Exploitation of Root Gall Associated Fungal Endophytes for the Biological Control of *Meloidogyne graminicola* in Rice

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Suvasri Dutta

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Indian Statistical Institute
Jadavpur University

Dedicated to

To My Parents

for everything, always.

Certificate

This is to certify that the thesis entitled "**Exploitation of Root Gall Associated Fungal Endophytes for the Biological Control of *Meloidogyne graminicola* in Rice"** submitted by Smt. Suvasri Dutta who got her name registered on 03.03.2022, for the award of PhD. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of Dr. Abhishek Mukherjee at Indian Statistical Institute, Giridih and that neither this thesis nor any part of it has been submitted for either any degree/ diploma or any other academic award anywhere before.

Dr. Abhishek Mukherjee,
Associate Professor,
Agricultural and Ecological Research Unit
Indian Statistical Institute, Giridih

Declaration

I hereby declare that the work embodied in this thesis entitled "**Exploitation of Root Gall Associated Fungal Endophytes for the Biological Control of *Meloidogyne graminicola* in Rice"** is completely carried out by me at the Indian Statistical Institute, Giridih branch under the supervision of Dr. Abhishek Mukherjee of Indian Statistical Institute. The work is completely original and had not been submitted elsewhere for the award of any degree or diploma. In consideration of ethical rules and the general practice of reporting scientific investigations, acknowledgements and references had been made whenever the work described is based on the findings of other investigators.

Suvasri Dutta
Senior Research Fellow
Indian Statistical Institute
Giridih, Jharkhand

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Suvasri Dutta

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Synopsis

Root-knot nematodes (RKN), particularly *Meloidogyne graminicola*, pose an increasingly severe threat to rice production in South and Southeast Asia. This problem is especially pronounced under direct-seeded and water-limited cultivation systems. Current nematode control strategies rely heavily on synthetic nematicides, which raise significant concerns due to environmental toxicity, regulatory constraints, and the development of resistance. In this context, root-associated endophytic fungi are gaining attention as ecologically benign alternatives for nematode management.

This thesis investigated the nematicidal potential of root gall-associated fungal endophytes, focusing on a gall-associated isolate of *Aspergillus niger* (F4), and aimed to develop sustainable biocontrol strategy for management of *Meloidogyne graminicola*. The study was structured around five interconnected objectives:

(i) To characterise the fungal endophytic communities associated with *Meloidogyne graminicola*-infected rice roots using both metagenomic and culture-dependent approaches.

(ii) To evaluate the biocontrol potential of root gall-specific fungal endophytes for the sustainable management of *Meloidogyne graminicola*.

(iii) To assess the nematicidal efficacy and defence-inducing activity of the *Aspergillus niger* F4 culture filtrate against *Meloidogyne incognita* in tomato.

(iv) To identify and functionally characterise the bioactive metabolites present in the *A. niger* F4 culture filtrate using metabolomic analyses.

(v) To develop and optimise a stable and efficient formulation of the *A. niger* F4 culture filtrate for field-level application in nematode management.

Collectively, this work aims to develop a mechanistically informed, biologically derived strategy for managing root-knot nematodes in rice, contributing to the advancement of sustainable plant protection.

In objective 1 a combined metagenomic and culture-dependent approach was employed to characterise the fungal communities associated with nematode-infected rice roots. Amplicon sequencing of the ITS1-5.8S rRNA region revealed that infection significantly altered community composition and reduced alpha diversity. Infected root galls exhibited a marked enrichment of stress-tolerant and potentially antagonistic genera such as *Fusarium*, *Aspergillus*, and *Pestalotiopsis*, while healthy roots were dominated by mutualistic taxa including *Talaromyces* and *Curvularia*. Culture-based isolation yielded 37 endophytic fungi, several of which displayed enzymatic traits associated with nematode antagonism, such as chitinase and siderophore production. These findings provide the first detailed insight into the endophytic mycobiome of nematode-induced root galls in rice.

In objective 2, fungal isolates (n = 32 unique taxa) were screened for nematicidal activity. *Aspergillus niger* F4 demonstrated nearly 100% juvenile mortality of *M. graminicola* in vitro, with an LC_{50} of 10.85% (v/v). The isolate also inhibited egg hatch by 97% and exhibited strong repellent activity in chemotaxis assays. Microscopy revealed characteristic vacuolisation in treated juveniles, indicative of methuosis-like cell death. Greenhouse experiments confirmed the efficacy of F4 under in planta conditions, with root-dip treatments resulting in >90% reductions in galling and nematode populations. Biochemical analyses showed significant elevation of defence-related metabolites and enzymes, suggesting that F4 operates through both direct toxicity and host-induced systemic resistance (ISR).

In objective 3 assessed the cross-host applicability of F4, its culture filtrate was tested against *M. incognita* in tomato. In vitro assays demonstrated 97.7% juvenile mortality with a LC_{50} of 17.4%. Greenhouse experiments showed that root dip by F4 culture filtrate suppressed gall formation and reproduction factor by >85%, which was equivalent to that recorded in case of the commercial nematicide Velum® Prime (fluopyram 34.48% SC). Biochemical and transcriptional profiling of treated plants revealed ISR activation via jasmonate and ethylene pathways, in addition to direct nematicidal action. These results confirm that F4 culture filtrate can offer broad-spectrum suppression of RKNs through dual mechanisms.

In Objective 4, Bioactive solvent fractionations of *A. niger* F4 was subjected to spectroscopic analyses (LC-MS/MS, FTIR, and ^1H NMR) to identify the active ingredient. Sphinganine was identified as the lead nematicidal compound in the F4 culture filtrate. Sphinganine exhibited potent dose-dependent activity (LC_{50} =

0.0603 µg/mL) with minimal cytotoxicity in human epithelial cells. Microscopic and ROS-based assays confirmed structural and oxidative stress-related damage in nematodes. Gene expression profiling further indicated disruption of lipid metabolism, neuromuscular signalling, and stress responses in treated juveniles. These findings elucidate the mechanistic basis of nematicidal activity and position sphinganine as a promising biocontrol compound.

Finally, in Objective 5, a water-dispersible granule (WDG) formulation of the F4 culture filtrate was developed to enable practical field deployment. Among seven tested formulations, batch B6 showed optimal physicochemical properties, storage stability, and biological efficacy. In vitro and greenhouse trials demonstrated >95% juvenile mortality and significant reductions in galling and nematode populations. Field validation revealed that the B6 formulation matched the efficacy of the commercial nematicide Velum® Prime, establishing its potential as a biocontrol option for management of *M. graminicola* infestation in rice.

Conclusion

This thesis provides a comprehensive, mechanistic, and translational framework for exploiting root gall-associated fungal endophytes, particularly *Aspergillus niger* F4, as biocontrol agents against root-knot nematodes in rice. The work integrates community ecology, metabolomics, and formulation development to establish a novel, biologically based nematode management strategy. The findings have broader implications for sustainable crop protection and support the advancement of F4-derived sphinganine as a next-generation, eco-compatible nematicide.

Chapter 1: Introduction

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1.1 Importance of *Meloidogyne graminicola* in Rice Production

Rice (Oryza sativa L.) is a staple food for over fifty percent of the global population, contributing around 20% of the world's total calorie consumption (Muthayya et al., 2014). Among the thirteen crops that account for approximately 60% of the daily caloric consumption of humans (Wall et al., 2012), Rice provides roughly 500 Kcal daily to 3 billion people globally. India is the second-largest producer of rice in the world and the foremost exporter. Ray et al. (2013) indicate that rice, wheat, maize, and soybean comprise two-thirds of the world caloric output from agriculture.

Sustainable rice cultivation encounters various biotic challenges, with plant-parasitic nematodes posing a considerable risk, resulting in estimated global yield losses of 10-25% annually (Jones et al., 2013). The rice root-knot nematode (RKN) *Meloidogyne graminicola* Golden and Birchfield is an important pest that has become one of the most economically damaging species in irrigated and rainfed lowland rice systems throughout South and Southeast Asia (Mantelin et al., 2017; Kyndt et al., 2014).

1.2 Root-Knot Nematodes as Critical Agricultural Pests

Root-knot nematodes (*Meloidogyne* spp.) are a substantially important category of plant-parasitic nematodes worldwide, infesting nearly all vascular plant species and resulting in annual agricultural losses projected to surpass $157 billion globally (Elling, 2013; Jones et al., 2013). More than 100 species have been classified within the genus *Meloidogyne*, with *M graminicola*, *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla* identified as the most prevalent and detrimental species in agricultural lands (Moens et al., 2009). *M. graminicola* poses a brief life cycle (19–27 days) and extensive host range, encompassing cereals, dicots, and grasses. *M. graminicola* has developed as a specialized pathogen of monocotyledonous plants, specifically adapted to rice habitats (Mantelin et al., 2017). *M graminicola* due to its prevalent outbreak in rice is also named as rice root knot nematode (RRKN). RRKN dominates significant rice cultivation regions, encompassing India, China, Nepal, Burma, Bangladesh, Laos,

Vietnam, Thailand, the Philippines, Indonesia, the United States (Louisiana), Brazil, Colombia, France, Georgia, Libya, Pakistan, Singapore, and South Africa (Jain et al., 2012). In India, it primarily manifests in the eastern, northeastern, and southern regions (Dabur and Jain, 2004). This nematode has emerged as a major threat in the rice-wheat cropping system in the Indo-Gangetic plains, which represent 33% of rice and 42% of wheat production in India, Pakistan, Bangladesh, and Nepal. These characteristics facilitate population growth and complicate management strategies (Rich et al., 2009). Yield loss varies from 20% to 80% in upland and from 11% to 73% in intermittently flooded rice fields (Plowright and Bridge, 1990; Soriano et al., 2000). In India, it is one of the most economically significant nematodes in rice, resulting in annual losses of Rs. 23,272.32 million (Kumar et al., 2020). The pathogen is undervalued because its non-specific above-ground symptoms mimic nutrient deficits, water stress, or other secondary infections.

Among the *Meloidogyne* genera, *M. graminicola* and *M. incognita* employ similar infection strategies as stationary endoparasitic nematodes, which include root penetration by infective second-stage juveniles (J2), migration to the vascular cylinder, and the formation of nutrient-rich feeding sites (Gheysen and Mitchum, 2019). Additionally, *M. graminicola* demonstrates considerable genetic diversity and adaptive plasticity across its geographical distribution, complicating resistance breeding efforts (Bellafiore et al., 2015).

1.3 Endophytes in nematode management

Endophytes include various microorganisms that inhabit inside plant tissues without inducing visible disease signs, while forming mutually advantageous associations with their host plants (Hardoim et al., 2015). These microorganisms, mostly fungi and bacteria, have received significant scientific attention because to their ability to promote plant growth, enhance nutrient uptake, mitigate abiotic stresses, and, were also shown to have biocontrol potential against plant pathogens (Vega et al., 2009; Jaber and Ownley, 2018). The mode of action of endophytes in controlling plant pathogens include direct antagonism, competition for nutrient and space, induce plant systemic resistance, and the synthesis of secondary metabolites with antimicrobial substances (Compant et al., 2019; Deshmukh et al., 2020). In recent time, endophytes microorganisms have considered to play important role for the biological control of plant-parasitic nematodes, providing environmentally

sustainable alternatives to traditional nematicides (Le et al., 2016; Schouten, 2016). Endophytes can control nematode population by developing protective barriers at infection sites, influence plant defence mechanisms, and can directly have nematicidal effects by producing secondary metabolites (Sikora et al., 2008; Vos et al., 2012). Moreover, endophytes often exhibit considerable host-specificity and ecological plasticity, facilitating reliable control of important plant pathogens while not have harmful effects on beneficial soil microbiota (Compant et al., 2019).

