performed by serial dilution, 1 mL of the product was added to 9 mL  
512 of autoclaved distilled water. The same process was repeated 8 times ( $10^{-8}$ ). Subsequently,  
513 100  $\mu$ L of the  $10^{-6}$ ,  $10^{-7}$ , and  $10^{-8}$  solutions were transferred to 9 cm Petri dishes containing  
514 culture medium. The culture media used were Potato Dextrose Agar (PDA) (Potato  
515 starch, dextrose, and agar - BD Becton, Dickinson and Company USA) for the fungus  
516 and Nutrient Agar (NA) (Peptic Digest of animal tissue, beef extract, and agar - Millipore,

517 Sigma Aldrich) for the bacterium. After growth (two days for the bacterium and seven  
518 days for the fungus), the microorganisms were identified morphologically (shape, color,  
519 and size) and by PCR, using specific primers for each.

520

## 521 **2.2 Obtaining *M. enterolobii* inoculum**

522 The inoculum used in the experiments was freshly collected from two farms  
523 located in Wimauma, Florida - USA (27° 42' 15.732" N, 82° 14' 20.472" W and 27° 45'  
524 10.296" N, 82° 15' 43.92" W). The species identification was performed by PCR using  
525 the primer Me (F- AAC TTT TGT GAA AGT GCC GCT G; R- TCA GTT CAG GCA  
526 GGA TCA ACC). The *M. enterolobii* egg suspension was obtained from sweet potato  
527 (*Ipomoea batatas*) c.v., Thai basil (*Ocimum basilicum* var. thyriflora), and pumpkin  
528 (*Cucurbita* sp) roots using the technique described by Hussey and Barker (1973). For that,  
529 the roots were carefully washed in tap water, cut into pieces of approximately 0.5 to 1  
530 cm, and crushed in a blender with a 0.5% sodium hypochlorite solution for about 20  
531 seconds. The egg and root suspension were poured into a 0.074 mm (200 mesh) sieve,  
532 attached to another 0.025 mm (500 mesh) sieve, with the eggs retained on the latter sieve.  
533 For experiments using second-stage juvenile (J2), the egg suspension was incubated in a  
534 hatching chamber at 28 °C, and only J2 hatched after 48 hours were used in the assays.

535

## 536 **2.3 Efficacy of the *P. lilacinum* plus *B. amyloliquefaciens* formulations against *M.* 537 *enterolobii* in plant**

538 To determine the efficacy of the combination *P. lilacinum* and *B.*  
539 *amyloliquefaciens*, the following treatments were used: I) MeloCon®; II) Double  
540 Nickle®; III) MeloCon® + Double Nickle®; IV) Positive control (Velum® - active  
541 ingredient: Fluopyram: N-[2-[3-chloro-5-(trifluoromethyl)-2-pyridinyl]ethyl]-2-  
542 (trifluoromethyl)benzamide); and V) Negative control (inoculated only with eggs). The  
543 assay was conducted in 1.8L pots filled with pasteurized (80 °C for 2 hours using the  
544 SST-15 electric pasteurizer) and natural local soil (Myakka fine sand, 98% sand, <0.5%  
545 organic matter, pH 6.5). Soil moisture was adjusted to 60% of the field capacity.  
546 Subsequently, the pots were infested with 4 mL of a suspension containing approximately  
547 6000 eggs of *M. enterolobii*, excluding treatment VI. After 24 hours, the solutions of the  
548 products used were prepared according to the recommendations and instructions on the

549 label. For treatment III, both products were mixed at the recommended concentrations.  
550 Subsequently, all formulations were manually homogenized and applied to the soil by  
551 drench. After 24 hours, one cucumber seed cultivar ‘Dasher II F1’ was added to the soil.  
552 The biological formulations were reapplied 15 and 30 days after sowing. At 60 days after  
553 sowing, the root weight, gall index, and number of nematode eggs were evaluated. The  
554 evaluation of the gall index was carried out following the scale proposed by Bridge and  
555 Page (1980).

556

#### 557 **2.4 Effect of volatile organic compounds (VOCs) released by *P. lilacinum* and *B.*** 558 ***amyloliquefaciens* on *M. enterolobii* second-stage juveniles (J2)**

559 The toxicity of VOCs released by *P. lilacinum* and *B. amyloliquefaciens* was  
560 evaluated following the same methodology as described by Terra et al. (2018), with  
561 modifications. The treatments included: I) *P. lilacinum*, II) *B. amyloliquefaciens*, III) *P.*  
562 *lilacinum* + *B. amyloliquefaciens*, and IV) control (only medium). To conduct the  
563 experiment, a three-compartmental Petri dish measuring 90 mm x 15 mm was utilized.  
564 Two of the compartments were filled with NA medium to cultivate the bacterium, PDA  
565 medium to cultivate the fungus, or a combination of both in the treatment involving the  
566 two microorganisms (one compartment with NA and the other with BDA). The third  
567 compartment was intentionally left empty for future utilization. For the control, dishes  
568 were filled with both mediums. The bacterium was transferred to the NA medium using  
569 a platinum loop. The fungus was transferred to the PDA medium using 100 µL of dilution  
570 from the commercial product (1 µL product per 50 mL of autoclaved distilled water). In  
571 the treatment where both microorganisms were used, they were placed in their preferred  
572 medium. On the lid, a hole was made using a 2-mm diameter needle and sealed using  
573 adhesive tape. Afterwards, the plate covers were arranged in a way that the hole remained  
574 in the empty compartment, and then sealed with a PVC plastic film. The plates were kept  
575 in the dark at 28 °C for seven days, for all treatments. Subsequently, an aqueous  
576 suspension containing 300 J2 of *M. enterolobii* (3 mL) was injected into the empty  
577 compartment using a syringe through the hole in the lid, and the remaining hole was  
578 quickly sealed again with plastic adhesive. After 48 hours, the plates were opened, and  
579 100 µL of the J2 suspension was transferred to a polypropylene plate containing 96 wells  
580 for quantifying the number of mobile, immobile, and dead J2. An inverted objective

581 microscope (Nikon TMS-F No. 211213, New York, USA) was utilized for this purpose.  
582 To determine the number of dead nematodes, 20  $\mu\text{L}$  of a 1.0 mol  $\text{L}^{-1}$  sodium hydroxide  
583 (NaOH) suspension was added to each well of the plate (Chen and Dickson 2000). The  
584 J2s that remained motionless for approximately 3 minutes were considered dead.

585

### 586 **2.5 *M. enterolobii* J2 hatching after egg exposure to liquid cultures of *P. lilacinum*** 587 **and *B. amyloliquefaciens***

588 This experiment was conducted using Supelco<sup>®</sup> tubes (80  $\times$  28 mm, Sigma-  
589 Aldrich, Bellefonte, PA, USA) filled with 25 g of autoclaved sand (120  $^{\circ}\text{C}$  for 20 min).  
590 The treatments included: I) *P. lilacinum*; II) *B. amyloliquefaciens*; III) *P. lilacinum* + *B.*  
591 *amyloliquefaciens*; IV) control (water without any microorganism was added). To obtain  
592 the microbe suspension, the microorganisms were multiplied using liquid media. Nutrient  
593 Broth (NB) (Peptone, Yeast extract, Sodium Chloride and D(+)-Glucose - Millipore,  
594 Sigma Aldrich) for the bacterium, and Potato Dextrose Broth (PDB) (Potato Infusion and  
595 Dextrose - Alpha Biosciences) for fungus. For each medium, 250 mL was prepared,  
596 following the instructions given by the manufacturer. The microorganisms were  
597 transferred to the proper medium following the same process as described before.  
598 Subsequently, the Erlenmeyers flasks were incubated at 25  $^{\circ}\text{C}$  with shaking (150 rpm).  
599 After 2 days the bacteria concentration was determined using a spectrophotometer at  
600 600nm ( $\text{OD}_{600}$ ), and the suspension was adjusted for  $10^8$  CFU  $\text{mL}^{-1}$ . For the fungus, after  
601 7 days, the number of spores was determined and adjusted for  $6.1 \times 10^6$  spores  $\text{mL}^{-1}$  using  
602 a Neubauer chamber. Subsequently, the tubes were infested with an aqueous suspension  
603 containing 500 eggs of *M. enterolobii* (100  $\mu\text{L}$ ) plus 200  $\mu\text{L}$  of each microorganism  
604 suspension. Distilled water (3 mL) was added, and the tubes were closed. Fifteen tubes  
605 were prepared for each treatment as replicates. Hatching was evaluated after 12 days, by  
606 opening the tubes, transferring the sand to sieves with 0.074 mm (200 mesh) attached to  
607 0.025 mm (500 mesh), collecting the J2 suspension, and quantifying the number of  
608 hatched ones.

609

610 **2.6 Effect of cell-free supernatant produced by *P. lilacinum* and *B. amyloliquefaciens***  
611 **on *M. enterolobii* J2**

612 To produce the cell-free supernatants, *B. amyloliquefaciens* was grown in NB as  
613 described above, 48 h later the cultures were adjusted to the same cell concentration ( $10^8$   
614 CFU mL<sup>-1</sup>) and centrifuged twice at 4500 rpm for 5 min. The pelleted cells were  
615 discarded, and the cell-free supernatants were used in the experiments. To produce the  
616 cell-free supernatants of *P. lilacinum*, a filter with a 0.22 µm opening was used. To  
617 confirm the absence of cells, the supernatants were transferred to plates with medium  
618 (PDA or NA) and incubated for 24 h at 28 °C. After confirmation, the cell-free  
619 supernatants were either directly used or frozen (-18°C) for later use. The experiment was  
620 carried out using a polypropylene plate with 96 wells. Four concentrations of both cell-  
621 free supernatants or the mixture were used: 50%, 25%, 10%, and 5%. For the mixture,  
622 half of each supernatant was used. Water, PDB, and NB were employed as negative  
623 controls. To prevent the growth of other microorganisms, streptomycin sulfate (0.01%  
624 w/v) and ampicillin (0.01% w/v) were used in all treatments. Afterwards, an aqueous  
625 suspension (100 µL) containing 40 J2 of *M. enterolobii* was poured into each well. Each  
626 treatment and control were prepared with double of the final desired concentration.  
627 Subsequently, an aliquot of 100 µL was used, resulting in a final aliquot of 200 µL. Then,  
628 when necessary, distilled water was used for reaching the final concentrations.  
629 Subsequently, the plates were covered and incubated at 28 °C for 48 h. After this period,  
630 the number of mobile and dead J2s was evaluated following the methodology already  
631 described.

632

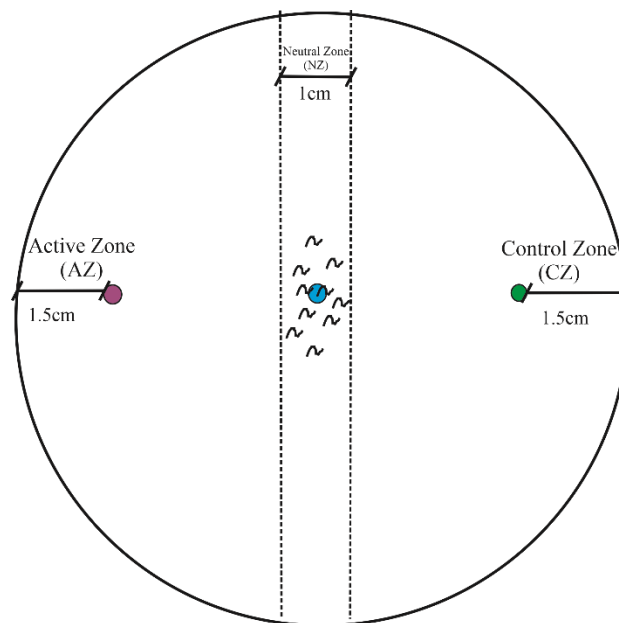
633 **2.7 *M. enterolobii* hatching after egg exposure to cell-free supernatant produced by**  
634 ***P. lilacinum* and *B. amyloliquefaciens***

635 The procedure for the preparation of the cell-free supernatant and controls (water,  
636 NB, and PDB) were followed as described above. In 600 µL microtubes, 100 µL of an  
637 aqueous suspension containing 500 *M. enterolobii* eggs and 100 µL of the cell-free  
638 supernatants or controls were added. For the mixture of supernatants, 50 µL of each was  
639 used. The microtubes were closed and incubated at 28 °C for 12 days. After this period,  
640 the suspension was transferred to a nematode counting dish and the total of J2 was  
641 evaluated.