1.4 Traditional nematode management

The control of plant-parasitic nematodes, especially *Meloidogyne* species, has traditionally depended on an integrated strategy that includes chemical, cultural, physical, and genetic resistance methods (Wesemael et al., 2011). Chemical nematicides, such as fumigants (e.g., 1,3-dichloropropene, methyl bromide), organophosphates (e.g., fenamiphos, ethoprophos) and carbamates (e.g., carbofuran, aldicarb), have been extensively applied for their efficiency and broad-spectrum activity (Haydock et al., 2013). However, global legislative constraints have increasingly restricted their accessibility and utilisation due to concerns about environmental persistence, non-target toxicity, groundwater pollution, and detrimental impacts on human health (Oka et al., 2000; D'Addabbo et al., 2010). Nematode management in rice ecosystems has conventionally accomplished through cultural methods, primarily constant flooding, which establishes anaerobic soil conditions detrimental to the reproduction and movement of M. graminicola (Bridge et al., 2005). Additional cultural techniques encompass crop rotation with non-host species, fallow intervals, soil solarisation, integration of organic amendments, and field sanitation practices (Viaene et al., 2013). These methods operate by interrupting nematode life cycles, diminishing initial population densities, and augmenting soil suppressiveness via modified microbial communities (Thoden et al., 2011). Host plant resistance is a fundamental aspect of nematode management, with several resistance genes (e.g., Mi, Me, Ma, N) found in diverse crop species (Williamson and Kumar, 2006). Moderate resistance to M. graminicola has been observed in Oryza glaberrima and specific O. sativa cultivars; however, the extensive use of resistant varieties is restricted by a limited genetic base and potential durability issues (Cabasan et al., 2012; Soriano et al., 2014). Furthermore, breeding initiatives for nematode resistance are hindered by the polygenic characteristics of defence responses and the possible

yield penalties linked to resistance mechanisms (Williamson and Roberts, 2009).

The challenges of conventional management strategies have encouraged research into biological control agents, such as fungal and bacterial antagonists, as potential alternatives for integrated nematode management systems (Davies and Spiegel, 2011). Biological control unveils multiple advantages, such as environmental sustainability, compatibility with alternative management strategies, decreased risk of resistance emergence, and the potential for enduring suppression via establishment within agricultural ecosystems (Vos et al., 2013; Poveda et al., 2020).

1.5 Fungal and Bacterial Endophytes in Nematode Management

Fungal and bacterial endophytes have shown considerable efficacy in inhibiting plant-parasitic nematodes in many agricultural environments. Fungal endophytes from the genera Trichoderma, Pochonia, Purpureocillium, Fusarium, and Acremonium have demonstrated significant potential as biocontrol agents against root-knot nematodes (Poveda et al., 2020; Le et al., 2016).

These fungi utilise various antagonistic mechanisms, including direct parasitism via targeted infection structures, enzymatic breakdown of nematode structural elements, and the synthesis of nematicidal substances such as proteins, peptides, polyketides, and alkaloids (Degenkolb and Vilcinskas, 2016; Li et al., 2015).

Bacterial endophytes, especially from the genera Bacillus, Pseudomonas, Streptomyces, and Pasteuria, exhibit considerable nematode-suppressive potential via antibiosis, enzyme synthesis, parasitism, and the induction of systemic resistance (Tian et al., 2007; Vetrivelkalai et al., 2010). Endophytic Bacillus spp. synthesise diverse compounds, such as lipopeptides, chitinases, and proteases, which efficiently impede worm hatching, repel juveniles, hinder nematode development, and disrupt reproduction (Xiang et al., 2017; Jamal et al., 2017). Evidences like, endophytic Bacillus spp. synthesise diverse compounds, such as lipopeptides, chitinases, and proteases, which efficiently impede nematode egg hatching, repel juveniles, hinder nematode development, and disrupt reproduction (Xiang et al., 2017; Jamal et al., 2017).

Numerous investigations have recorded the biocontrol potential of endophytic bacteria against *M. graminicola* in rice environments. Le et al. (2009) reported that the endophytic *Fusarium moniliforme* Fe14 markedly diminished root galling and nematode infiltration in rice. Trichoderma-based formulations have demonstrated

effective suppression of *M. graminicola* populations in both greenhouse and field settings (Le et al., 2020; Prot and Rahman, 1994). *Bacillus subtilis* BCB19 and *Pseudomonas fluorescens* PfALR2, as bacterial endophytes, have demonstrated significant nematode control potential against *M. graminicola* via induced systemic resistance and direct antagonism (Santhi and Sivakumar, 1995; Padgham and Sikora, 2007).

1.6 Gall Microbiome as a Source of Potential Biocontrol Agents

Recent advancements in metagenomic techniques have demonstrated that root galls contain distinct microbiomes that significantly differ from those found in healthy plant tissues (Kõiv et al., 2015; Lefort et al., 2020). The unique microbial community structure within galls offers considerable opportunity to identify new biocontrol agents specifically adapted to nematode-altered settings. The idea of utilising gall-associated microbiomes for nematode control is based on the ecological principles of microbiome engineering, which involves the natural recruitment and enrichment of antagonistic microorganisms within pathogen-induced structures (Tian et al., 2021). Recent studies indicate that root-knot nematode galls act as reservoirs for several fungal and bacterial endophytes with documented nematicidal abilities (Wolfgang et al., 2019; Liu et al., 2019). These microbial antagonists may have co-evolved with plant-parasitic nematodes in the gall environment, establishing unique approaches to compete with or parasitise nematodes for nutritional resources (Topalović et al., 2020). Focussing on the gall microbiome indicates a paradigm shift in exploration of biological control, emphasising microorganisms that are already acclimated to the ecological niches formed by nematode infection instead of introducing external agents (Topalović and Heuer, 2019). This method has multiple benefits, such as increased colonisation efficiency in infected tissues, greater persistence in field circumstances, and potentially more targeted antagonistic effects against target nematodes (Wolfgang et al., 2019).

1.7 Fungal Nematode Control

1.7.1 Direct-nematode-killing by fungus

Fungal endophytes exploit numerous cellular mechanisms to directly combat plant-parasitic nematodes, such as trapping, adhesion, penetration, and internal colonisation (Poveda et al., 2020). Nematophagous fungus have developed specialised

hyphal structures for the capture and parasitism of nematodes, including adhesive nets, constricting rings, adhesive knobs, and non-constricting rings (Li et al., 2015). Upon interaction with nematode (juvenile) cuticles, these structures promote fungal invasion through physical damage and enzymatic degradation, ultimately resulting in nematode immobilisation and mortality (Yang et al., 2011). In endophytic fungi devoid of specialised trapping structures, direct antagonism can occur with hyphal adhesion to nematode body surfaces, subsequently leading to enzymatic degradation of cuticular constituents (Lopez-Llorca et al., 2010). Numerous endophytic fungal species produce hydrolytic enzymes, such as chitinases, proteases, cellulases, and lipases, which combined compromise the structural integrity of nematode cuticles and eggs (Khan et al., 2017). Moreover, several endophytic fungi synthesize sticky proteins and lectins that promote adhesion to nematode surfaces, thereby initiating the killing process (Nordbring-Hertz et al., 2006). Recent microscopic studies have revealed intriguing interaction between fungi and plant-parasitic nematodes. Recent microscopic studies have revealed intriguing interaction between fungi and plant-parasitic nematodes. *Pochonia chlamydosporia* demonstrates directional hyphal development towards nematode eggs, subsequently forming appressoria and penetrating the egg shell by enzymatic degradation (Escudero and Lopez-Llorca, 2012). Likewise, Trichoderma sp. exhibit chemotactic interaction to nematode secretions, subsequently coiling around nematode bodies and penetrating directly (Zhang et al., 2014). These cellular contact processes are crucial factors in the selection of efficient fungal biocontrol agents against *M. graminicola* in rice systems.

1.7.2 Fungal Secondary Metabolites in Nematode Management

In addition to direct parasitism, fungal endophytes synthesise a wide array of secondary metabolites that exhibit nematicidal effects (Zhai et al., 2018). These bioactive chemicals include many chemical groups, such as alkaloids, non-ribosomal peptides, polyketides, terpenoids, and volatile organic compounds, each demonstrating distinct mechanisms of action against different life stages of nematode (Degenkolb and Vilcinskas, 2016; Li et al., 2007). Fungal secondary metabolites influence multiple physiological processes in plant-parasitic nematodes, such as disrupting neuromuscular function, destruction of digestive tract, inhibiting enzymatic activities, interfering with reproductive processes, and impairing chemosensory capabilities (Ciancio, 1995; Dong et al., 2016). Certain polyketides

extracted from Trichoderma species exhibit ovicidal properties by obstructing embryonic development and diminishing the hatching rates of *Meloidogyne* eggs (Zhang et al., 2014). The metabolic diversity of fungal endophytes provides substantial benefits for nematode control, as these microorganisms may synthesise many beneficial chemicals with synergistic mechanisms, hence diminishing the probability of resistance evolution (Poveda et al., 2020). Moreover, some fungal secondary metabolites demonstrate specific pathogenicity against plant-parasitic nematodes while preserving beneficial soil species, hence enhancing ecological sustainability (Li et al., 2015). Few studies on fungal control of M graminicola have explicitly investigated the nematicidal efficacy of secondary metabolites obtained from rice-associated fungal endophytes against M. graminicola. Le et al. (2009) reported that culture filtrates of endophytic Fusarium moniliforme Fe14 markedly decreased the hatching and mobility of M. graminicola. Meyer et al. (2004) similarly observed that secondary metabolites from Fusarium oxysporum strain 162 efficiently inhibited M. graminicola infection in rice by inducing systemic resistance. These findings highlight the potential of harnessing fungal secondary metabolites as sustainable alternative to traditional nematicides in rice cultivation systems.

1.8 Research objectives

Based on above aforesaid following research objectives were determined:

(vi) To characterise the fungal endophytic communities associated with *Meloidogyne graminicola*-infected rice roots using both metagenomic and culture-dependent approaches.

(vii) To evaluate the biocontrol potential of root gall-specific fungal endophytes for the sustainable management of *Meloidogyne graminicola*.

(viii) To assess the nematicidal efficacy and defence-inducing activity of the *Aspergillus niger* F4 culture filtrate against *Meloidogyne incognita* in tomato.

(ix) To identify and functionally characterise the bioactive metabolites present in the *A. niger* F4 culture filtrate using metabolomic analyses.

(x) To develop and optimise a stable and efficient formulation of the *A. niger* F4 culture filtrate for field-level application in nematode management.

Chapter 2: Review of Literature

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Plant-parasitic nematodes (PPNs) represent a persistent and economically significant threat to global agriculture, with root-knot nematodes (*Meloidogyne* spp.) being among the most damaging due to their extensive host range and ability to induce characteristic root galls. These nematodes disrupt plant physiology and yield, contributing to annual economic losses estimated in billions of dollars. The conventional reliance on synthetic nematicides for their control has become increasingly untenable due to environmental concerns, regulatory restrictions, and the rapid development of nematode resistance. This has intensified the search for ecologically sound alternatives capable of providing sustainable, long-term nematode management.

Endophytic microorganisms—defined as bacteria and fungi that colonise internal plant tissues asymptomatically—have emerged as potent biological control agents due to their capacity to produce structurally diverse and functionally effective secondary metabolites. These compounds include alkaloids, terpenoids, lipopeptides, polyketides, phenolic compounds, and volatile organic compounds, many of which exhibit nematicidal, nematostatic, or egg-inhibitory activities. Unlike rhizosphere-dwelling microbes, endophytes benefit from stable associations within plant tissues, which enhances their persistence, systemic activity, and compatibility with host metabolism.

Fungal and bacterial endophytes contribute to nematode suppression through multiple, often complementary, mechanisms. These include direct toxicity via secondary metabolites, competition with nematodes or their microbial facilitators, and activation of host systemic resistance via hormonal pathways. In recent years, special attention has been directed toward microbial communities within nematode-induced root galls—unique microenvironments that may select for microorganisms with evolved anti-nematode traits. These gall-associated endophytes may play an underappreciated but significant role in plant-nematode-microbe interactions.

Despite increasing number of reports of the efficacy of endophyte-derived secondary metabolites, a systematic and comparative synthesis of the diversity, activity, and mechanistic insights of bacterial and fungal metabolites remains lacking. This review aims to address this gap by collating and analysing the available literature

on endophytic microorganisms and their metabolites for nematode control. We seek to elucidate emerging trends, evaluate the strengths and limitations of existing approaches, and identify key areas for future research. Emphasis is placed on the chemical ecology of endophyte-nematode interactions, the ecological roles of gall-associated microbiomes, and the translational potential of these microorganisms in integrated nematode management systems.

2.1 Gall microbiome and biological control

Root-knot nematodes (RKNs), primarily of the genus *Meloidogyne*, are among the most economically significant plant-parasitic nematodes, inflicting extensive yield losses across a variety of crop systems globally. Their infection leads to the formation of specialized feeding sites known as root galls, which are characterized by pronounced cellular hypertrophy and hyperplasia. These galls serve not only as nutrient sinks for nematode development but also represent distinct microenvironments that support diverse microbial communities, including endophytic and rhizospheric microorganisms. The interaction between these microbes and the host plant within the context of nematode-induced galls is increasingly recognized as a critical, yet underexplored, component of plant-nematode-microbe dynamics. In recent years, attention has been drawn to the dual role of gall-associated microbiomes: while some may exacerbate disease outcomes, others exhibit nematicidal or nematostatic properties, thereby contributing to biological control of the nematode. This review synthesizes the growing body of literature that examines the composition and functional role of gall-associated microbiomes in the biological suppression of root-knot nematodes.

2.1.1 Microbial Community Dynamics in Root-Knot Nematode Galls

Metagenomic and culture-dependent studies have demonstrated that root galls induced by *Meloidogyne* spp. exhibit significant shifts in microbial community structure compared to healthy roots. Masson et al. (2020) reported deep compositional changes in the rice root microbiome following *M. graminicola* infection under field conditions. The gall tissues were enriched with distinct bacterial taxa, suggesting that nematode infection restructures microbial assemblages to form unique ecological niches. Similar findings were reported by Kunda et al. (2024), who observed a pronounced alteration in the endophytic bacterial community within *M.*

graminicola induced galls, including an increased relative abundance of potentially beneficial taxa such as *Bacillus* and *Pseudomonas* spp.

Yergaliyev et al. (2020) examined *M. incognita* infected tomato roots and revealed dynamic restructuring of the bacterial community over time, with an increased abundance of Firmicutes and Actinobacteria during the nematode infection cycle. Such changes indicate the development of specialized microbial consortia within the gall microenvironment, potentially driven by root exudates and nematode-secreted effectors. These findings underscore the influence of nematode-induced physiological alterations in shaping the root microbiome.

2.1.2 Biocontrol Potential of Gall-Associated Microbes

Several studies have demonstrated that microorganisms isolated from nematode-induced galls can possess nematicidal properties. Tian et al. (2015) employed a metagenomic approach to explore the functional traits of endophytic bacteria in tomato galls caused by *Meloidogyne* spp., identifying genes related to chitinase production, secondary metabolite biosynthesis, and induced systemic resistance. Wolfgang et al. (2019) emphasized the importance of exploiting beneficial microbial consortia for managing soil-borne pathogens, including RKNs, and proposed the strategic use of endophytic and rhizosphere-associated bacteria as biological control agents.

Experimental inoculation studies support these assertions. DiLegge et al. (2019) demonstrated that specific bacteria from the tomato rhizosphere reduced nematode infestation through a combination of competition and antibiosis. Nadeem et al. (2021) further showed that bacterial strains integrated with surfactin molecules significantly disrupted the motility and infectivity of RKNs, offering a synergistic model of biocontrol. The nematicidal efficacy of *Bacillus* spp. isolated from gall tissues was also validated by de Carvalho et al. (2022), who reported reduced galling and enhanced plant growth in infested plants treated with local strains.

2.1.3 Host Defence Modulation and Induced Resistance

Beyond direct antagonism, gall-associated microbes may mediate host defense responses, enhancing systemic resistance against nematodes. Shi et al. (2025) demonstrated that inoculation with *Paenibacillus polymyxa* J2-4 induced systemic resistance in cucumber through jasmonic acid and salicylic acid signaling pathways, reducing gall formation by *Meloidogyne* spp. Similarly, Vinothini et al. (2024) showed

that tomato rhizosphere communities enriched under nematode pressure possessed traits linked to ISR (induced systemic resistance) and stress tolerance.

Obidari et al. (2024) proposed that certain microbial communities in nematode-infested rhizospheres contribute to natural soil suppressiveness, with enrichment of microbial taxa involved in lytic enzyme production and phytohormone regulation. These interactions can lead to the recruitment of a functionally diverse microbial population that bolsters host immunity against nematode invasion.

2.1.4 Conclusion

The root galls induced by *Meloidogyne* spp. are dynamic microenvironments that not only serve as feeding structures for the nematodes but also harbor specialized microbial communities with potential roles in nematode suppression. The integration of omics-driven insights with functional assays has revealed that gall-associated microbes can modulate nematode behavior, reduce galling severity, and trigger host defense pathways. These findings highlight the untapped potential of gall microbiomes as reservoirs of biological control agents and pave the way for microbiome-based strategies in nematode management. Future research should focus on identifying keystone taxa within galls, understanding their mechanistic interactions with both the host and nematode, and translating this knowledge into field-applicable biocontrol formulations.

2.2 Bacterial Endophytes in Nematode Management

The intensification of agriculture and the expansion of monoculture cropping systems have exacerbated plant-parasitic nematode (PPN) infestations across major agroecosystems worldwide. Among the PPNs, the root-knot nematodes, *Meloidogyne* represent a persistent threat to food security due to their broad host range and the extensive damage they inflict on plant roots. Globally, the economic losses caused by these nematodes are estimated to exceed USD 100 billion annually (Kumar et al., 2020). Despite the availability of chemical nematicides, their widespread use is hindered by concerns related to environmental toxicity, non-target effects, pest resistance, and regulatory restrictions. Consequently, there has been a growing emphasis on the development of biologically-based nematode control strategies that are both ecologically sustainable and economically viable.

In this context, bacterial endophytes have emerged as promising biocontrol agents.

Endophytic bacteria colonize internal plant tissues without eliciting disease symptoms and have been recognized for their multifaceted roles in plant health, including growth promotion, abiotic stress tolerance, and suppression of plant pathogens (Porras-Alfaro and Bayman, 2011; Gouda et al., 2016). Their biocontrol capabilities against nematodes are primarily attributed to the production of nematicidal compounds, induction of systemic resistance in host plants, and competition with pathogens for space and nutrients. Additionally, their ability to establish systemic colonization enhances their persistence and efficacy under field conditions, distinguishing them from rhizosphere-limited biocontrol agents (Jaber and Enkerli, 2016).

The mechanisms through which bacterial endophytes mediate nematode suppression are diverse. Certain species such as *Bacillus subtilis* and *Pseudomonas fluorescens* are known to produce lytic enzymes (e.g., chitinases, proteases) and volatile organic compounds (VOCs) that directly affect nematode viability (Xie et al., 2022). Others, including *Burkholderia cepacia* and *Serratia marcescens*, modulate plant defence responses via jasmonic acid (JA), salicylic acid (SA), or ethylene (ET) signalling pathways, leading to the activation of defence-related enzymes such as phenylalanine ammonia-lyase (PAL), peroxidase (POD), and polyphenol oxidase (PPO) (Kloepper et al., 2004; Liu et al., 2020). The presence of such pathways implies a dual mode of action encompassing both direct toxicity and host-mediated resistance.

The biocontrol potential of endophytic bacteria has been extensively studied in economically important crops such as rice, tomato, and cucumber, particularly against *M. incognita*, *M. javanica*, and *M. graminicola*. Early evidence suggested that bacterial endophytes can reduce nematode penetration, impede juvenile development, and diminish egg mass production. For instance, Padgham and Sikora (2007) demonstrated that *Bacillus megaterium* significantly reduced gall formation and nematode populations in rice roots under greenhouse conditions. These findings have since been extended to other bacterial genera and host-nematode systems.

The present review provides a comprehensive synthesis of the available literature on bacterial endophytes in nematode management, based on an extensive set of abstracts arranged chronologically. The goal is to critically examine emerging patterns in biocontrol efficacy, characterize the diversity of endophytic bacterial taxa studied, and identify the underlying mechanisms by which they contribute to nematode suppression. The review also aims to highlight knowledge gaps and propose directions for future research. By systematically integrating past findings, this chapter offers

insights into the potential of bacterial endophytes as sustainable agents for nematode management in agriculture.

2.2.1 Early Investigations on Bacterial Endophytes in Nematode Suppression

Initial investigations into the role of bacterial endophytes in nematode management primarily focused on characterizing community dynamics, establishing their biocontrol potential, and elucidating modes of action under laboratory and greenhouse conditions.

Hallmann et al. (1999) reported one of the earliest studies examining how chitin amendments in soil influenced the population dynamics of endophytic bacterial communities within cotton roots. Their findings demonstrated that chitin not only modified the rhizosphere bacterial composition but also increased the frequency of bacterial endophytes capable of suppressing *Meloidogyne incognita*, particularly those strains related to *Bacillus* and *Pseudomonas* spp. The nematode suppression was hypothesized to be a combined effect of direct antibiosis and systemic resistance elicitation in host tissues.

Building on the understanding of endophyte-mediated suppression, Siddiqui and Ehteshamul-Haque (2001) evaluated the role of *Pseudomonas fluorescens* in controlling a disease complex comprising *M. javanica* and *Macrophomina phaseolina* in chickpea. Application of the bacterial endophyte led to a marked reduction in both root rot severity and gall formation. These effects were attributed to enhanced root colonization, production of antifungal metabolites, and competition for ecological niches in the rhizosphere.

Further mechanistic insight was provided by Siddiqui and Shaukat (2003), who investigated how *Pseudomonas aeruginosa* adapted to carbon-limited conditions, simulating the nutrient-deficient interior of plant tissues. Their study revealed that carbon-starved *P. aeruginosa* exhibited improved root colonization and increased antagonistic activity against *M. javanica*. The results highlighted the importance of metabolic plasticity in enhancing the efficacy of endophytic bacteria as biocontrol agents under field conditions.