642

643 **2.8 Chemotaxis of *M. enterolobii* towards the isolates of *P. lilacinum* and *B.***  
 644 ***amyloliquefaciens***

645 To find the chemotaxis index, the methodology described by Wang et al. (2019)  
 646 and Pacheco et al. (2021), with modifications, was performed. The treatments included:  
 647 I) *B. amyloliquefaciens*; II) *P. lilacinum*; III) *B. amyloliquefaciens* + *P. lilacinum*; IV)  
 648 *B. amyloliquefaciens* against *P. lilacinum* and V) control. The experiment was conducted  
 649 using microtube (2 mL) caps (8 x 8 mm) filled with PDA where the fungus was grown or  
 650 NA for the bacteria. The infested caps with the microorganisms were incubated for 4 days  
 651 at 28 °C. Then, Petri dishes (9 cm) were filled with water-agar (2%) and divided into  
 652 three zones: middle (1 cm) = neutral zone; left (1.5 cm from the border) = control zone;  
 653 right (1.5 cm from the border) = active zone (Fig.1). A 20 µL suspension containing 100  
 654 J2 of *M. enterolobii*, was deposited in the middle of the neutral zone. In the active zone  
 655 (right), one cap of bacteria or fungi or both were put and in the control area, only water  
 656 (20 µL) was used. For treatment 4, the caps containing bacteria were placed in the active  
 657 zone and the ones with fungus were put in the control area. As a control, in another dish,  
 658 water was added in both zones (control and active). Then, the plates were closed and kept  
 659 in the dark for 16 hours. By using an inverted objective microscope (Nikon TMS-F No.  
 660 211213, New York, USA), the number of J2s in each zone was counted.



661

662 **Figure 1:** Schematic representation of the plate used for the chemotaxis experiment. AZ:  
 663 Active zone; NZ: Neutral zone; CZ: Control zone.

664 The chemotaxis index (CI) was calculated by subtracting the number of J2 in the  
665 active zone from that in the control zone, and dividing by the total J2 outside of the neutral  
666 zone. According to Wang et al. (2019) and Pacheco et al. (2021), the CI values for  
667 attraction and repellency are:  $CI \geq 0.2$  highly attractive;  $0.1 < CI < 0.2$  slightly attractive; -  
668  $0.1 \leq CI < 0.1$  random response;  $-0.2 \leq CI < -0.1$  repellent; and  $CI \leq -0.2$  highly repellent.  
669

## 670 **2.9 Chemotaxis response of *M. enterolobii* to the root exudates**

671 Cucumber cultivar 'Dasher II F1' were seeded inside test tubes filled with  
672 autoclaved sand (120 °C for 20 min). After 20 days, the plants were carefully removed  
673 from the tubes, and the roots were washed with tap water. Two cucumber plants were  
674 placed in a 30 mL beaker. The beaker opening was sealed with parafilm around the stem  
675 keeping the leaves outside. For this experiment the treatments were: I) MeloCon®; II)  
676 Double Nickel®; III)- a mixture of both; IV) roots without treatment; and V) water. The  
677 solutions were prepared following the label instructions. In each beaker, 6 mL of the  
678 respective treatment was added, ensuring that the roots were completely covered by the  
679 solution. For treatment IV, only water was used. Afterwards, the liquid from the beakers  
680 was collected and transferred to 2 mL microtubes. The microtubes samples were frozen  
681 at -18 °C, lyophilized, and stored in a freezer. To run the experiment, the remaining  
682 content of each microtube was resuspended into 80 µL of distilled water. The chemotaxis  
683 assay was conducted using the same methodology as described earlier. For the active  
684 zone, 20 µL of each solution was used, while for the control zone, only water (20 µL) was  
685 used. After 16 hours, the number of J2 nematodes in both the active and control zones  
686 was counted.

687

## 688 **2.10 Statistics and data analysis**

689 All trials were conducted in a completely randomized design with five replicates  
690 per treatment. The experiments were performed twice, and the repetitions (experiments 1  
691 and 2) were analyzed through a two-way analysis of variance (ANOVA). If there is no  
692 significantly different among repetitions, a combined analysis was performed. Before  
693 analysis, the data were subjected to normality tests (Shapiro-Wilk) and to homogeneity  
694 tests (Bartlett). Data that fail to meet these assumptions were transformed. Once the  
695 assumptions were met, the ANOVA was conducted using the F test. If the F test showed

696 significance ( $P < 0.05$ ), the means of the different treatments were compared using  
 697 Tukey's test ( $P < 0.05$ ).

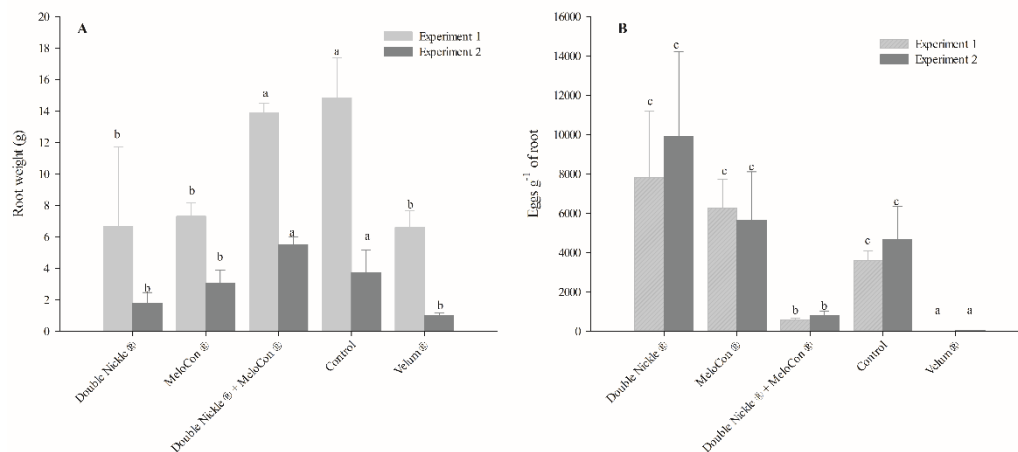
698

### 699 3. Results

700

#### 701 3.1 Efficacy of the *P. lilacinum* plus *B. amyloliquefaciens* formulations in *M.* 702 *enterolobii* control

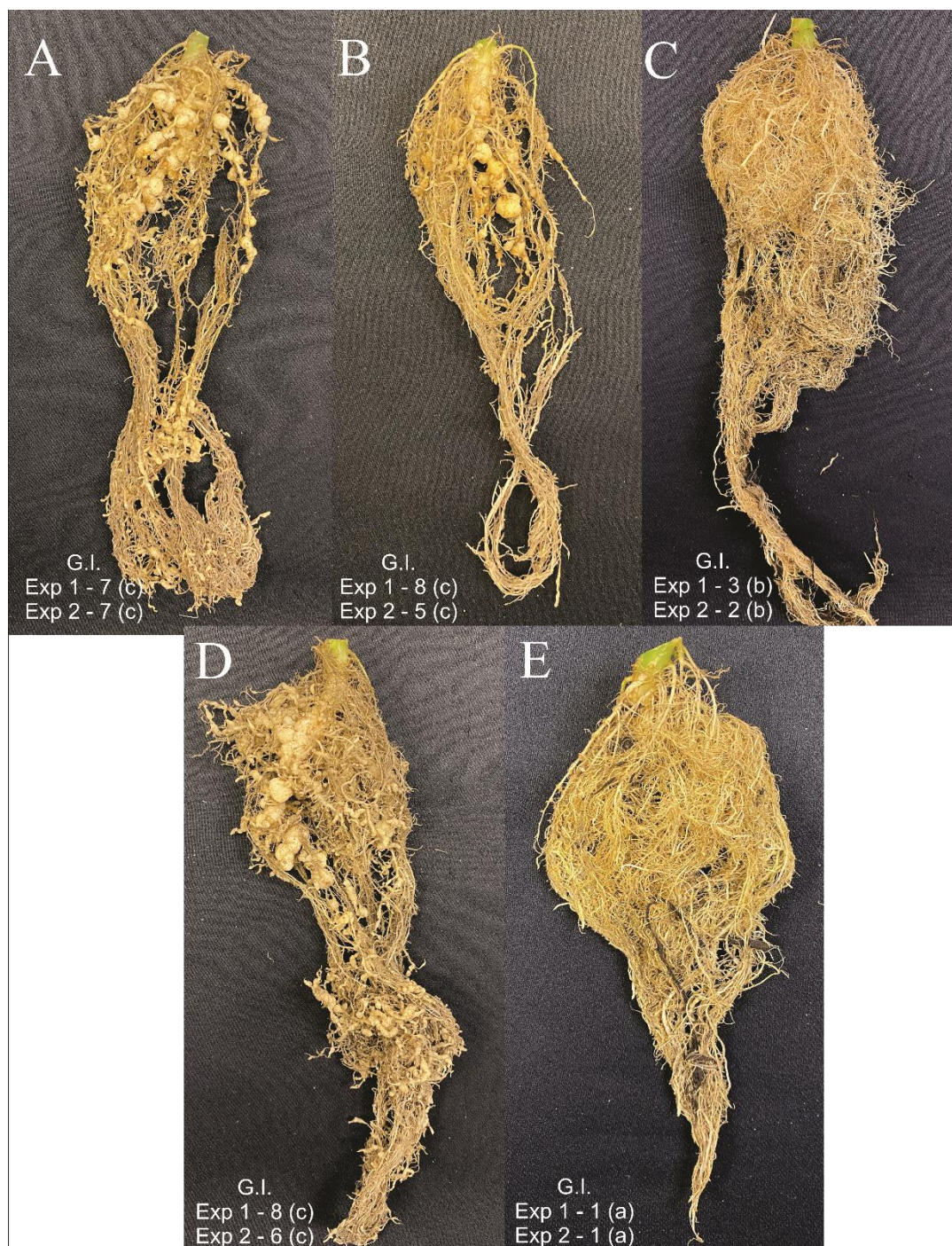
703 Application of MeloCon® (*P. lilacinum*) or Double Nickle® (*B.*  
 704 *amyloliquefaciens*) did not affect the gall index or the number of *M. enterolobii* eggs per  
 705 gram of root, being statistically ( $P > 0.05$ ) similar to the control (Fig. 2). However, in both  
 706 experiments, when Double Nickle® and MeloCon® were used together, the number of  
 707 eggs per gram of root decreased significantly ( $P < 0.05$ ) by 84% and 83% respectively, as  
 708 compared to the control group, with the gall index showing the same trend. In both  
 709 experiments, the combination of Double Nickle® and MeloCon® resulted in a significant  
 710 increase in the fresh weight of the roots. The root weight showed a remarkable  
 711 improvement of 117% and 150% compared to the control group, which did not receive  
 712 any inoculation. On the other hand, when MeloCon® or Double Nickle® was applied  
 713 individually, the fresh root weight was comparatively lower (Fig. 3).



714

715 **Figure 2:** Root weight (g) and reproduction (eggs g<sup>-1</sup> of root) of *Meloidogyne enterolobii*  
 716 on cucumber roots after 3 applications of the commercial products Double Nickel®  
 717 (*Bacillus amyloliquefaciens*) and MeloCon® (*Purpureocillium lilacinum*) in soil infested  
 718 with eggs. Negative control: Water. Positive control: Velum®. Bars represent the standard

719 error of the mean. Means with the same letter do not differ statistically ( $P < 0.05$ ) using  
 720 Tukey's test.



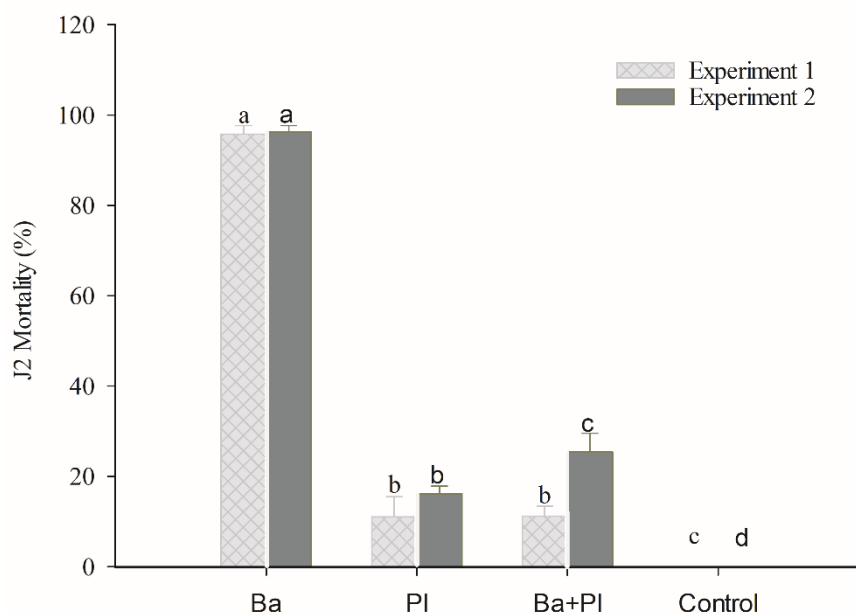
721

722 **Figure 3:** Cucumber roots after 60 days of planting and treatment with MeloCon<sup>®</sup>,  
 723 Double Nickle<sup>®</sup> or the mixture. (A) Root treated with Double Nickle<sup>®</sup>, (B) root treated  
 724 with MeloCon<sup>®</sup>, (C) root treated with the mixture of MeloCon<sup>®</sup> and Double Nickle<sup>®</sup>, (D)  
 725 negative control (Without treatment with infestation of *M. enterolobii*), and (E) Positive

726 control (Velum®). Gall Index (GI) evaluation was conducted using the scale proposed by  
 727 Bridge and Page (1980). Means with the same letter do not exhibit statistically significant  
 728 differences ( $P < 0.05$ ) based on Tukey's test.

729 **3.2 Effect of volatile organic compounds emitted by *P. lilacinum* and *B.***  
 730 ***amyloliquefaciens* on *M. enterolobii* J2**

731 *B. amyloliquefaciens* released VOCs that were toxic to *M. enterolobii*, resulting  
 732 in J2 mortality of 92% in the first experiment and 96% in the second compared with the  
 733 control. In the first experiment, the mortality average for *P. lilacinum* and the mixture of  
 734 both microorganisms were statistically similar ( $P > 0.05$ ) at 6% and 9%, respectively. In  
 735 the second experiment, the mortality average for the mixture was statistically different  
 736 from that of the fungus, with a mortality rate of 25% for the mixture and 16% for the  
 737 fungus (Fig. 4).



738

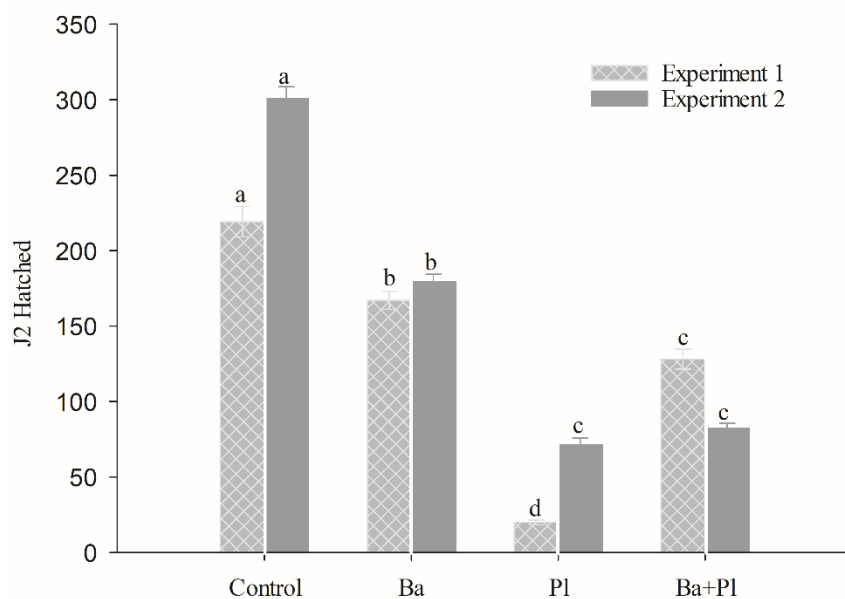
739 **Figure 4:** Meloidogyne enterolobii mortality after 48 hours of J2 exposure to volatile  
 740 organic compounds (VOCs) emitted by Bacillus amyloliquefaciens (Ba), Purpureocillium  
 741 lilacinum (PI), and the mixture of both (Ba+PI). Negative control: water. Bars represent  
 742 the standard error of the mean. Means with the same letter do not differ statistically ( $P <$   
 743  $0.05$ ) using Tukey's test.

744

745

746 **3.3 *M. enterolobii* hatching after egg exposure to liquid culture of *P. lilacinum* and**  
 747 ***B. amyloliquefaciens***

748 *B. amyloliquefaciens* and *P. lilacinum* alone or mixture resulted in a significant  
 749 reduction ( $P < 0.05$ ) in the number of hatched J2 compared to the control (water). In the  
 750 first experiment, the reduction of J2 hatched using *B. amyloliquefaciens* was 13%, while  
 751 in the second experiment it was 40%, both compared to the control. The VOCs released  
 752 by *P. lilacinum* caused a reduction of 90% and 76% in the first and second experiments,  
 753 respectively. When both microorganisms were mixed, the reduction of J2 hatched  
 754 observed in the first experiment was 41%. For the second experiment, the number of J2  
 755 hatched was similar ( $P > 0.05$ ) to the treatment using *P. lilacinum*, showing a reduction  
 756 of 72% when compared with the control (Fig. 5).



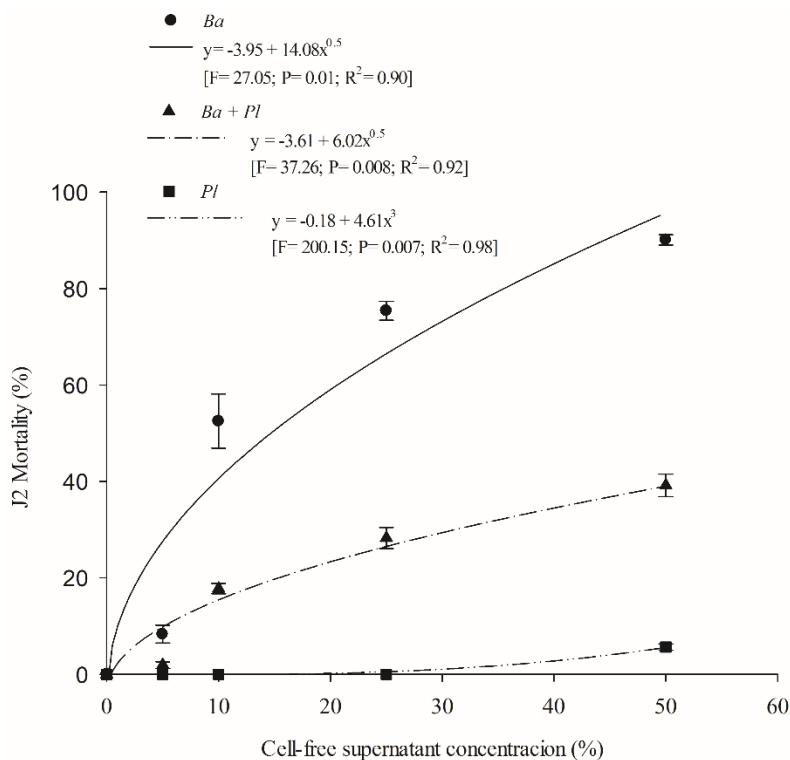
757

758 **Figure 5:** Number of *Meloidogyne enterolobii* J2s hatched 12 days after exposure of the  
 759 eggs to liquid culture of *Bacillus amyloliquefaciens* (Ba), *Purpureocillium lilacinum* (Pl),  
 760 and the mixture of both (Ba+Pl). Negative control: water. The bars indicate the standard  
 761 error of the mean. Means with the same letter do not differ statistically ( $P < 0.05$ ) by  
 762 Tukey's test.

763

764 **3.4 Effect of cell-free supernatant produced by *P. lilacinum* and *B. amyloliquefaciens***  
 765 **on *M. enterolobii* J2**

766 Although the cell-free supernatant obtained from *B. amyloliquefaciens* was found  
 767 to be toxic to *M. enterolobii* J2 in all concentrations tested, the cell-free supernatant of *P.*  
 768 *lilacinum* exhibit a low toxicity towards the nematode. Furthermore, the combination of  
 769 cell-free supernatants obtained from the mixture of both microorganisms showed toxicity  
 770 toward *M. enterolobii* J2. The cell-free supernatant of *B. amyloliquefaciens* at a  
 771 concentration of 50% exhibited the highest effectiveness, resulting in a mortality rate of  
 772 90% for *M. enterolobii* J2. In contrast, when a mixture of cell-free supernatants was used,  
 773 the highest concentration only achieved a mortality rate of 40%. Furthermore, the cell-  
 774 free supernatant produced by *P. lilacinum* at the highest concentration (50%) resulted in  
 775 a relatively lower mortality rate of 6%. (Fig. 6).



776

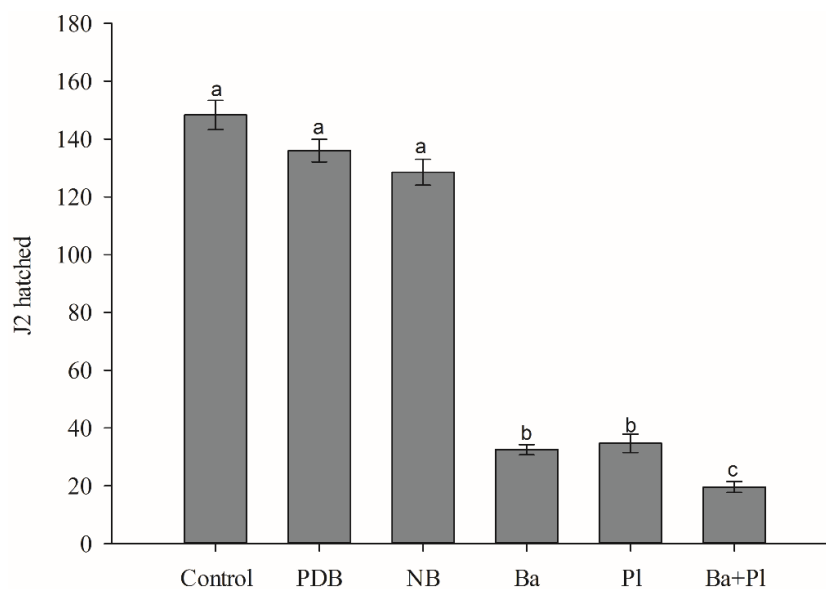
777 **Figure 6:** Meloidogyne enterolobii mortality after 48 hours of second-stage juvenile (J2)  
 778 exposure to the cell-free supernatant extracted from Bacillus amyloliquefaciens (Ba),  
 779 Purpureocillium lilacinum (Pl) and the mixture of the cell-free supernatant extracted from  
 780 B. amyloliquefaciens and Purpureocillium lilacinum (Ba+Pl). Negative controls: for Ba  
 781 the control used was Nutrient agar; for Ba+Pl the control used was water. Bars represent  
 782 the standard error of the mean (P < 0.05). No statistically significant difference (P > 0.321)

783 was observed among the experiments, and thus, the mortality average was determined  
784 based on 10 repetitions per treatment.