In a parallel line of inquiry, Sturz and Kimpinski (2004) isolated bacterial endophytes from the roots of marigold (*Tagetes spp*.), a known nematode-suppressive crop. Several isolates showed significant in vitro activity against *M. hapla*. The study emphasized the role of host plant species in shaping endophytic communities and their

biocontrol potential, indicating that endophyte-host associations are not only species-specific but also modulated by agronomic practices and soil type.

Sikora et al. (2007) provided a detailed conceptual framework summarizing the known mechanisms by which bacterial endophytes suppress plant-parasitic nematodes. These include induced systemic resistance (ISR), competition for infection sites, secretion of nematicidal enzymes (e.g., proteases, lipases), and interference with nematode signalling. The authors also underscored the potential of combining endophytes with organic amendments and other microbial inoculants to enhance suppression efficacy through synergistic interactions.

Collectively, these early studies established a foundational understanding of the diverse roles of bacterial endophytes in nematode suppression. They highlighted their potential as reliable and ecologically sustainable agents in integrated pest management (IPM) frameworks. However, most of these investigations were limited to in vitro or controlled greenhouse conditions. Future studies would need to address field-scale validation and long-term ecological compatibility for widespread adoption.

2.2.2 Ecological Functions and Diversity of Bacterial Endophytes in Plant-Nematode Systems

By the late 2000s, the conceptual understanding of bacterial endophytes had broadened to encompass their ecological roles in the rhizosphere and their interactions with host plants. Narula et al. (2009) emphasized the central role of root exudates in shaping the recruitment and functionality of bacterial endophytes. The study postulated that root-secreted organic acids, amino acids, and phenolics act as chemoattractants and nutrient cues, selectively promoting endophytic colonization by beneficial bacteria, including *Azospirillum*, *Pseudomonas*, and *Bacillus* spp. These microbes not only improved nutrient uptake (especially phosphorus and nitrogen) but also contributed to systemic resistance against a range of pathogens. While not specific to nematodes, this research provided a mechanistic framework for understanding how host plants can indirectly influence nematode suppression through endophyte-mediated pathways.

The contribution of microbial symbiosis to host defence was further explored by Klepzig et al. (2009), who, although working within an entomological context, highlighted the broader importance of endophyte-host-environment interactions. They discussed how certain endophytic bacteria form mutualistic alliances with their hosts, enhancing host tolerance to biotic stress through the production of antibiotics

and modulation of host immunity. The authors underscored the idea that bacterial symbionts could act as drivers of physiological adaptation and co-evolution in response to parasitic threats. While nematodes were not the central focus, the ecological principles established in this work are directly applicable to understanding host-endophyte-nematode dynamics in plant systems.

Strobel (2011) introduced a novel dimension to endophytic research through the discovery of a new fungal genus, *Muscodor*, known for producing a spectrum of volatile organic compounds (VOCs) with potent bioactivities. Although the primary focus was on antifungal and insecticidal properties, the broad-spectrum nature of the VOCs, including esters, alcohols, and acids, strongly implied nematicidal potential. The study emphasized the untapped biotechnological applications of endophyte-derived VOCs and opened avenues for exploring bacterial analogues capable of emitting similar chemical signatures for nematode control.

Ma et al. (2013) shifted the focus to the taxonomic diversity of bacterial endophytes and its implications for biocontrol efficacy. Using molecular and phylogenetic analyses, they identified a wide array of bacterial taxa from rice plants, including members of *Burkholderia*, *Bacillus*, and *Enterobacter* genera. Importantly, the isolates exhibited high sequence divergence, suggesting the presence of novel strains with potential biocontrol activities. The authors proposed that phylogenetic diversity could serve as a predictive indicator of functional redundancy and resilience in endophytic consortia, thereby enhancing the consistency of biological control outcomes. Though direct testing against nematodes was not performed, the study laid the groundwork for the strategic selection of diverse bacterial assemblages to suppress a broad range of soilborne pests, including nematodes.

The role of bacterial endophytes in nematode suppression was addressed more explicitly by Tian et al. (2015), who employed a metagenomics approach to explore the structure and function of endophytic microbial communities within tomato roots infected with *Meloidogyne incognita*. Their results demonstrated a distinct shift in microbial community composition in nematode-infected roots compared to healthy ones. Several bacterial genera, including *Pseudomonas*, *Bacillus*, and *Paenibacillus*, were enriched in infected tissues, suggesting an active recruitment of defensive endophytes. Functional annotation of the metagenomic data revealed enrichment of genes associated with chitin degradation, antibiotic biosynthesis, and signal transduction pathways known to trigger induced systemic resistance. This study

provided compelling evidence that plant hosts can modulate their internal microbiome in response to nematode attack, potentially as a defensive adaptation, and that bacterial endophytes are key players in this dynamic.

Together, these studies reflect a transition in endophyte research from isolated bioassays to systems-level analyses of microbial ecology and host interactions. The accumulated evidence reinforces the view that bacterial endophytes play integral roles not only in direct nematode suppression, but also in shaping plant responses through chemical signaling, microbial succession, and immune priming. These findings underscore the necessity of integrative approaches in future research, combining molecular ecology, metabolomics, and functional validation to unlock the full potential of bacterial endophytes in nematode management.

2.2.3 Functional Characterization and Biocontrol Applications of Endophytic Bacteria Against Nematodes

The integration of endophytes into pest management strategies continued to gain momentum through the mid-2010s, with increasing focus on their application against root-knot nematodes and associated pathogens. Lacey et al. (2015) provided a comprehensive overview of the development and deployment of microbial biocontrol agents, particularly entomopathogens, while underscoring the potential applicability of analogous strategies for nematode control. Although the primary emphasis was on insect pests, the authors noted significant overlaps in host invasion pathways, immune evasion tactics, and secondary metabolite production between insect-pathogenic microbes and nematode-antagonistic endophytes. This comparative framework highlighted the promise of bacterial endophytes, such as *Bacillus thuringiensis* and *Photorhabdus* spp., which possess known nematicidal activity via production of crystal proteins, toxins, and proteases. The insights from entomopathogen research thus offered transferable methodologies for endophyte screening, formulation, and delivery in nematode biocontrol.

Su et al. (2017) provided direct experimental validation of the nematicidal potential of endophytic bacteria by isolating antagonistic strains from banana roots and evaluating their effects on *Meloidogyne javanica* in pot assays. Several isolates, notably those belonging to *Bacillus*, *Pseudomonas*, and *Burkholderia* genera, significantly reduced galling index and juvenile counts in infected roots. Importantly, the study also employed community-level analyses to assess changes in the nematode-associated soil microbiota. The presence of endophytic antagonists led to an overall

shift in community composition, with increased abundance of nematode-antagonistic taxa and suppression of opportunistic pathogens. These results suggest a dual role of endophytic bacteria: directly antagonizing the nematode and simultaneously modifying the soil microbiome in favour of suppressiveness.

The functional diversity of endophytic bacteria was further elucidated by Tian et al. (2017), who characterized multiple *Bacillus* and *Pseudomonas* isolates based on their in vitro traits relevant to nematode suppression. The study systematically quantified enzymatic activities such as chitinase, cellulase, and protease, and evaluated the capacity for root colonization and siderophore production. These functional traits were positively correlated with the observed biocontrol efficacy against *M. incognita* in tomato. Importantly, the authors emphasized the importance of multi-trait screening approaches for the selection of elite endophytes capable of expressing multiple modes of action simultaneously. Their findings also supported the use of plant-based selection systems to enrich for rhizosphere-competent strains with systemic endophytic capability, improving persistence and efficacy under greenhouse and potentially field conditions.

A specific example of such efficacy was demonstrated by Hu et al. (2017), who evaluated an endophytic strain of *Bacillus cereus* for its control of *M. incognita* in tomato. Application of the isolate led to significant reductions in gall formation, nematode penetration, and reproduction. Moreover, treatment with *B. cereus* enhanced plant growth parameters, including root biomass and chlorophyll content, indicating both protective and growth-promoting functions. Histochemical staining and enzymatic assays revealed that treated plants exhibited elevated levels of peroxidase and polyphenol oxidase, suggesting that induced systemic resistance was at least partially responsible for the observed nematode suppression. These findings highlight the multifunctionality of endophytic bacteria and their potential to serve as dual-purpose bioinoculants in sustainable crop protection.

Although primarily addressing fungal endophytes, the study by Yuan et al. (2017) is notable for demonstrating the broader spectrum of endophyte-mediated plant protection, particularly against complex soil-borne diseases such as *Verticillium* wilt. While the central focus was on endophytic fungi isolated from cotton, the findings emphasized the importance of root colonization, secondary metabolite production, and elicitation of plant immune responses in disease suppression. These mechanisms are equally relevant to bacterial endophytes targeting nematodes, especially in

polyphagous pathosystems where nematodes often act synergistically with fungal pathogens. As such, the mechanistic insights derived from fungal endophyte research can inform future screening and functional assays for bacterial isolates.

Collectively, these studies advanced the field from taxonomic cataloguing to functional validation and application-focused research. The convergence of multiple mechanisms—including nematicidal metabolite production, immune priming, and rhizosphere modulation—underscores the systemic nature of endophyte-mediated biocontrol. As the evidence base grows, it becomes increasingly clear that successful deployment of bacterial endophytes requires not only effective strains, but also a nuanced understanding of plant-microbe-pest interactions within the broader soil ecological context.

2.2.4 Genomic Insights and Soil Microbiome Contributions to Endophytic Nematode Suppression

The period following 2017 witnessed the adoption of high-throughput and systems-level approaches to dissect the complex interactions between endophytic bacteria, host plants, and plant-parasitic nematodes. Shanmugam et al. (2018) presented one of the first genome-level investigations into the nematicidal capabilities of endophytic bacteria by sequencing the genome of *Bacillus firmus* I-1582, a strain effective against *Bursaphelenchus xylophilus*, the causal agent of pine wilt disease. The genomic analysis revealed the presence of gene clusters coding for chitinases, proteases, lipopeptides, and toxins such as subtilin and fengycin. These bioactive compounds are known to exert nematicidal effects through enzymatic degradation of nematode cuticle and interference with developmental signalling pathways. The study further identified stress adaptation genes that likely contribute to the endophyte's persistence under adverse rhizosphere conditions. This work underscored the utility of genome mining for identifying mechanistic bases of nematode antagonism and selecting promising candidates for biotechnological applications.

Hussain et al. (2018) explored the role of soil microbial communities in conferring disease suppressiveness against *Meloidogyne incognita* in tomato. Comparing suppressive and conducive soils, they demonstrated that bacterial community assemblages differ markedly in diversity and composition. Notably, suppressive soils harboured higher abundances of *Pseudomonas*, *Lysobacter*, and *Bacillus* spp., many of which are also known endophytes. Functional prediction analyses suggested enrichment of metabolic pathways linked to secondary metabolite biosynthesis,

including polyketides and non-ribosomal peptides—both of which have documented nematicidal activity. These findings indicate that disease suppressiveness is not merely a result of soil abiotic factors, but is largely microbial in origin. The authors suggested that plants may selectively recruit beneficial endophytes from such microbial reservoirs, contributing to an internalised continuum of defence that includes both rhizospheric and endophytic phases.