785

### 786 **3.5 *M. enterolobii* J2 hatching after egg exposure to cell-free supernatant produced** 787 **by *P. lilacinum* and *B. amyloliquefaciens***

788 The cell-free supernatant produced by *P. lilacinum* and *B. amyloliquefaciens*  
789 significantly ( $P < 0.05$ ) decreased the number of *M. enterolobii* J2 hatched compared to  
790 the control. Both cell-free supernatants were statistically ( $P > 0.05$ ) similar in their ability  
791 to reduce hatching, with an average reduction of 76% for *P. lilacinum* and 78% for *B.*  
792 *amyloliquefaciens*. When, the combination of both cell-free supernatants was tested  
793 occurred the highest efficacy in reducing J2 hatching by 87% compared to the control  
794 (Fig. 7).

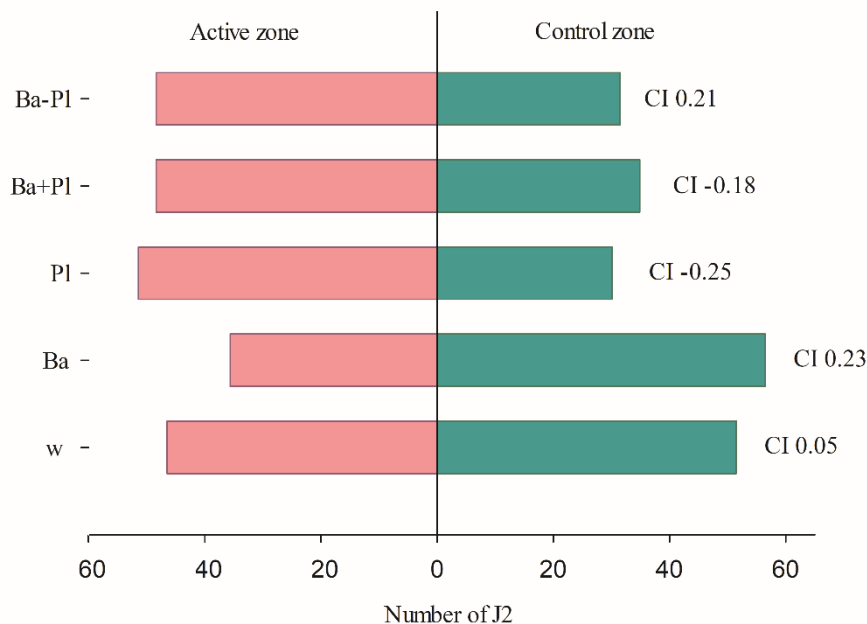


795

796 **Figure 7:** Number of second-stage juveniles (J2) of *Meloidogyne enterolobii* hatched 12  
797 days after exposure of the eggs to the cell-free supernatant extracted from *Bacillus*  
798 *amyloliquefaciens* (Ba), *Purpureocillium lilacinum* (Pl), and the mixture of both (Ba+Pl).  
799 Negative controls: water (control), Potato Dextrose Broth (PDB), and Nutrient Broth  
800 (NB). The bars indicate the standard error of the mean. Means with the same letter do not  
801 differ statistically ( $P < 0.05$ ) by Tukey's test. No statistically significant difference  
802 ( $P > 0.382$ ) was observed among the experiments, and thus, the average of J2 hatched was  
803 determined based on 10 repetitions per treatment. The data was transformed for  $\log(x)$ .

804 **3.6 Chemotaxis of *M. enterolobii* towards the isolates produced by *P. lilacinum* and**  
 805 ***B. amyloliquefaciens***

806 The strains of *B. amyloliquefaciens* and *P. lilacinum* were found to have distinct  
 807 effects on the attraction of *M. enterolobii* J2. While the bacterium showed high levels of  
 808 attraction ( $CI \geq 0.2$ ) to the nematode, the fungus isolate displayed a strong repellent ( $CI$   
 809  $\leq -0.2$ ) effect. When both microorganisms were placed on the same side of the plate, they  
 810 showed to be repellent to the nematode. Moreover, when the microorganisms were  
 811 positioned on the same plate but on opposite sides, the nematode demonstrated a strong  
 812 preference for the bacteria (Fig. 8).

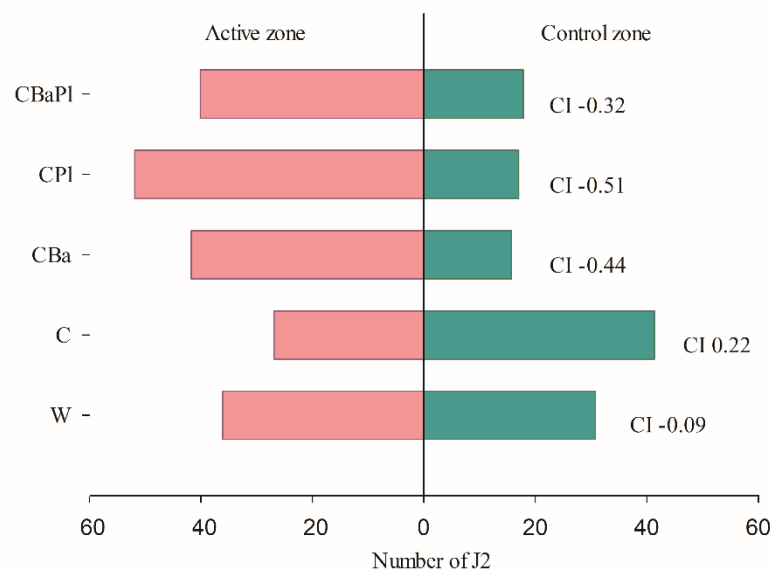


813  
 814 **Figure 8:** Chemotaxis index of *Meloidogyne enterolobii* second-stage juveniles (J2)  
 815 towards the isolates of *Bacillus amyloliquefaciens* (Ba), *Purpureocillium lilacinum* (Pl),  
 816 the mixture of both (Ba+Pl), and one against other (Ba-Pl). Control: Water (w). No  
 817 statistically significant difference ( $P > 0.660$ ) was observed among the experiments, and  
 818 thus, the chemotaxis index average was determined based on 10 repetitions per treatment.  
 819  $CI \geq 0.2$  highly attractive;  $0.1 < CI < 0.2$  slightly attractive;  $-0.1 \leq CI < 0.1$  random  
 820 response;  $-0.2 \leq CI < -0.1$  repellent; and  $CI \leq -0.2$  highly repellent

821

### 822 3.7 Chemotaxis response of *M. enterolobii* J2 to the root exudates of cucumber 823 treated or not with microorganisms

824 The J2 of *M. enterolobii* was highly attracted by the cucumber roots exudates free  
825 of microorganisms. In contrast, after the treatments with *B. amyloliquefaciens*, *P.*  
826 *lilacinum*, and the mixture of both, the exudates showed to be highly repellent to the *M.*  
827 *enterolobii* J2. A random response was observed in the control (water) (Fig. 9).



828

829 **Figure 9:** Chemotaxis index of *Meloidogyne enterolobii* second-stage juveniles (J2)  
830 towards the exudates of cucumber roots without (C) and with treatment of *Bacillus*  
831 *amyloliquefaciens* (CBa), *Purpureocillium lilacinum* (CPI) and the mixture of both  
832 (CBaPI). Control: Water (w). The chemotaxis index average was determined based on 10  
833 repetitions per treatment, as there was no statistically significant difference observed ( $P >$   
834  $0.482$ ) among the experiments.  $CI \geq 0.2$  highly attractive;  $0.1 < CI < 0.2$  slightly  
835 attractive;  $-0.1 \leq CI < 0.1$  random response;  $-0.2 \leq CI < -0.1$  repellent; and  $CI \leq -0.2$   
836 highly repellent

837

## 838 4. Discussion

839 The application of bioproducts based on antagonist microorganisms for the  
840 management of plant-parasitic nematodes has increased exponentially in recent years  
841 (Machado, 2022). In the present study, *B. amyloliquefaciens* strain D747 and *P. lilacinum*

842 strain PL251 alone were not able to reduce the nematode infectivity (gall index) or  
843 reproduction (eggs per gram of root). However, the combination of the bio-nematicides  
844 effectively decreased the number of galls and eggs in experiments *in vivo*. Therefore, in  
845 this study, we further investigated how the compatibility between these bio-nematicides  
846 affects some of their beneficial activities. Based on our results the cell-free supernatant  
847 produced by the mixture of *B. amyloliquefaciens* strain D747 and *P. lilacinum* strain  
848 PL251 seems to potentiate the effect of bionematicides when applied together.

849 In certain cases, other mixtures of bio-nematicides may be detrimental, which can  
850 lead to a reduction in their effectiveness in controlling diseases (Lucon et al., 2009;  
851 Fernandes et al., 2014; Oliveira et al., 2019). Here, the combination of *B.*  
852 *amyloliquefaciens* and *P. lilacinum* showed a cooperative effect that enhanced the control  
853 of *M. enterolobii* by reducing the nematode's hatching, infectivity, and reproduction. In  
854 the literature, similar outcomes were observed with the application of the *B. subtilis* plus  
855 *P. lilacinum*, which increased the microbial biomass in the soil, and induced changes in  
856 the soil physicochemical properties. These findings suggest that this kind of combination  
857 has the potential to modulate the soil-plant system, reducing nutrient losses and changing  
858 the soil physicochemical properties (Mecante et al., 2004; Oliveira et al., 2019; Chen,  
859 2023).

860 *In vitro* trials using *M. enterolobii* J2 showed that the VOCs and cell-free  
861 supernatant produced by *B. amyloliquefaciens* are highly toxic to the nematode. Strains  
862 of *B. amyloliquefaciens* and OGBa can produce compounds such as 2-undecanone, 2-  
863 nonanone, benzeneacetaldehyde, and 2-heptanone, which have been demonstrated to be  
864 toxic to PPNs (Wu et al., 2019; Grahovac et al., 2023; Paula et al. 2023). Furthermore, *B.*  
865 *amyloliquefaciens* and other bacteria from the OGBa group can produce enzymes, such  
866 as surfactin, iturin, bacilomycin, and fungycin (Wang et al., 2022). Nevertheless, it  
867 distinguishes itself from other strains by producing subtilisin, an enzyme that plays a role  
868 in the digestion of nematode cuticles (Ahman et al., 1996; Kerry, 2000; EFSA, 2014).

869 In contrast to the bacteria, the predominant mode of action of *P. lilacinum* is by  
870 the production of molecules that inhibit the nematode hatching, and by the direct  
871 parasitism of eggs. These characteristics are attributed to the capacity of the fungus to  
872 produce enzymes that can degrade the eggshell, helping them to colonize the eggs (Khan  
873 et al., 2006; Giné and Sorribas, 2016). This ability has a considerable impact on the

874 development of nematode J2, disrupting their life cycle. Additionally, our findings are  
875 consistent with earlier studies, indicating that this strain of the fungus produces  
876 metabolites or VOCs with lower toxicity to J2 of *Meloidogyne* spp. (Khan et al., 2003;  
877 Rousidou et al., 2013).