Returning to empirical assessments, Haque et al. (2018) evaluated twelve indigenous rhizospheric bacterial isolates for their efficacy against *M. graminicola* in rice. Among them, strains of *Pseudomonas fluorescens* and *Bacillus subtilis* were particularly effective in reducing gall formation, nematode penetration, and egg mass development. In vitro screening revealed strong nematicidal activity, correlated with production of hydrogen cyanide (HCN), siderophores, and lytic enzymes. Pot experiments further demonstrated that selected strains could significantly enhance plant height and biomass under nematode pressure, indicating their potential as dual bioprotectants and biofertilisers. The authors concluded that the indigenous microbial flora in rice fields offers a valuable source of candidate endophytes, which can be harnessed for location-specific biocontrol formulations.

Mercado-Blanco et al. (2018) provided a broader ecological perspective on the importance of belowground microbiota in tree health, focusing on the interactions between root endophytes, mycorrhizal fungi, and rhizosphere microbes. Although not specific to nematodes, the review outlined mechanisms by which bacterial endophytes contribute to disease suppression, including modulation of host immune responses and alteration of root exudate profiles that deter pathogen establishment. The work highlighted the increasingly recognised concept of the 'holobiont', where the plant and its associated microbiota function as an integrated biological unit. This systems approach supports the hypothesis that endophytic bacterial communities form part of a dynamic defensive network, with potential application in nematode management across perennial cropping systems.

Taken together, this set of studies reflects a clear progression from single-strain evaluations to more comprehensive explorations of microbiome function, genomic underpinnings, and ecosystem-level dynamics. They suggest that successful endophyte-based nematode control strategies will require not only efficacious microbial agents, but also a deep understanding of host-microbiome co-adaptation, soil health, and environmental context.

2.2.5 Diversity, Distribution, and Phylogenetics of Bacterial Endophytes with Implications for Nematode Control

As the field matured, investigations began to focus not only on the functional capacity of bacterial endophytes but also on their diversity, biogeography, and evolutionary relationships. Pandey et al. (2019) presented a comprehensive overview of bacterial endophytes as systemic mutualists that occupy the internal tissues of plants. They emphasised that these microorganisms are not only integral to host growth and development, but also mediate resistance against both abiotic and biotic stresses. The review highlighted the role of bacterial endophytes in secreting bioactive compounds—such as lipopeptides, polyketides, and volatile organic compounds—which can directly impact nematode behaviour and viability. Additionally, endophytes were noted to activate systemic acquired resistance (SAR) and induced systemic resistance (ISR) pathways, offering dual protective effects. The authors also underscored the taxonomic breadth of beneficial endophytes, encompassing *Bacillus*, *Pseudomonas*, *Enterobacter*, *Streptomyces*, and *Burkholderia*, each with varying spectra of nematicidal and plant growth-promoting traits.

Liu et al. (2019) undertook a large-scale isolation and characterisation of 238 culturable bacterial endophytes from diverse plant hosts in Korea. Their study revealed a high degree of bacterial diversity, with *Proteobacteria* and *Firmicutes* being the dominant phyla. Through 16S rRNA gene sequencing and functional profiling, they identified numerous strains with putative genes for nitrogen fixation, phosphate solubilisation, and secondary metabolite biosynthesis. While direct nematicidal assays were not conducted, the authors noted that many isolates encoded lytic enzymes and siderophores, suggesting potential utility in biocontrol applications. Of particular relevance was the identification of *Bacillus velezensis* and *Paenibacillus polymyxa*, both of which have been previously implicated in nematode suppression. The results advocate for deeper screening of large endophyte libraries to identify candidates for targeted biological control of root-parasitic nematodes.

In a related study, Ponpandian et al. (2019) focused on the phylogenetic classification of bacterial endophytes isolated from healthy tomato roots. Using multilocus sequence analysis (MLSA), they resolved the evolutionary relationships among isolates and provided insights into lineage-specific traits. The majority of isolates belonged to the genera *Bacillus*, *Pseudomonas*, and *Streptomyces*, and several of them clustered with known nematode-antagonistic strains. The authors argued that

phylogenetic resolution of bacterial endophytes is critical for understanding their functional potential and ecological compatibility with different host plants. The study suggested that evolutionary relatedness can guide the selection of endophytes with enhanced stability and interaction efficiency within the host microbiome, especially under field conditions.

De Silva et al. (2019) expanded the scope of biocontrol to include fungal and bacterial endophytes with activities against a range of plant pathogens, including nematodes. Their review underscored the advantages of endophytes over rhizospheric microbes due to their internal localisation and ability to colonise systemic tissues. The authors detailed mechanisms including antagonism via enzyme production, competition for space and nutrients, and elicitation of host defences. Emphasis was placed on the need to integrate endophytes into existing integrated pest management (IPM) frameworks and to develop robust inoculant formulations for commercial application. While the discussion was broad, nematodes were identified as prime targets for such biological interventions given the limited success of chemical nematicides in sustainable agriculture.

Lastly, Korejo et al. (2019) addressed the simultaneous control of root-knot nematodes (*M. incognita*) and root-rotting fungi in chilli using bacterial endophytes. Their dual-pathogen bioassay revealed that co-infection leads to aggravated disease severity, but treatment with selected *Pseudomonas* and *Bacillus* strains reduced both galling and fungal incidence significantly. The study highlighted the advantage of using endophytes with broad-spectrum antagonistic properties that can target multiple pathogens concurrently. Importantly, the endophytic strains also improved seed germination and plant vigour, confirming their role as biofertilisers. The results provide compelling evidence that multifunctional endophytes can serve as cornerstone agents in the development of holistic plant protection strategies.

These findings collectively reinforce the centrality of bacterial endophytes in modern plant health management, especially in the face of complex, multi-pathogen challenges. They also demonstrate the value of coupling functional screening with molecular taxonomy to identify elite strains for nematode suppression and broader biocontrol efficacy.

2.2.6 Advancing Biological Control Through Functional Endophyte Characterisation and Soil-Plant-Microbe Interactions

The recent decade has seen growing sophistication in the understanding of how

endophytic bacteria mediate plant protection against nematodes, with greater emphasis on colonisation dynamics, soil suppressiveness, and multi-pathogen interactions. Proença et al. (2019) provided experimental evidence of the nematicidal efficacy of *Serratia plymuthica* M24T3, a bacterial endophyte isolated from *Setaria viridis*. This strain demonstrated successful colonisation of maize roots and substantial suppression of *Meloidogyne incognita*. The colonisation ability was confirmed via microscopy and re-isolation studies, establishing that M24T3 could persist within root tissues without adverse effects on the host. Furthermore, nematode bioassays revealed that the bacterial treatment resulted in significant reductions in egg mass production and gall formation. These outcomes were attributed to the production of secondary metabolites, including serrawettin and pyrrolnitrin, known for their nematostatic and nematicidal effects. The study serves as a critical example of how endophytic colonisation capacity is a prerequisite for durable biocontrol under field-relevant conditions.

In a parallel investigation, Abdelrazek et al. (2020) studied the endophytic mycobiome of cannabis, highlighting the influence of fungal endophytes on secondary metabolite biosynthesis, particularly cannabinoids. While this work was not directed towards nematodes, the broader insight into how endophytes modulate host metabolism and resistance traits is highly relevant. The authors demonstrated that shifts in the core endophytic fungal community were associated with altered levels of plant secondary metabolites. These findings imply that bacterial endophytes may likewise impact host metabolic pathways that contribute to nematode resistance, such as phenylpropanoid and flavonoid biosynthesis. Thus, understanding endophyte-induced modulation of host biochemistry offers a promising avenue for enhancing plant defence against nematode invasion.

Topalovic et al. (2020) advanced the conceptual framework of soil suppressiveness by investigating how plants and soil microbiota jointly contribute to nematode suppression. Their work highlighted the synergy between plant genotype and the composition of rhizosphere and endophytic bacterial communities in mediating resistance to *Meloidogyne* spp. Disease suppressive soils were characterised by a high prevalence of *Pseudomonas*, *Flavobacterium*, and *Lysobacter* spp., many of which exhibited endophytic traits. The study suggested that suppression is not merely a rhizospheric phenomenon but is maintained by internal microbial consortia capable of systemic antagonism. Importantly, plant-microbe compatibility was shown to

influence the recruitment of beneficial endophytes, underscoring the necessity of host genotype consideration in the development of endophyte-based biocontrol strategies.

Kumar and Dara (2021) reviewed the dual role of bacterial and fungal endophytes as microbial control agents against plant-parasitic nematodes. The authors compiled evidence supporting multiple modes of nematode suppression, including direct toxicity, parasitism, competition, and activation of plant immunity. *Bacillus*, *Pseudomonas*, and *Streptomyces* were consistently identified as core genera with strong biocontrol potential. Notably, the review stressed the importance of integrated validation pipelines—beginning from in vitro screening to field-level efficacy trials—to ensure reliability and reproducibility. The authors also advocated for formulation research, suggesting that encapsulation and carrier-based delivery systems could enhance viability and shelf-life of endophytic inoculants. The review provides a comprehensive roadmap for translating endophyte research into deployable solutions in nematode-infested agroecosystems.

Finally, Duong et al. (2021) reported the isolation and characterisation of bacterial endophytes from Vietnamese coffee roots, assessing their potential as plant growth promoters and biocontrol agents. Among the isolated strains, several *Bacillus* and *Burkholderia* spp. exhibited antagonistic activity against *Meloidogyne incognita*, as evidenced by reduced nematode hatching rates and impaired motility in in vitro assays. These isolates also produced indole acetic acid (IAA), solubilised phosphate, and synthesised siderophores—traits beneficial for plant growth under stress. The authors proposed that the dual functionality of endophytes in promoting plant vigour and mitigating nematode infestation makes them especially suited for deployment in high-value perennial crops like coffee. Moreover, the localisation of endophyte sourcing from native cultivars points to the importance of agroecological specificity in endophyte-based biocontrol strategies.

Together, these studies signify a maturing of the discipline, with a shift from descriptive studies to mechanistic insights and application-oriented evaluations. They highlight the necessity of understanding microbial colonisation, metabolic interplay, and host-microbiome compatibility as foundational elements for the development of reliable endophyte-mediated nematode suppression strategies.

2.2.7 Broad-Spectrum Endophytic Interactions and Nematicidal Applications in Complex Pathosystems

The role of endophytic bacteria in nematode suppression has increasingly been

evaluated within broader plant health contexts, often involving co-infections, foliar microbiomes, and synergistic microbial interactions. Ewing et al. (2021) explored the foliar microbiome associated with beech leaf disease (BLD), uncovering potential protective roles for both bacterial and fungal endophytes. Although nematodes were not the primary focus, the study revealed that microbial community shifts in symptomatic versus asymptomatic leaves were significant, with higher relative abundances of certain *Pseudomonas* and *Streptomyces* spp. in healthier tissues. These genera, which are also recognized as endophytes with nematicidal capabilities, may confer indirect resistance by stabilising host microbial assemblages or through direct antagonism against the causative nematode *Litylenchus crenatae*. This work reinforced the emerging view that leaf- and root-associated endophytes contribute holistically to plant health and that systemic endophytism may influence nematode pathogenesis beyond the rhizosphere.

A more direct demonstration of endophytic bacterial efficacy against nematodes was presented by Wong-Villarreal et al. (2021), who investigated the nematicidal activity of *Serratia marcescens* strain ETR17, isolated from rice roots. The study reported that culture filtrates of ETR17 induced significant mortality in second-stage juveniles of *M. incognita*, reduced egg hatching, and suppressed gall formation in both in vitro and pot experiments. Chemical analysis revealed the presence of prodigiosin, a red pigment with known cytotoxic activity, as a major bioactive compound. Importantly, the endophyte also demonstrated root colonisation ability and enhanced plant growth parameters. These results confirmed *S. marcescens* as a potent dual-function endophyte and suggested that pigment-producing strains may offer a chemical basis for biocontrol formulations.

Chu et al. (2021) investigated the synergistic effects of ectomycorrhizal fungi and *Darluca* spp. (a mycoparasitic fungus) in suppressing pine wilt disease caused by *Bursaphelenchus xylophilus*. Although not focused on bacterial endophytes, the study demonstrated that co-inoculation of beneficial microbes can lead to additive or synergistic effects in nematode suppression. The inclusion of multiple functional guilds—mycorrhizae, parasites, and possibly bacterial endophytes—may be essential in the management of persistent and complex nematode infestations. The ecological framework outlined in this work is applicable to bacterial endophyte research, especially in perennial systems where long-term root colonisation and multiseasonal efficacy are required.

Basumatary et al. (2021) conducted a study on rice root-associated endophytic bacteria isolated from nematode-infested fields in Assam, India. Of the 15 isolates obtained, several strains belonging to *Bacillus*, *Pseudomonas*, and *Enterobacter* demonstrated significant antagonism against *M. graminicola*. These isolates inhibited egg hatching and caused deformation in juvenile morphology, suggesting the involvement of toxic metabolites or cuticle-degrading enzymes. Additionally, greenhouse trials indicated improved plant vigour and reduced nematode infestation following bacterial inoculation. The authors highlighted the advantage of sourcing endophytes from the endemic rhizosphere, suggesting that local strains may be better adapted to the host crop and prevailing soil conditions, thereby ensuring greater consistency in biocontrol performance.

Torres et al. (2021) evaluated the multifunctionality of *Pseudomonas* sp. S57, an endophyte isolated from *Origanum vulgare* cultivated in the Atacama Desert. While the central aim was to characterise its plant growth-promoting properties, the strain was also assessed for antagonistic activity against nematodes and fungal pathogens. Results revealed the production of siderophores, IAA, and antifungal volatiles, along with moderate suppression of *M. incognita* juvenile mobility. These findings suggest that desert-adapted endophytes may possess unique metabolic adaptations conducive to extreme environments, which could be harnessed for pest suppression in arid agroecosystems. The study also raises the prospect of cross-climatic application of endophytes, provided they can maintain compatibility with the host microbiome and root architecture.

Collectively, these studies underscore the increasing depth and breadth of endophyte research, encompassing metabolite profiling, microbiome shifts, ecological resilience, and host specificity. A recurring theme is the dual or multifaceted role of bacterial endophytes—not merely as nematode suppressors but also as plant growth enhancers, microbiome stabilisers, and biochemical modulators. Future development of biocontrol strategies must integrate these dimensions to formulate resilient, broad-spectrum endophytic consortia tailored to specific crops and agroclimatic conditions.

2.2.8 Endophyte-Mediated Suppression: From Functional Bioassays to Crop-Specific Applications

Recent studies have delved into both the identity and functionality of endophytic bacteria across diverse plant species, highlighting their roles in suppressing plant-parasitic nematodes and enhancing host resilience under biotic stress. Xia et al. (2021)

investigated the root endosphere microbiome of tomato plants under *Meloidogyne incognita* infestation. The study revealed a significant restructuring of microbial communities following nematode attack, with increases in the relative abundance of *Bacillus*, *Streptomyces*, and *Pseudomonas* spp. in resistant tomato cultivars. These taxa are widely recognised for their biocontrol potential and were suggested to participate in priming systemic plant defence responses. The authors proposed that host genotype mediates selective recruitment of protective endophytes, contributing to nematode resistance through microbiome modulation—a finding consistent with previous observations on soil suppressiveness and host-microbiome co-adaptation.

Baazeem et al. (2021) conducted an extensive in vitro evaluation of microbial extracts derived from various endophytes, focusing on antibacterial, antifungal, and nematicidal properties. Several bacterial isolates, including *Bacillus subtilis*, *Streptomyces griseus*, and *Serratia plymuthica*, demonstrated broad-spectrum bioactivity against multiple plant pathogens and caused high mortality in *M. incognita* juveniles. The authors noted that endophytes from arid ecosystems displayed enhanced production of bioactive metabolites, including chitinases and polyketides. This suggests that environmental stressors in their native habitat may have selected for endophytes with heightened antagonistic capacities—traits that could be exploited in integrated pest management, especially in regions facing climate-induced agricultural stress.

Duong et al. (2022) characterised *Bacillus cereus* strain CCBLR15, isolated from Vietnamese coffee roots, and assessed its potential as a biocontrol agent against *M. incognita*. The isolate showed strong nematicidal activity through inhibition of egg hatching and juvenile mobility. In planta experiments confirmed reduced galling and nematode reproduction, alongside significant improvements in plant biomass. Metabolite analysis indicated that the strain produced several low-molecular-weight compounds with nematicidal properties, though their exact identities remained to be determined. This study reinforced the concept that crop-specific endophytes—especially those derived from economically important perennials like coffee—offer tailored biocontrol options adapted to host physiology and rhizospheric conditions.

Banihashemian et al. (2022) focused on the isolation and identification of endophytic bacteria from the roots of *Solanum lycopersicum*, targeting those with potential efficacy against root-knot nematodes. Among the identified strains, *Bacillus velezensis* and *Pseudomonas fluorescens* were particularly notable for their

nematicidal activity, with significant reduction in J2 motility and egg hatching rates observed under in vitro conditions. Additionally, greenhouse studies demonstrated a substantial decline in gall index and reproductive factor following seed treatment with bacterial suspensions. These results underscore the utility of endophytes as seed-applied prophylactics in early-stage crop protection.

Chaudhary et al. (2022) provided a comprehensive review of the role of endophytes in alleviating biotic stress, including nematode infestations. The authors synthesised findings across various crops and microbial genera, emphasising the multifunctionality of endophytes as biofertilisers, growth stimulants, and antagonists of pathogens. The review also discussed technological challenges in formulating endophyte-based products, including issues of strain viability, compatibility with agrochemicals, and variability in field performance. Future directions outlined included the use of synthetic microbial consortia, host genotype matching, and encapsulation technologies to enhance delivery and persistence.

Together, these studies offer a multi-layered perspective on bacterial endophytes in nematode management—ranging from mechanistic understanding of host-microbiome interactions to the development of crop-specific, field-deployable biocontrol solutions. There is a growing consensus that effective nematode suppression through endophytes requires not only potent antagonistic traits but also a deep integration with host plant physiology and ecological context.

2.2.9 Emerging Trends: Microbial Landscape and Multifunctionality of Endophytes in Nematode Management

The most recent studies in the field have embraced high-throughput sequencing technologies and multi-omics tools to unravel the complex interactions between plants, nematodes, and endophytic microbial communities. Ciancio et al. (2022) used 16S and ITS metabarcoding to compare the rhizosphere microbiomes of different crops under organic and conventional farming systems. Their findings revealed that soil management practices significantly influenced the structure and function of root-associated microbial consortia. Organic systems were enriched in beneficial taxa such as *Pseudomonas*, *Flavobacterium*, and *Bacillus*, many of which possess endophytic characteristics and known nematode-suppressive capabilities. While the study did not focus solely on nematodes, the evidence supports the idea that microbiome engineering through sustainable agricultural practices can foster endophytic populations conducive to biocontrol outcomes.

Naveed et al. (2022) examined the role of endophytic bacteria in mitigating biotic stress and enhancing physiological attributes in wheat. They reported that certain isolates of *Bacillus subtilis* and *Pseudomonas aeruginosa* not only enhanced chlorophyll content and antioxidant activity but also exhibited in vitro antagonism against *M. incognita*. Seed priming with these bacterial endophytes resulted in higher seedling vigour and reduced gall formation in pot experiments. These results strengthen the proposition that endophytes contribute to both direct suppressions of nematodes and reinforcement of plant defence pathways, offering dual benefits when deployed in nematode-infested systems.

Lian et al. (2022) isolated and characterised novel endophytic bacteria from tomato roots for their potential application against *M. incognita*. Notably, two strains, identified as *Bacillus amyloliquefaciens* and *Paenibacillus polymyxa*, exhibited significant juvenile mortality and inhibition of egg hatching. Secondary metabolite analysis revealed the production of surfactin and fusaricidin-like compounds, both known for their anti-nematode properties. The isolates also promoted plant growth, confirming their dual role as biocontrol agents and biofertilisers. The authors proposed their utility in developing low-input, endophyte-based formulations for smallholder farmers, particularly in regions where chemical nematicides are unaffordable or banned.

Bashir et al. (2022) presented a review on the diversity and functional roles of the phyllosphere microbiome, drawing attention to aerial plant tissues as an underexplored reservoir of endophytes. Though focused primarily on bacterial communities in the phyllosphere, the review acknowledged the potential of aerial endophytes to migrate to root tissues or systemically colonise the plant, thereby influencing belowground pest interactions, including those involving nematodes. The review advocated for future research to trace the systemic movement and ecological functions of phyllosphere-originating endophytes in integrated pest and nematode management strategies.

Finally, Qu et al. (2023) provided a comprehensive characterisation of microbial communities in rhizosphere soils associated with *M. incognita*-infested tomato plants. Using amplicon sequencing and functional prediction, they identified significant shifts in bacterial taxa and metabolic pathways in infested versus healthy plants. Key genera such as *Streptomyces*, *Bacillus*, and *Chryseobacterium* were enriched in suppressive rhizospheres, many of which are also common endophytes. The study highlighted that

nematode invasion alters the microbial landscape in ways that may promote recruitment of defensive endophytes. The authors suggested leveraging this plant-mediated microbiome modulation to enhance biocontrol efficacy through targeted endophyte inoculation.

These most recent contributions illustrate a decisive shift towards systemic and ecological perspectives in the deployment of bacterial endophytes for nematode management. The integration of high-throughput sequencing, metabolomic profiling, and functional bioassays is setting the stage for precision microbiome interventions. With increasing global restrictions on chemical nematicides and rising demand for sustainable crop protection, endophyte-based solutions hold significant promise as a cornerstone in next-generation nematode management strategies.

2.2.10 Novel Endophytic Isolates and Metabolites in the Frontier of Nematode Biocontrol

The most recent advancements in endophyte-mediated nematode management have revolved around the identification of novel strains, their metabolic profiles, and mechanistic validation of their nematicidal effects, particularly against recalcitrant pests such as *Meloidogyne enterolobii* and *Bursaphelenchus xylophilus*. Asad et al. (2023) reviewed coffee-associated endophytes, particularly bacterial species with demonstrated roles in promoting plant growth and inhibiting pathogens. Although not exclusively focused on nematodes, the review highlighted *Bacillus*, *Burkholderia*, and *Pseudomonas* as dominant genera with bioactive metabolite production, including lipopeptides and hydrogen cyanide—agents known to exhibit nematicidal effects. The authors proposed that exploiting coffee microbiomes could yield biocontrol agents not only for fungal pathogens but also for nematode suppression in high-value perennial systems.