878 Furthermore, there is a hypothesis that the synergic effect of the mixture may be  
879 attributed to the combined modes of action offered by the bacteria and the fungus  
880 (Stirling, 1991; Siddiqui & Shaukat, 2003; Roberts et al., 2005; Ferraz et al., 2010). *In*  
881 *vitro* tests revealed that the combination of cell-free supernatant can boost the mortality  
882 rate of J2 while also reducing the number of J2 hatched. In addition, the *in vivo*  
883 experiments provided further evidence that the mixture can effectively control nematode  
884 populations by decreasing their infectivity and reproductive capacity. These findings  
885 clarify the additive effect, which probably involves a combination of enzymatic activity,  
886 the release of beneficial compounds by the microorganisms, and direct parasitism.  
887 Moreover, the metabolites produced by the microorganisms can be mutually beneficial.  
888 Some findings have indicated that the indole acetic acid (IAA) produced by the *Bacillus*  
889 group can function as a signaling molecule for both the fungus and the plant (Sedhupathi  
890 et al., 2022).

891 Besides the effect, when each microorganism was applied alone to the soil, they  
892 were unable to reduce nematode damage and reproduction. One of the facts that can help  
893 to explain the result is the composition of the soil used in the experiment. Florida is well  
894 known for having soils with more than 90% sand and a low content of material organic  
895 (Desaeger, 2021). This factor can impact the persistence and density of *P. lilacinum*  
896 spores in the soil due to the saprophytic nature of the fungus (Rumbos & Kiewnick, 2006).  
897 Moreover, studies using the same strain have demonstrated that after 5 days, the number  
898 of spores decreases gradually, and in 90 days, the level of spores in the soil can be reduced  
899 by more than 70% (Rumbos et al., 2008; Rousidou et al., 2013). As a result, the reduction  
900 of spores in the soil may contribute to the lower control rate observed in the trials. For *B.*  
901 *amyloliquefaciens*, there is a lack of information about its persistence in the soil.

902 Chemotaxis is the term used to describe the movement of nematodes toward  
903 exudates released by plants (Wang et a., 2021). This behavior can be influenced by  
904 nematode species, rhizosphere microorganisms, and plants (Kihika et al., 2017; Wang et  
905 al., 2019; Wang et al., 2021; Pacheco et al., 2021; Cavalcanti, 2023). In addition, the

906 biocontrol agents can modulate the plant exudate composition, resulting in the host  
907 becoming less attractive or even repulsive to the nematode (Zhao et al., 2022; Cavalcanti  
908 et al., 2023). Our results indicated that while the nematode was highly attracted to *B.*  
909 *amyloliquefaciens*, it was repelled by *P. lilacinum*. However, when the microorganisms  
910 were inoculated in the cucumber roots, the host became repellent for J2 of *M. enterolobii*  
911 before being attracted to it. Similar results were demonstrated using the exudates  
912 extracted from lettuce roots, with and without the inoculation of *B. subtilis*. The plants  
913 without bacteria were attractive to J2 of *M. incognita*. However, after *B. subtilis*  
914 inoculation, the exudates became repellent to it (Cavalcanti et al., 2023).

915         Based on our results and the mode of action of the microorganisms, a more  
916 suitable timing for product application, for sand soils and vegetables, can be suggested.  
917 The label instructions for both products suggest the first application before planting  
918 (Double Nickel® LC 2(ee) Nematodes (TX), 2021; MeloCon® LC, 2020). suggest the first  
919 application before planting. Specifically, for the mixture, we recommend the initial  
920 application to be conducted between 7 to 14 days before planting. This early application  
921 can effectively reduce the initial nematode population while promoting the growth of  
922 beneficial microorganisms. Based on our findings, a second application should be  
923 scheduled 10 days after planting. At this stage, the microorganisms can form associations  
924 with the plant roots, releasing repellent compounds to protect the roots. To maintain an  
925 adequate population of beneficial microorganisms in the soil, it could also be  
926 recommended to make an additional 2 of the mixture at intervals of 20, and 30 days after  
927 planting. This approach will help to ensure that the microorganisms remain active and  
928 effective in supporting plant growth and health throughout the growing season. We intend  
929 to test and hopefully confirm these findings in naturally root-knot infested vegetable  
930 fields in Florida.

931

## 932 **5. Conclusions**

933         Our findings demonstrate the potential efficacy of a mixture comprising *P.*  
934 *lilacinum* and *B. amyloliquefaciens* in controlling *M. enterolobii*. The observed  
935 interactive effects suggest that the mixture acts at various stages of the nematode's life  
936 cycle. Additionally, the chemotaxis experiment showed that the microorganism mixture  
937 can modulate the nematode's host preference by making attractant the root exudates

938 repellent to the nematode. These results highlight the potential of the mixture as a  
939 valuable tool for managing nematode infestations in agriculture. However, it is  
940 important to note that further experiments are needed to better understand how the  
941 mixture may act in different soil types, with various plant and nematode species. These  
942 experiments would help to enhance our knowledge of the effectiveness of the product  
943 and enable more targeted and efficient use in diverse agricultural settings.

944

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949

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1211 **Abstract**

1212 Nematodes from the *Meloidogyne* genus present a major challenge to agricultural  
1213 progress, requiring control methods that are environmentally safe and human friendly.  
1214 This study assessed the nematicidal effects of papaya latex and papain, both *in vitro* and  
1215 *in vivo*, on tomato plants, targeting second-stage juveniles (J2s) and eggs of *Meloidogyne*  
1216 *javanica*. The results demonstrated that papaya latex effectively increased J2 mortality,  
1217 reaching 100% at the highest concentration (1%). In contrast, papain resulted in a  
1218 maximum J2 mortality of 72% at its highest concentration (3.5%). Additionally, papaya  
1219 latex consistently inhibited J2 hatching, with an average reduction of 45% in the first  
1220 experiment and reductions of 39% and 61.9% in the second experiment at concentrations  
1221 of 1.5% and 3%, respectively. Similarly, papain suppressed J2 hatching at all  
1222 concentrations, leading to an average reduction of 44% in the first experiment and 55%  
1223 in the second. In the first replication of the *in vivo* experiment with tomato plants, the  
1224 highest concentration of papaya latex (4%) reduced *M. javanica* infectivity by 68% and  
1225 reproduction by 89%. In the second experiment, this concentration reduced infectivity by  
1226 70% and reproduction by 80%. For papain, infectivity was reduced by 55% in the first  
1227 experiment and 40% in the second. While reproduction did not differ significantly  
1228 between treatments and the control in the first experiment, it decreased by 30% in the  
1229 second. Further experiments showed that both papaya latex and papain effectively  
1230 activated the plant defense system in tomato. Overall, these findings add to the growing  
1231 body of evidence supporting the use of natural compounds for sustainable nematode  
1232 management.

1233 **Keywords:** Sustainable pest management, root-knot nematodes, cysteine, plant defense  
1234 induction, natural compounds.

## 1235 **1 Introduction**

1236 Year by year the global crop production has been challenged by the attack of plant-  
1237 parasitic nematodes (PPNs) (Sikora et al., 2018). These soil-borne pathogens pose a  
1238 significant threat to global agriculture, resulting in annual losses of approx.130 billion  
1239 dollars (Sikora et al., 2022). Among the PPNs, the *Meloidogyne* genus, commonly known  
1240 as root-knot nematodes (RKNs), stands out as the most important (Jones et al., 2013). The  
1241 RKNs are obligate, polyphagous endoparasites that induces changes in the roots  
1242 (Castagnone-Sereno, 2002). In addition to direct damage, the injury caused during  
1243 juvenile penetration can also facilitate the development of other disease complexes (Sarri  
1244 et al., 2024). Of the approximately 100 described RKN species, *M. arenaria*, *M.*  
1245 *incognita*, *M. javanica*, and *M. hapla* are the most damaging. These species are widely  
1246 distributed globally and can parasitize numerous plant species (Subbotin et al., 2021).

1247 Nematode control is an issue for farmers worldwide and can be achieved through  
1248 various techniques, including crop rotation, solarization, soil fumigation, chemical  
1249 applications, and biological control (Sarri et al., 2024). In past decades, chemicals used  
1250 for nematode control were broad-spectrum products with high toxicity to soil microbiota,  
1251 the environment, humans, and animals (Desaeger et al., 2020). However, due to their  
1252 harmful effects on the environment and health, many of the most effective chemical  
1253 nematicides have been withdrawn from the market in recent years (Noling, 2002; U.S.  
1254 Environmental Protection Agency, 2008; Anvisa, 2017; Wram and Zasada, 2019).  
1255 Currently, the most effective approach involves an integrated strategy that combines  
1256 chemical treatments with genetic resistance, biological control, and the use of natural  
1257 extracts, such as latex from various plant species, which exhibit nematocidal properties.

1258           Latex is a substance produced by specialized cells called laticifers, which plants  
1259 generate as a defense mechanism against insects, animals, and environmental stresses  
1260 (Macalood, 2014). Several types of latex, obtained from different species, demonstrate  
1261 toxic effects on plant-parasitic nematodes (PPNs) and may contribute to the plant's  
1262 defense system against them (Anver & Alam, 1992; Lopes et al., 2004; Kalaiselvi et al.,  
1263 2019). Among latex-producing species, the most common and well-studied is *Carica*  
1264 *papaya* L. (Caricaceae). Papaya is typically consumed fresh and is rich in sugars,  
1265 vitamins, and volatile compounds (Embrapa, 2013). As a laticiferous plant, papaya  
1266 produces latex throughout its entire structure, with the highest concentration of bioactive  
1267 compounds and enzymes found in immature fruits. Papain [EC 3.4.22.2] is one of the  
1268 most well-studied enzymes in papaya latex, primarily because it is the easiest to isolate  
1269 (Moussaoui et al., 2001; Castro et al., 2023). This enzyme is stable at high temperatures,  
1270 easily dissolved in water, and low-cost. In addition to papain, other bioactive compounds  
1271 are also present in papaya latex (Moussaoui et al., 2001).

1272           Several studies have shown the high toxicity of papaya latex and papain against  
1273 various nematode species that infect both animals and plants (Satrija et al., 1995; Satrija  
1274 et al., 1994; Saravanapriya & Sivakumar, 2004; Saravanapriya et al., 2004; Peachey et  
1275 al., 2016; Stepek et al., 2004; Soares et al., 2019). However, these effects have mainly  
1276 been investigated in vitro, emphasizing the need for further research to understand the  
1277 stability of these compounds in soil and their potential impact on nematode disease  
1278 establishment, particularly in terms of plant defense. To address this gap, the present  
1279 study aimed to evaluate the nematicidal potential of papaya latex and papain against  
1280 second-stage juveniles (J2s) and eggs of *Meloidogyne javanica*. In vitro experiments  
1281 using various concentrations of papaya latex and papain were conducted to assess their

1282 effects on J2s and egg hatching. *In vivo* experiments examined the impact of different  
1283 concentrations of latex and papain on the infectivity and reproduction rates of the  
1284 nematode. Additionally, the effects of these treatments on the tomato plant's defense  
1285 system against *M. javanica* were evaluated using the split-root technique.

1286

## 1287 **2 Material and methods**

### 1288 **2.1 Extraction of Papaya Latex and Papain, and Assessment of Enzymatic** 1289 **Concentration and Activity**

1290 Green papaya fruits of the *Formosa* variety were sourced from a local producer in  
1291 Lavras, MG. The fruits were surface cleaned using 70% alcohol, afterward, the latex was  
1292 extracted by making longitudinal cuts. The collected latex was stored in Falcon tubes, and  
1293 to preserve it, the water content was removed through lyophilization. The soluble protein  
1294 content was measured using the Bradford method (1976), with a standard curve  
1295 constructed using bovine serum albumin (BSA). Protease activity (U/mg) was determined  
1296 by casein hydrolysis, as described by Gomes et al. (2018). One enzymatic unit (U) was  
1297 defined as the amount of enzyme required to release 1.0 µg of tyrosine per minute under  
1298 assay conditions. Pure papain, obtained from *Dinamica Laboratory (Dinamica Química*  
1299 *Contemporânea LTDA – Indaiatuba, São Paulo)*, was used at an approximate  
1300 concentration of 100%. Both protein content and enzymatic activity were measured using  
1301 the same methodology as for the latex.