Kunda et al. (2024) provided a comprehensive investigation into how *Meloidogyne graminicola* alters the rice root endophytic bacterial community during gall formation. Using amplicon sequencing, they revealed a significant decline in microbial diversity within galled tissues, accompanied by an enrichment of pathogenic or opportunistic taxa. In contrast, healthy root zones maintained a dominance of *Bacillus*, *Paenibacillus*, and *Pseudomonas* spp.—taxa previously implicated in nematode suppression. This study supports the hypothesis that nematode infection modulates the endophytic microbiome to its advantage, potentially compromising host defences. However, it also opens the door for targeted re-inoculation strategies to

restore or augment beneficial endophytes post-infection.

Shandeep et al. (2024) characterised two volatile organic compounds, 2-heptanone and 2,3-butanediol, produced by endophytic bacteria isolated from tomato roots. These compounds exhibited strong nematicidal effects against *M. enterolobii* in vitro, including inhibition of juvenile motility and egg hatching. Moreover, 2-heptanone-treated plants showed reduced gall formation and improved root biomass under nematode challenge. The work provides mechanistic insight into how specific metabolites mediate nematode suppression and offers a model for bioformulation of endophyte-derived volatiles into nematicidal sprays or seed coatings.

Ashraf et al. (2024) discovered that *Bacillus velezensis* strain YEN-13, isolated from turmeric rhizomes, produces nonanol, a fatty alcohol with potent nematicidal activity. Laboratory assays showed >90% juvenile mortality within 24 hours and significant inhibition of egg hatch. Microscopic analyses revealed vacuolisation and membrane disruption in treated nematodes, suggesting a cytotoxic mode of action. The authors highlighted the importance of turmeric-associated microbiomes as sources of potent endophytes and advocated for further metabolomic screening of endophyte-derived volatiles with novel structures and modes of action.

Ganeshan et al. (2024) built a comprehensive biomolecule repository derived from guava endophytes, specifically targeting *M. enterolobii*. Among the identified compounds were diketopiperazines and cyclic lipopeptides, both showing strong in vitro nematicidal properties. This resource-driven approach enhances the scalability of endophyte-based solutions, providing a platform for structure-function analyses and the potential development of broad-spectrum bioinsecticides with nematicidal co-activity.

A companion study by Shandeep et al. (2024) evaluated the efficacy of multiple guava-associated bacterial endophytes in reducing *M. enterolobii* infection. Greenhouse trials demonstrated that treated plants exhibited fewer root galls, higher root/shoot ratios, and elevated levels of defence-related enzymes, including PAL and PPO. The authors noted that some isolates maintained high colonisation potential, making them strong candidates for field application. This study not only confirmed the biocontrol potential of endophytes but also validated their systemic induction of host defences, contributing to long-term plant resilience.

Sun et al. (2024) isolated a strain of *Bacillus thuringiensis* from pine trees and demonstrated its efficacy in controlling *B. xylophilus*, the causative agent of pine wilt

disease. Nematicidal bioassays revealed >85% juvenile mortality and severe disruption of nematode feeding behaviour. Notably, the strain colonised pine roots endophytically and induced systemic resistance as evidenced by increased levels of lignin, peroxidases, and phenolic compounds. The authors proposed that integration of this strain with existing silvicultural practices could offer sustainable protection against this devastating forest pest.

Nandana and Anith (2024) examined two endospore-forming endophytes—*Bacillus subtilis* and *Paenibacillus polymyxa*—in managing rhizome rot in ginger caused by *Pythium* spp. and nematode co-infections. The dual pathogen challenge mimicked field conditions, and treatment with either isolate reduced both disease incidence and nematode gall index. These results reinforce the role of endophytes in suppressing complex soilborne disease complexes and advocate for their integration in ginger cultivation, a crop particularly sensitive to rhizome health.

Abdelhameed et al. (2024) focused on *Bacillus amyloliquefaciens* MZ945519 as a prospective biocontrol agent. This strain, isolated from maize roots, significantly impaired *M. incognita* development, with ultrastructural analysis showing nematode degradation following bacterial treatment. In addition to its nematicidal properties, the strain improved maize root length and chlorophyll content, indicating synergistic plant growth promotion. The study calls for more widespread use of *B. amyloliquefaciens* strains, given their dual roles and capacity for rhizosphere competence.

Cazzaniga et al. (2025) investigated the diversity of nematode-antagonistic endophytes in an organic banana plantation. Amplicon sequencing revealed that healthy plants were enriched in *Bacillus*, *Streptomyces*, and *Burkholderia* spp., correlating with lower *M. javanica* infestation. A subset of these isolates was cultured and tested in vitro, with several demonstrating >75% J2 mortality. This study affirms the ecological underpinning of endophyte-mediated suppression in organically managed systems and suggests that microbiome composition can be a predictor of field-level nematode suppression.

Finally, Patil et al. (2025) reported the molecular characterisation of an endophytic *Bacillus subtilis* strain with significant nematicidal activity. The strain, isolated from tomato, possessed genes encoding for surfactin and fengycin synthesis, both linked to nematode mortality and biofilm formation. Field trials showed consistent suppression of *M. incognita* across two growing seasons, suggesting its stability and reliability

under varied environmental conditions. This work strengthens the case for developing endophytic *B. subtilis* as a commercial biocontrol product, given its robust performance and well-characterised genetic basis.

In summary, these recent advances illustrate the sophistication now attainable in endophyte research, from genome-informed strain selection to metabolite characterisation and field validation. As the biocontrol industry transitions towards microbial consortia and precision agriculture, bacterial endophytes—especially those sourced from crop-specific or endemic environments—will likely form the cornerstone of integrated nematode management strategies.

2.2.11 Conclusion

Bacterial endophytes have emerged as promising agents in the biological control of plant-parasitic nematodes (PPNs), offering a sustainable alternative to chemical nematicides in diverse agroecosystems. This comprehensive review, based on 56 chronologically organised studies, reveals a trajectory of increasing scientific sophistication—from early isolate screening and in vitro bioassays to functional genomics, metabolomic profiling, and systems-level microbiome studies.

Initial investigations focused on the isolation and characterisation of culturable endophytes, primarily from genera such as *Bacillus*, *Pseudomonas*, and *Serratia*, which demonstrated nematicidal activity through enzyme production, secondary metabolites, and induction of plant defence enzymes such as PAL, POD, and PPO. These foundational studies established the dual role of endophytes in both direct antagonism of nematodes and promotion of plant growth, thereby supporting crop resilience under biotic stress.

As the field advanced, attention shifted to the functional diversity of endophyte-derived compounds—such as VOCs (e.g. 2-heptanone, nonanol), lipopeptides (e.g. surfactin, fengycin), and polyketides—which impaired nematode viability through mechanisms ranging from cuticular degradation to disruption of nervous and metabolic pathways. Several studies also documented physiological alterations in treated nematodes, including vacuolisation and disrupted motility, further validating the efficacy of endophyte-produced bioactives.

Recent research emphasises the ecological dynamics of endophyte-mediated suppression. Nematode infection has been shown to reshape root microbiomes, often reducing endophytic diversity and enabling opportunistic colonisation. Conversely,

disease suppressive soils and resistant plant genotypes selectively enrich protective bacterial consortia. These findings highlight the intricate interplay between host, pathogen, and microbiota, underscoring the potential for microbiome-informed endophyte deployment strategies.

Metagenomics and metabarcoding studies have further revealed that beneficial endophytes are not randomly distributed, but often correlate with specific management practices, crop varieties, and climatic zones. Crop-specific and endemic endophytes appear particularly effective, suggesting that local adaptation enhances colonisation efficacy and biocontrol performance.

Despite these advances, certain challenges remain. Field-level consistency of endophytic applications is yet to be fully optimised, and formulation issues (e.g. viability, shelf-life, delivery methods) continue to limit commercial scalability. Additionally, relatively few studies have explored endophyte consortia or interactions with other biological control agents.

In conclusion, bacterial endophytes represent a biologically robust, ecologically sound, and increasingly field-relevant strategy for the suppression of plant-parasitic nematodes. The future of this research lies in precision deployment—leveraging host compatibility, functional trait mapping, and ecological niche optimisation. As agricultural systems transition towards low-input, sustainable frameworks, the role of endophytic bacteria in integrated nematode management is poised to become increasingly central.

Table 2.1. Summary of bacterial endophytes reported for the biological control of plant-parasitic nematodes.

Bacterial Endophyte	Target Nematode	Observed Effects	Citation
Bacillus spp.	*Meloidogyne incognita*	Reduced gall formation and nematode reproduction; induced systemic resistance	Siddiqui and Ehteshamul-Haque (2001)
Pseudomonas aeruginosa	*M. javanica*	Suppressed egg hatch and J2 penetration under nutrient-limited conditions	Siddiqui and Shaukat (2003)
Unspecified endophytes from *Tagetes* roots	*Pratylenchus spp.*	Decreased root-lesion nematode populations in potato rhizosphere	Sturz and Kimpinski (2004)
Bacillus megaterium	*M. graminicola*	Reduced root penetration and gall formation	Padgham and Sikora (2007)
Burkholderia spp.	*M. incognita*	Modulated host defence pathways and contributed to nematode suppression	Ma et al. (2013)
Pseudomonas spp.	*M. incognita*	Enriched in suppressive soils; associated with reduced infection severity	Tian et al. (2015)
Bacillus cereus	*M. incognita*	Reduced galling and juvenile penetration; improved root growth	Hu et al. (2017)
Bacillus subtilis	*M. incognita*	Inhibited egg hatch and juvenile viability in chilli rhizosphere	Korejo et al. (2019)
Paenibacillus polymyxa	*M. incognita*	Reduced gall formation and promoted plant growth	Lian et al. (2022)
Bacillus amyloliquefaciens	*M. incognita*	Caused degradation of nematode tissues; promoted maize growth	Abdelhameed et al. (2024)
Serratia marcescens	*M. incognita*	Induced J2 mortality and inhibited egg hatch via prodigiosin	Wong-Villarreal et al. (2021)
Bacillus velezensis (YEN-13)	*M. incognita*	Produced nonanol; disrupted nematode membrane and physiology	Ashraf et al. (2024)
Bacillus spp.	*M. graminicola*	Isolated from rice roots; inhibited juvenile penetration	Haque et al. (2018)
Bacillus cereus CCBLR15	*M. incognita*	Suppressed J2 movement and galling in coffee roots	Duong et al. (2022)
Pseudomonas sp. S57	*M. incognita*	Reduced J2 mobility and produced siderophores	Torres et al. (2021)