1302

### 1303 **2.2 Obtaining the nematode inoculum**

1304 The initial population of *M. javanica* was obtained from lettuce (*Lactuca sativa*)  
1305 roots at a farm located in Ijaci, MG, Brazil (latitude 21°16'37" S; longitude 44°91'66" W).

1306 The purity of the inoculum was confirmed by isoenzyme electrophoresis (Carneiro and  
1307 Almeida, 2001). The pure population was then cultivated in pots filled with commercial  
1308 substrate (Multiplant®) and tomato seedlings (*Solanum lycopersicum* L. cv. Santa Clara).  
1309 The pots were kept in a greenhouse for at least two months at 28 °C, with soil moisture  
1310 maintained at 60% of field capacity. Afterward, an egg suspension was prepared using  
1311 the technique described by Hussey and Barker (1973). For the experiments with J2, the  
1312 egg suspension was placed in a hatching chamber and incubated at 28 °C. Only J2s that  
1313 hatched after 48 hours were used in the subsequent tests.

1314

### 1315 **2.3 Toxic Effects of Papaya Latex and Papain on Second-Stage Juveniles of** 1316 ***Meloidogyne javanica***

1317 For this experiment, five concentrations of dry papaya latex (0.0%, 0.25%, 0.35%,  
1318 0.5%, 0.75%, and 1% weight/volume) were used. In a separate experiment with papain,  
1319 the concentrations tested were 0%, 1.5%, 2%, 2.5%, 3%, and 3.5% (weight/volume). The  
1320 solutions were prepared at twice the desired final concentration using distilled water. To  
1321 eliminate dirt and large particles, the solutions were centrifuged at 10,000 rpm for 5  
1322 minutes. In 600 µL microtubes, 300 J2 nematodes were combined with 300 µL of the  
1323 selected papaya latex or papain solutions to achieve the desired final concentrations. For  
1324 the control group, 300 µL of distilled water replaced the test solutions. The microtubes  
1325 were sealed and incubated at 28 °C for 24 hours. After incubation, the microtubes were  
1326 homogenized and opened, and 100 µL of the suspension was transferred to a 96-well  
1327 polypropylene plate. The numbers of mobile, immobile, and dead J2 nematodes were  
1328 estimated using an inverted microscope (Nikon TMS-F, New York, USA). To confirm  
1329 nematode death, 20 µL of a 1.0 mol L<sup>-1</sup> sodium hydroxide (NaOH) solution was added to

1330 each well, following the procedure of Chen and Dickson (2000). J2 nematodes that  
1331 remained immobile for approximately 3 minutes were recorded as dead.

1332

#### 1333 **2.4 Hatching of *Meloidogyne javanica* Second-Stage Juveniles Following Egg** 1334 **Exposure to Papaya Latex and Papain**

1335 This experiment was conducted using 5.5 cm polypropylene Petri dishes. Latex  
1336 solutions (0%, 0.25%, 1%, 1.5%, and 3% weight/volume) and papain solutions (0%, 1%,  
1337 2%, 4%, and 8% weight/volume) were prepared in distilled water, with distilled water  
1338 serving as the control. Each dish was filled with 10 mL of the respective solution. A 500-  
1339 mesh PVC sieve (pore size 0.025 mm) was placed in each dish to hold the eggs. An  
1340 aqueous suspension of approximately 5,000 eggs in 50  $\mu$ L was added to each sieve. The  
1341 dishes and sieves were covered and incubated at 28 °C for 48 hours.

1342 After the incubation period, the sieves were removed, and the liquid in the dishes  
1343 was collected to quantify the number of hatched J2 nematodes. The sieves were then  
1344 carefully washed to remove residual solution, and 10 mL of distilled water was added to  
1345 each dish. The sieves containing the eggs were returned to their respective dishes.  
1346 Hatching evaluations were conducted every 48 hours over a 10-day period, resulting in a  
1347 total of five evaluations. The procedure outlined above was repeated for each evaluation.

1348

#### 1349 **2.5 Application of Papaya Latex and Papain to Substrates Infested with *Meloidogyne*** 1350 ***javanica* Second-Stage Juveniles**

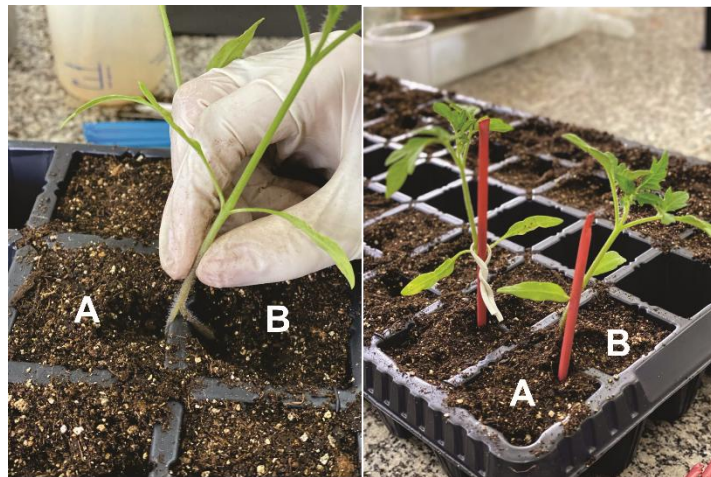
1351 In this experiment, different concentrations of papaya latex (0%, 1.5%, 2%, 2.5%,  
1352 and 3% weight/volume) and papain (0%, 1%, 2%, 4%, 6%, and 8% weight/volume) were  
1353 tested. The solutions were prepared using distilled water. Twenty-day-old tomato

1354 seedlings of the Santa Clara variety were transplanted into 300 mL plastic pots filled with  
1355 Multiplant® substrate. Using a glass stick, four holes were made near the stem of each  
1356 seedling, and 4 mL of a suspension containing 600 J2 nematodes were distributed across  
1357 the holes within the same pot. Immediately after infestation, 4 mL of the respective latex  
1358 solution was applied into the holes. The holes were then covered with substrate, and the  
1359 pots were placed in a greenhouse. After 45 days, the infectivity (number of galls) and  
1360 reproduction (number of eggs per gram of root) were evaluated.

1361

## 1362 **2.6 Induction of Plant Defense Mechanisms by Papaya Latex and Papain**

1363 This assay followed the methodology described by Medeiros et al. (2017). A 15-  
1364 day-old tomato seedling was carefully removed from the substrate, and its roots were  
1365 gently washed. The root system was then divided into two parts by making a vertical cut  
1366 at the base of the stem using a scalpel. Each split root was transplanted into separate  
1367 border cells (A and B) of a 50-cell planting tray (Fig. 1).



1368

1369 **Figure 1.** Transplantation of tomato seedling after the vertical cut in the stem (1) and  
1370 the treatment portions A and B (1 and 2).

1371 Five days after transplantation, the split root in cell A was treated with 2 mL of  
1372 one of the following treatments: (i) water (control), (ii) 2% latex, (iii) 8% latex, (iv) 8%  
1373 latex + 2 mL containing 500 J2, (v) 2% papain, (vi) 8% papain, or (vii) 8% papain + 2  
1374 mL containing 500 J2. Five days after the treatments were applied to the roots of part A,  
1375 the roots in cell B were infested with 2 mL of a suspension containing 500 J2 of  
1376 *Meloidogyne javanica*.

1377 Forty-five days later, the number of galls per gram of root and eggs per gram of  
1378 root were evaluated for both parts A and B. For treatments (i), (ii), (iii), (v), and (vi), only  
1379 the roots of part B were analysed, while for treatments (iv) and (vii), both root portions  
1380 (A and B) were analysed. To complete the evaluation, the tomato plants were removed,  
1381 and the two root portions were separated for analysis.

1382

## 1383 **2.7 Experimental design and statistical analysis**

1384 The experiments were conducted in a completely randomized design with five replicates  
1385 per treatment, and each experiment was performed twice to ensure result reliability. Data from  
1386 the two experimental repetitions were analysed using Two-way ANOVA. If no significant  
1387 interaction was detected ( $P > 0.05$ ), the results were combined ( $N = 10$ ). Normality and  
1388 homogeneity assumptions were verified using the Shapiro-Wilk test and Bartlett test,  
1389 respectively. For data that did not meet these assumptions, transformations were applied before  
1390 analysis.

1391 Once the statistical assumptions were satisfied, an F-test was performed as part of the  
1392 ANOVA. When the F-test indicated significance ( $P < 0.05$ ), treatment means were compared  
1393 using Tukey's test ( $P < 0.05$ ). For quantitative variables, regression analyses were conducted  
1394 using linear or non-linear models. Statistical analyses were performed in R (version 3.6.0), while  
1395 graphics were generated using SigmaPlot (version 12.0) and TableCurve 2D (version 5.0).

1396

1397 **3 Results**1398 **3.1 Protein Content and Enzymatic Activity of Papaya Latex and Papain**

1399 Biochemical assays conducted on the extracted latex samples revealed differences  
 1400 in soluble protein concentrations: 392.1  $\mu\text{g/mL}$  in the first sample and 558.2  $\mu\text{g/mL}$  in  
 1401 the second. Despite this variation, the protease activity of the extracts was similar,  
 1402 measuring 7.86 U/mg in the first collection and 7.73 U/mg in the second. For papain, the  
 1403 same product was used in all experiments. After approximately seven months of storage  
 1404 at  $-20\text{ }^{\circ}\text{C}$ , a slight decline in protease activity was observed, from 2.07 U/mg in the first  
 1405 assay to 1.89 U/mg in the second (Table 1).

1406

1407 **Table 1.** Soluble protein content of Latex (PCL) and Papain (PCP) and protease activity  
 1408 of Latex (EAL) and Papain (EAP). Papaya latex was obtained from green papaya fruits  
 1409 of the Formosa variety. Papain was obtained from a commercial product.

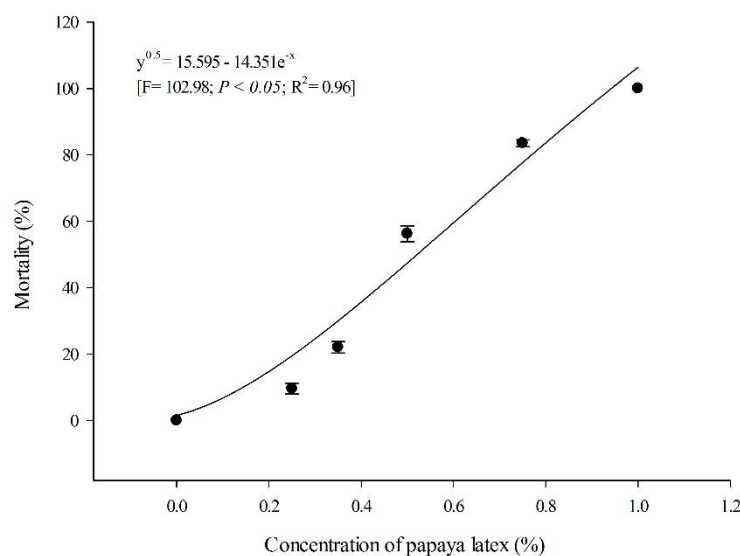
Initial determination				7 months later		
PCL	EAL	PCP	EAP	PCL	EAL	EAP
( $\mu\text{g/mL}$ )	(U/mg)	( $\mu\text{g/mL}$ )	(U/mg)	( $\mu\text{g/mL}$ )	(U/mg)	(U/mg)
392.1	7.86	671.96	2.070	558.2	7.73	1.89

1410

1411

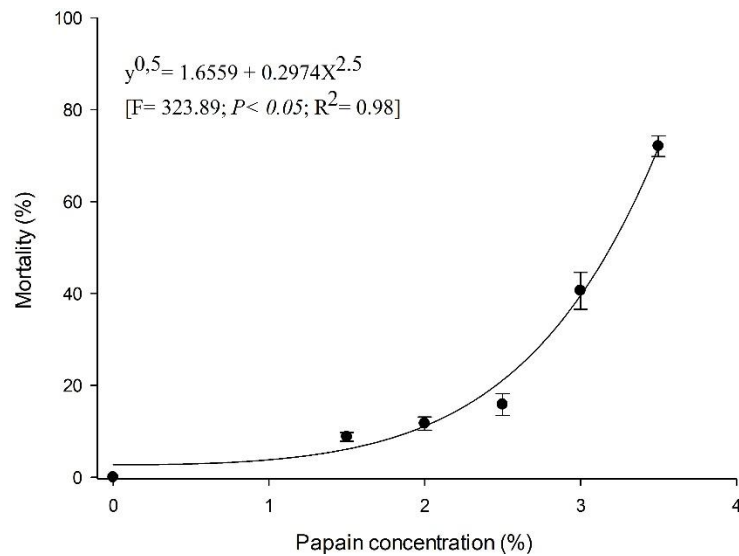
1412 **3.2 Toxic Effects of Papaya Latex and Papain on Second-Stage Juveniles of**  
 1413 ***Meloidogyne javanica***

1414 An interaction was observed between the concentration of papaya latex and the  
 1415 mortality rate of *Meloidogyne javanica* J2s. Even at the lowest tested concentration  
 1416 (0.25%), a mortality rate of 12.7% was recorded, increasing to 100% at the highest  
 1417 concentration (1%) (Fig. 2).



1418  
 1419 **Figure 2.** *Meloidogyne javanica* mortality after 24 hours of J2 exposure to different  
 1420 papaya latex concentrations (0%, 0.25%, 0.35%, 0.5%, 0.75%, and 1%). The data  
 1421 presented are the average of 10 repetitions, as there was no significant difference ( $P >$   
 1422 0.213) between the first and second repetition in time. Error bars represent the standard  
 1423 error of the mean.

1424 Papain demonstrated toxicity toward *Meloidogyne javanica* J2s. Similar to latex,  
 1425 an increase in papain concentration corresponded to higher mortality rates (Fig. 3). The  
 1426 highest tested concentration (3.5%) resulted in a mortality rate of 72%.



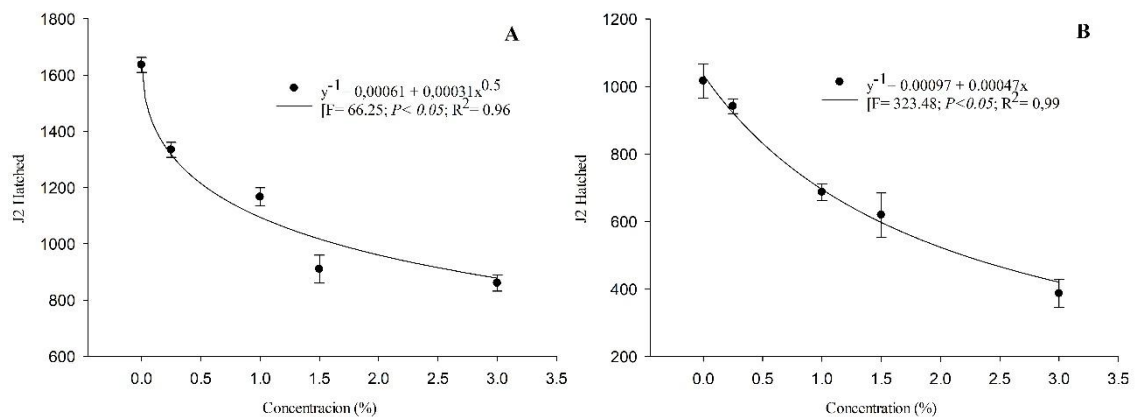
1427

1428 **Figure 3.** *Meloidogyne javanica* mortality after 24 hours of second stage juveniles (J2)  
 1429 exposure to different papain concentrations (0%, 1.5%, 2%, 2.5%, 3% and 3.5%). This  
 1430 trail was analyzed considering the average of 10 repetitions, as there was no significant  
 1431 difference ( $P > 0.05$ ) between the first and second repetition in time. Bars represent the  
 1432 standard error of the mean.

1433

### 1434 **3.3 Hatching of *Meloidogyne javanica* Second-Stage Juveniles Following Egg** 1435 **Exposure to Papaya Latex and Papain**

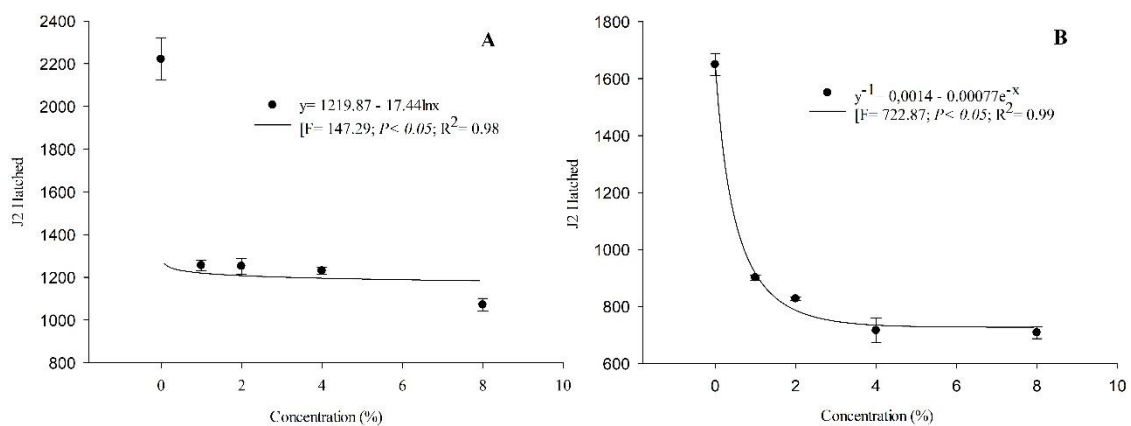
1436 All concentrations of papaya latex significantly reduced the hatching of *Meloidogyne*  
 1437 *javanica* J2s compared to the control. In the first experiment, no significant difference was  
 1438 observed between the 1.5% and 3% latex concentrations, both achieving an average hatching  
 1439 reduction of 45% relative to the control. In the second experiment, however, the highest  
 1440 concentrations (1.5% and 3%) demonstrated a significant difference, with hatching reductions of  
 1441 39% and 61.9%, respectively, compared to the control (Fig. 4).



1442

1443 **Figure 4.** Experiment 1 (A) and Experiment 2 (B). Number of *Meloidogyne javanica*  
 1444 second stage juveniles (J2s) hatched after exposure of the eggs to papaya latex (0%,  
 1445 0.25%, 1%, 1.5%, e 3%) for 48 hours. Negative controls: water (0%). The regression  
 1446 analysis was performed with the total number of J2s hatched after the tenth day of five  
 1447 evaluations.

1448 Similarly, all concentrations of papain effectively inhibited the hatching of *M. javanica*  
 1449 J2s, showing a consistent trend across both experiments. The average hatching reductions were  
 1450 44% and 55% in experiments one and two, respectively, compared to the control (Fig. 5).



1451

1452 **Figure 5.** Experiment 1 (A) and Experiment 2 (B). Number of *M. javanica* J2s hatched  
 1453 10 days after exposure of the eggs to papain (0%, 1%, 2%, 4% and 8%.) for 48 hours.

1454 Negative controls: water (0%). Statistical analysis was performed with the total number  
1455 of J2s hatched after the tenth day of evaluation.

1456

### 1457 **3.4 Application of Papaya Latex and Papain on Substrates Infested with** 1458 ***Meloidogyne javanica* Second-Stage Juveniles**

1459 The direct application of papaya latex to *Meloidogyne javanica* J2-infested  
1460 substrate significantly reduced ( $P < 0.01$ ) nematode infectivity (galls per gram of root)  
1461 and reproduction (eggs per gram of root) (Table 2). In the first experiment, an increase in  
1462 the concentration of papaya latex was positively correlated with a reduction in nematode  
1463 infectivity and reproduction. At the highest concentration (4%), reductions of 68% in  
1464 infectivity and 89% in reproduction were observed compared to the control. However, in  
1465 the second experiment, the averages of galls per gram of root and eggs per gram of root  
1466 were similar across all concentrations used. When comparing the treatments with the  
1467 control, the overall averages showed reductions of 70% in infectivity and 80% in  
1468 reproduction.

1469

1470 **Table 2.** Infectivity (galls/g of root) and reproduction (eggs/g of root) of *Meloidogyne*  
1471 *javanica* resulting from the application of different papaya latex concentrations (0%,  
1472 1.5%, 2%, 3% and 4%) on *M. javanica* J2s infested substrate. Means with the same letter  
1473 do not differ statistically ( $P > 0.05$ ) using Tukey's test.

Treatments	Galls/g root		Eggs/g root	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
<b>Water</b>	25.5 c	32.82 b	714.13 c	4868.04 b
<b>1%</b>	16.07 b	10.77 a	392.27 b	1437.59 a
<b>2%</b>	10.95 a	10.36 a	342.33 b	1520.67 a
<b>3%</b>	11.27 a	11.08 a	394.73 b	1187.8 a
<b>4%</b>	8.86 a	11.55 a	92.76 a	1682.5 a

F=27.8/ CV=10,1 P<0.001	F=16.3/ CV=16,0 P<0.001	F=22.2/ CV=29,7 P<0.001	F=6.7/ CV=27,8 P<0.001
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1474

1475 For papain, infectivity (galls per gram of root) was significantly higher ( $P < 0.05$ )  
1476 at concentrations above 4% in both experiments. A similar trend was observed for  
1477 reproduction (eggs per gram of root) reduction in the second experiment (Table 3).

1478

1479 **Table 3.** Infectivity (galls/g of root) and reproduction (eggs/g of root) of *Meloidogyne*  
1480 *javanica* resulting from the application of papain on substrate infested with 600 *M.*  
1481 *javanica* J2s (0%, 1%, 2%, 4%, 6% and 8%). Negative control: water. Means with the  
1482 same letter do not differ statistically ( $P > 0.05$ ) using Tukey's test.

Treatments	Galls/g root		Eggs/g root	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Water	15.01 b	15.69 b	1100.97 a	3463.68 b
2%	6.70 a	13.32 b	1271.10 a	3368.39 b
4%	6.30 a	8.78 a	597.14 a	1928.51 a
6%	8.76 a	9.78 a	1093.60 a	2715.29 a
8%	5.62 a	10.15 a	1117.70 a	2597.87 a
	F=1.9/ CV=25,3 P<0.001	F=9.5 / CV=9,5 / P<0.001	F=0.9/ CV=56,1 P<0.001	F= 2.2/ CV=36.8 P<0.05

1483

1484 In the first experiment, all concentrations showed a statistically significant reduction in  
1485 galls per gram of root compared to the control. However, no significant differences were  
1486 observed among the 2%, 4%, 6%, and 8% concentrations. On average, these treatments  
1487 resulted in a 55% reduction in infectivity relative to the control.

1488 In the second experiment, the lowest concentration (2%) was statistically similar ( $P >$   
1489 0.05) to the control. Conversely, the 4%, 6%, and 8% concentrations did not differ  
1490 significantly from each other but achieved an average reduction of 40% in galls per gram  
1491 of root compared to the control.

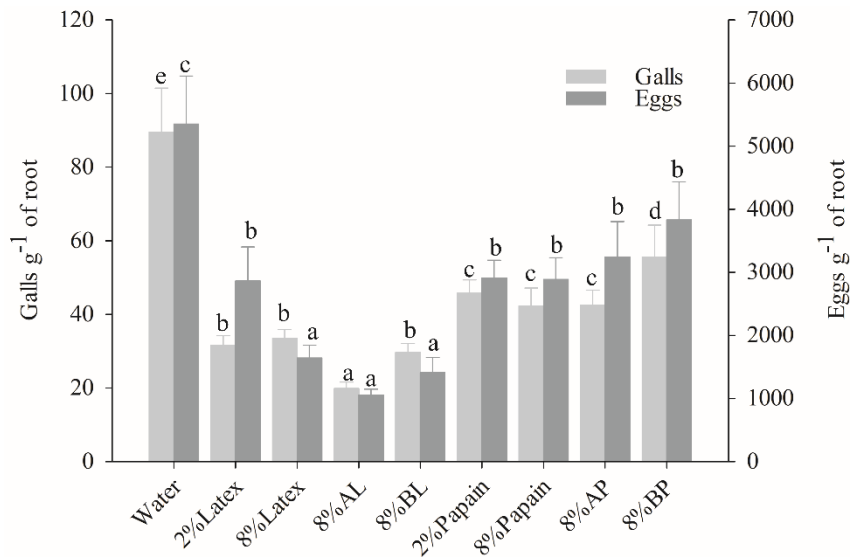
1492 Similarly, the trend observed in the second experiment for galls per gram of root  
1493 was also evident for eggs per gram of root. Only the lowest concentration (2%) did not  
1494 show a significant difference compared to the control. In contrast, the concentrations of  
1495 4%, 6%, and 8% did not differ significantly from each other. On average, these  
1496 concentrations resulted in a 30% reduction in nematode reproduction compared to the  
1497 control.

1498

### 1499 **3.5 Induction of Plant Defense by Papaya Latex and Papain**

1500 All aqueous concentrations of papaya latex and papain induced the tomato plant's  
1501 defense system, effectively reducing nematode infectivity and reproduction. Compared  
1502 to the control, the highest reduction in infectivity (78%) was observed with the treatment  
1503 of 8% papaya latex plus nematodes on portion A. Among the other papaya latex  
1504 treatments, no significant differences ( $P > 0.05$ ) were observed, with an average  
1505 infectivity reduction of 65%. For infectivity, the 8% papaya latex treatment, across all  
1506 conditions, demonstrated a consistent reduction, averaging 75%.

1507 For papain, infectivity reductions were similar across treatments, with an average  
1508 reduction of 44%. The only statistically significant difference was observed in portion B  
1509 treated with 8% papain plus nematodes, which showed a reduction of 37%. Regarding  
1510 reproduction, all papain treatments and the 2% papaya latex treatment were statistically  
1511 similar ( $P > 0.05$ ), resulting in an average reduction of 41% (Fig. 6).



1512

1513 **Figure 6.** Infectivity (galls  $g^{-1}$  of root) and reproduction (eggs  $g^{-1}$  of root) of *M. javanica*

1514 resulted of induction of defense system of plant by papaya latex and papain. Treatments

1515 on portion A and result from portion B: Water (control), 2%Latex, 8%Latex, 8%BL

1516 (Latex plus infestation with J2 of *M. javanica*), 2%Papain, 8%Papain and 8%BP (Papain1517 plus infestation with J2 of *M. javanica*); Treatment from portion A and result from portion1518 A: 8%AL (Latex plus infestation with J2 of *M. javanica*), 8%AP (Papain plus infestation1519 with J2 of *M. javanica*).

1520

1521 **4 Discussion**

1522 Plant incorporation, extracts, and plant residues have been shown to effectively

1523 reduce nematode population densities (Stepek et al., 2007; Carboni and Ntalli, 2014;

1524 Paula et al., 2023). Furthermore, the effectiveness of different latexes and their associated

1525 compounds in controlling plant-parasitic nematodes has been studied (Kumar et al., 2018;

1526 Kalaiselvi et al., 2019; Gomes et al., 2019). In this study, we demonstrated the toxicity of

1527 papaya latex and papain on various life stages of *Meloidogyne javanica*. Additionally, we

1528 highlighted the nematicidal activity of papaya latex and papain following soil application  
1529 and their potential to induce plant resistance.

1530         The slight variation in soluble protein content and protease activity of the latex  
1531 may be attributed to the timing of fruit collection. Latex production and soluble protein  
1532 content are influenced by several factors, including seasonality, plant sex (female or  
1533 hermaphrodite), and environmental fluctuations (Borella & Stevanato, 2015). When  
1534 comparing the soluble protein content and proteolytic activity of latex and papain, the  
1535 differences can be explained by the composition of both extracts. Papaya latex, prior to  
1536 water removal, consists of 85% water and 15% dry matter, which includes a soluble  
1537 fraction containing carbohydrates, salts, lipids, and biomolecules such as cysteine  
1538 proteinases, glutathione, and other proteins (Moussaoui et al., 2001). Therefore, it is  
1539 expected that the pure papain, purchased from the market, would have a higher  
1540 concentration of soluble proteins due to the absence of these other compounds. In  
1541 contrast, the higher proteolytic activity observed in the latex is likely due to the presence  
1542 of additional cysteine proteinases and other proteins (Moussaoui et al., 2001; Azarkan et  
1543 al., 2003; Premakumari et al., 2023).

1544         As observed in other studies in the literature, both papaya latex and papain  
1545 exhibited toxicity towards J2 of *Meloidogyne javanica*. The higher toxicity observed in  
1546 latex, such as 100% mortality at 1% compared to 20% mortality for papain at 2%, can be  
1547 attributed to the presence of additional enzymes and compounds like alkaloids,  
1548 terpenoids, tannins, flavonoids, and phenols (Chandrasekaran et al., 2018; Premakumari,  
1549 Gopinath, and Narmadha, 2023). Castro et al. (2023) demonstrated that even after  
1550 denaturation, latex still exhibited toxic effects on the free-living nematode *Panagrellus*  
1551 *sp.* Furthermore, the toxicity of both latex and papain is primarily linked to the disruption

1552 of the nematode's cuticle, which is mainly composed of proteins, collagens, and cuticulin  
1553 (Stepek et al., 2007; Njom et al., 2021; Castro et al., 2023).

1554 We further demonstrated the detrimental effect of papaya latex and papain on  
1555 nematode eggs, a crucial stage in the nematode life cycle that serves as an inoculum for  
1556 *Meloidogyne* in the soil. Increasing the concentration of papaya latex resulted in a  
1557 decrease in the hatching rate. However, this trend was not observed with papain. Similar  
1558 results were reported when different doses of latex were applied to egg masses and eggs  
1559 of *M. incognita* (Saravanapriya and Sivakumar, 2004; Neeraj et al., 2017). The nematode  
1560 eggshell differs in composition from the cuticle, being primarily composed of non-  
1561 proteinaceous compounds such as chitin (Bird and Bird, 1991). Stepek et al. (2007)  
1562 showed that after 4 hours of exposure to papain, the eggshell remained unaltered.

1563 For the first time, the nematicidal potential of papaya latex and papain applied to  
1564 nematode-infested soil was demonstrated. The study showed that both papaya latex and  
1565 papain effectively reduced nematode infectivity and reproduction. As discussed earlier,  
1566 this outcome can be attributed to the disruption of the nematode cuticle, which can lead  
1567 to death or impair motility, directly affecting the nematode's ability to penetrate plant  
1568 roots (Fioretti et al., 2002; Stepek et al., 2007).

1569 Furthermore, even in the absence of mortality, the nematode's vital functions  
1570 depend on the integrity of the cuticle. Disruption of this structure affects not only motility  
1571 but also the establishment of the parasitic relationship with the plant and the nematode's  
1572 reproductive capacity (Paula et al., 2023). In addition to its direct action on the nematode's  
1573 cuticle and eggshell, latex can also influence other physiological processes critical to the  
1574 nematode's survival. Gas chromatography-mass spectrometry (GC-MS) analysis of a  
1575 chloroform extract from papaya latex revealed various classes of compounds known for

1576 their fungicidal, nematocidal, and bactericidal effects (Chandrasekaran et al., 2018). For  
1577 instance, tetradecanoic acid, identified in the GC-MS analysis, was shown to be toxic to  
1578 *M. incognita* due to its affinity for the acetylcholinesterase (AChE) protein in the  
1579 nematode (Almutairi et al., 2022). AChE is the enzyme responsible for hydrolyzing the  
1580 neurotransmitter acetylcholine (ACh) in cholinergic synapses, where ACh transmits  
1581 signals between neurons. Cholinergic synapses are widespread in both the central and  
1582 peripheral nervous systems, playing a key role in maintaining various physiological  
1583 functions (Duerr et al., 2008).

1584         Despite several studies discussing the potential for nematodes to develop  
1585 resistance or tolerance after the application of various plant-derived latexes, this  
1586 phenomenon has not been extensively explored (Siddiqui and Alam, 1998; Anver and  
1587 Alam, 1992; Lopes et al., 2004). To investigate this, we examined the induction of plant  
1588 defense mechanisms by papaya latex and papain. Our research demonstrated that both  
1589 papaya latex and papain could effectively interfere with nematode infection and  
1590 reproduction, even when applied to separate portions of the roots. Among the proteins  
1591 and enzymes identified in papaya latex, glycosyl hydrolases are a diverse group with  
1592 crucial roles in plant metabolism and defense systems (Stinzi et al., 1993; Moussaoui et  
1593 al., 2001; Azarkan et al., 2003). One such enzyme,  $\beta$ -1,3-glucanase, plays a direct role in  
1594 plant defense by degrading the cell walls of fungal and bacterial pathogens. Additionally,  
1595 this enzyme contributes to the release of signals that activate immune responses by  
1596 triggering the activation of immunity elicitors (Stinzi et al., 1993; Moussaoui et al., 2001;  
1597 Azarkan et al., 2003).

1598         In summary, our study highlights the potential of papaya latex and papain as  
1599 effective agents in controlling nematode infestation and reproduction. Through in vivo

1600 experiments, we demonstrated their ability to reduce nematode populations, with their  
1601 toxicity primarily linked to the disruption of the nematode cuticle. Additionally, our  
1602 results suggest that papaya latex may be more toxic than papain, likely due to its complex  
1603 composition, which includes various compounds with fungicidal, nematicidal, and  
1604 bactericidal properties.

1605         For tomato growers, our findings offer a practical foundation for sustainable  
1606 nematode management strategies. Farmers can prepare cost-effective and easy-to-  
1607 produce aqueous extracts of papaya latex and apply them directly to tomato fields. This  
1608 method provides a natural alternative to chemical nematicides, promoting environmental  
1609 safety and reducing dependence on synthetic inputs. However, further research and field  
1610 trials are necessary to optimize application methods, concentrations, and frequencies for  
1611 large-scale use across various agricultural conditions.

1612         Looking ahead, the commercialization of papaya latex-based products for  
1613 nematode management presents substantial potential. Efforts should focus on developing  
1614 stable, easy-to-apply formulations that maintain the bioactivity of papaya latex  
1615 compounds while ensuring scalability for agricultural use. Additionally, further research  
1616 into the molecular mechanisms by which latex-derived compounds activate plant defense  
1617 systems could lead to more targeted and efficient biotechnological applications,  
1618 improving nematode control strategies and their impact on crop protection.

1619

## 1620 **Conclusion**

1621         Our findings highlight the potential efficacy of papain, and particularly papaya  
1622 latex, in controlling *M. javanica*, as evidenced by the reduction in the number of galls and  
1623 eggs in tomato roots. Under the conditions of this study, in vitro exposure of *M. javanica*

1624 J2 to natural compounds (papaya latex and papain) resulted in significant nematode  
1625 mortality and a reduced hatching rate. Furthermore, this study paves the way for further  
1626 investigation into how natural compounds, such as latex-derived substances, can activate  
1627 plant defense systems. Overall, these results contribute valuable insights into the use of  
1628 natural compounds for nematode management, underscoring the need for continued  
1629 exploration of the mechanisms and applications of plant-derived substances in sustainable  
1630 pest control strategies.

1631

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