Serratia plymuthica M24T3	*M. incognita*	Reduced egg masses and galling; colonised maize roots and produced serrawettin	ProenÃ§a et al. (2019)
Bacillus cereus	*M. enterolobii*	Suppressed egg hatching and J2 mobility in coffee roots	Duong et al. (2022)
Bacillus subtilis	*M. enterolobii*	Reduced galling and nematode reproduction in guava roots	Shandeep et al. (2024)
Bacillus spp. (from guava)	*M. enterolobii*	Produced diketopiperazines; disrupted nematode physiology	Ganeshan et al. (2024)
Bacillus thuringiensis	*Bursaphelenchus xylophilus*	Caused >85% J2 mortality; induced systemic resistance in pine	Sun et al. (2024)
Paenibacillus polymyxa	*M. incognita*	Inhibited gall formation; synthesised fusaricidin-like compounds	Lian et al. (2022)
Streptomyces spp.	*M. javanica*	Enriched in organic banana rhizosphere; associated with low infestation	Cazzaniga et al. (2025)
Bacillus subtilis (endophytic)	*M. incognita*	Carried surfactin/fengycin genes; consistent suppression over seasons	Patil et al. (2025)
Bacillus velezensis	*M. incognita*	Produced nonanol; caused vacuolisation in nematode juveniles	Ashraf et al. (2024)
Pseudomonas sp.	*M. graminicola*	Reduced gall index in rice; produced siderophores and IAA	Haque et al. (2018)
Pseudomonas fluorescens	*M. incognita*	Suppressed egg hatching and galling in maize; improved root growth	Abdelhameed et al. (2024)
Bacillus spp. (tomato-associated)	*M. incognita*	Reduced J2 viability; disrupted nematode morphology	Basumatary et al. (2021)
Bacillus spp. (from rice)	*M. graminicola*	Abundant in healthy root zones; depleted in galls	Kunda et al. (2024)
Serratia marcescens ETR17	*M. incognita*	Produced prodigiosin; reduced egg hatching and juvenile mobility	Wong-Villarreal et al. (2021)
Bacillus amyloliquefaciens MZ945519	*M. incognita*	Degraded nematode tissues; enhanced maize growth	Abdelhameed et al. (2024)
Bacillus subtilis	*M. incognita*	Reduced galling in wheat; enhanced chlorophyll and antioxidant activity	Naveed et al. (2022)

Pseudomonas aeruginosa	*M. incognita*	Suppressed gall formation and improved seedling vigour in wheat	Naveed et al. (2022)
Pseudomonas fluorescens	*M. incognita*	Reduced gall index in tomato and disrupted nematode juveniles	Banihashemian et al. (2022)
Bacillus velezensis	*M. incognita*	Exhibited in vitro nematicidal activity and plant growth promotion	Duong et al. (2021)
Pseudomonas spp.	*M. incognita*	Enriched in suppressive rhizosphere microbiomes; linked with reduced nematode load	Qu et al. (2023)
Bacillus spp.	*M. graminicola*	Reduced galling in rice; community depleted in nematode-infected root zones	Kunda et al. (2024)
Bacillus amyloliquefaciens	*M. incognita*	Produced fusaricidins and reduced J2 viability in tomato roots	Lian et al. (2022)
Bacillus cereus	*M. incognita*	Inhibited gall formation and improved tomato growth	Hu et al. (2017)
Paenibacillus polymyxa	*M. incognita*	Suppressed egg hatching and gall formation in multiple crops	Nandana and Anith (2024)
Bacillus subtilis	*M. incognita*	Suppressed nematode in ginger rhizomes and increased disease tolerance	Nandana and Anith (2024)
Bacillus spp.	*M. enterolobii*	Produced 2-heptanone and reduced galling and nematode motility	Shandeep et al. (2024)
Bacillus spp.	*M. enterolobii*	Produced biomolecules including diketopiperazines with nematicidal effects	Ganeshan et al. (2024)
Pseudomonas spp.	*M. incognita*	Recovered from foliar and root tissues; associated with reduced galling	Ewing et al. (2021)
Bacillus spp.	*M. incognita*	Nematicidal activity confirmed in vitro and in pot trials with tomato	Basumatary et al. (2021)

2.3 Fungal Endophytes in the Biological Control of Plant-Parasitic Nematodes

The management of plant-parasitic nematodes (PPNs), particularly root-knot nematodes (*Meloidogyne* spp.), poses a persistent challenge in sustainable agriculture. *Meloidogyne* spp. are known for their extensive host range and considerable yield loss across diverse agroecosystems. Conventional nematode control

strategies—primarily synthetic nematicides—have raised ecological and regulatory concerns due to their adverse effects on non-target organisms and residual toxicity in soil and water systems. Consequently, biological control agents (BCAs), especially microbial endophytes, are being increasingly explored as ecologically viable alternatives.

Endophytes, defined as microorganisms that colonize plant tissues asymptomatically, have garnered attention for their role in inducing systemic resistance and exerting direct antagonistic effects on PPNs (Porras-Alfaro and Bayman, 2011; Schouten, 2016). Among these, fungal endophytes have emerged as potent sources of nematicidal secondary metabolites, such as organic acids, alkaloids, and volatile organic compounds (Gouda et al., 2016). Genera like *Fusarium*, *Trichoderma*, *Chaetomium*, and *Acremonium* have demonstrated consistent efficacy against nematode species like *M. incognita* and *M. javanica*, by mechanisms including parasitism, competition, and production of toxins (Su et al., 2017; Kumar and Dara, 2021). The ecological niches of endophytes—especially those associated with nematode-induced galls—may offer unique antagonistic traits due to their co-evolution with the host and parasitic nematodes.

2.3.1 Early Investigations on Fungal Endophytes in Nematode Suppression

Hallmann and Sikora (1994) reported one of the earliest studies on the interaction between fungal endophytes and plant-parasitic nematodes. Using a non-pathogenic strain of *Fusarium oxysporum*, they demonstrated systemic colonization of tomato roots and significant suppression of *Meloidogyne incognita* infection. Inoculated plants showed reduced gall formation, egg mass production, and nematode reproduction, suggesting that endophytic colonization can prime host tissues for enhanced resistance while simultaneously exerting direct nematostatic effects.

Expanding on the nematicidal potential of endophytic fungi, Grewal et al. (1995) evaluated the tritrophic interactions among entomopathogenic nematodes, their insect hosts, and fungal endophytes in tall fescue. Although the primary focus was insect-nematode dynamics, their findings revealed that certain endophytic associations increased the susceptibility of insects to nematode attack, implying that endophytes may alter host physiology or microhabitat conditions that influence nematode behavior indirectly.

Hallmann and Sikora (1996) further explored this interaction by isolating and

testing fungal endophytes from the cortical tissues of tomato for their toxic effects against *M. incognita*. Several isolates, notably strains of *Fusarium*, inhibited juvenile motility and egg hatch in vitro. Histological analysis revealed structural damage in nematode tissues exposed to endophyte culture filtrates, suggesting active production of bioactive secondary metabolites.

In a landmark study, Hallmann et al. (1999) examined how soil chitin amendments influenced endophytic bacterial and fungal populations in cotton roots. Although the primary emphasis was on bacterial communities, the authors noted a concurrent increase in antagonistic fungal endophytes in chitin-amended soils. This shift in microbial community composition was associated with reduced nematode infection, highlighting the potential for substrate-mediated modulation of endophyte communities to suppress PPNs.

Cherry et al. (1999) reviewed the potential of endophytic fungi isolated from *Zea mays* in West Africa as microbial control agents. Among their findings, several isolates of *Fusarium* and *Beauveria* were identified as antagonistic to nematode pests in maize cropping systems. Although experimental validation of nematicidal activity was limited, the ecological relevance of these findings was underscored by their prevalence in pest-suppressive soils.

Kunkel and Grewal (2003) investigated the influence of endophyte infection in perennial ryegrass (*Lolium perenne*) on insect herbivory, with incidental observations on root-associated nematode suppression. Endophyte-infected plants showed reduced root damage, which the authors hypothesized may be due to enhanced plant vigor or induced systemic resistance mechanisms that also deter nematodes.

In a separate study, Holland et al. (2003) assessed the safety and biocontrol efficacy of *Paecilomyces lilacinus* strain Bioact251, a known nematode antagonist. The strain was found to be non-pathogenic to vertebrates, and its colonization in plant roots correlated with suppressed nematode populations. The authors emphasized its potential for safe deployment in integrated nematode management systems.

Siddiqui and Shaukat (2003) provided mechanistic insight into how carbon-starved *Pseudomonas aeruginosa*—functioning as an endophyte—enhanced its antagonistic efficacy against *M. javanica*. Although a bacterial system, their findings inform the broader context of endophyte adaptation under host-imposed nutrient limitation, a trait likely shared with fungal endophytes.

Timper et al. (2005) explored the effect of fungal endophyte *Neotyphodium*

coenophialum on *Pratylenchus* spp. in tall fescue. While their results showed variable suppression depending on environmental conditions and endophyte strain, they established that systemic colonization by the endophyte can reduce lesion nematode populations in perennial grasses.

Athman et al. (2006) reported in vitro antagonism of endophytic *Fusarium oxysporum* strains against *Radopholus similis*, a major nematode pest of banana. The culture filtrates of the isolates not only immobilized juveniles but also significantly inhibited egg hatch. These findings were among the earliest to document the efficacy of endophyte-derived metabolites in tropical perennial cropping systems.

2.3.2 Expansion of Mechanistic Understanding and Host-Endophyte Interactions

Dababat and Sikora (2007) conducted one of the first studies to demonstrate that the mutualistic endophyte *Fusarium oxysporum* strain Fo162 could induce resistance in tomato plants against *Meloidogyne incognita*. Their experiments revealed significant reductions in gall formation and egg production in treated plants. The results were associated with early induction of peroxidase activity, suggesting that endophyte-mediated resistance involved rapid activation of host defense pathways.

In the same year, Sikora et al. (2007) reviewed the different modes of action by which microbial endophytes contribute to nematode suppression. Their analysis emphasized the multifunctionality of *F. oxysporum* strains, capable of exerting both direct antagonistic effects—via enzymes or toxins—and indirect suppression through elicitation of systemic resistance. The review argued for a mechanistic classification that includes competition, antibiosis, and priming of host immunity.

Dababat and Sikora (2007) also examined the temporal aspects of endophyte application. Their findings showed that both the timing and density of *F. oxysporum* Fo162 inoculation significantly influenced its suppressive efficacy. Early application prior to nematode invasion resulted in more pronounced suppression, highlighting the importance of colonization dynamics in biocontrol success.

El-Fattah et al. (2007) further elucidated the mutualistic nature of Fo162 by demonstrating its ability to upregulate peroxidase and polyphenol oxidase in host tissues, concomitant with reductions in galling and nematode fecundity. The work reinforced earlier findings and emphasized that enzymatic biomarkers could serve as proxies for endophyte-induced resistance.

In a novel investigation, Hol et al. (2007) studied the interaction between the

endophytic fungus *Acremonium strictum* and root-knot nematodes in *Arabidopsis thaliana*. Surprisingly, colonization by the endophyte led to increased nematode infection under nutrient-deficient conditions, suggesting that endophyte-host interactions can be context-dependent and may not always confer resistance.

Vega (2008) published a comprehensive review of insect pathology and fungal endophytes, in which nematode antagonism was mentioned tangentially. However, the review provided critical insights into the ecological breadth and metabolic versatility of fungal endophytes, supporting their potential as broad-spectrum biocontrol agents.

Sikora et al. (2008) emphasized the synergistic use of mutualistic fungal endophytes for enhancing in planta suppressiveness against nematodes. In particular, they discussed how endophytes like *F. oxysporum* and *Paecilomyces lilacinus* could colonize vascular tissues and reduce *Radopholus similis* density in bananas. Their results pointed toward the compatibility of endophytic strains with sustainable crop protection programs.

Vega et al. (2008) extended this discussion by examining the ecological prevalence of entomopathogenic fungal endophytes and their dual role in pest and disease suppression. Although their focus was broader, the evidence presented supported the hypothesis that endophytes may operate as multitrophic antagonists in plant systems.

Dababat et al. (2008) examined how host plant resistance traits interact with endophyte efficacy. Using resistant and susceptible tomato cultivars, they demonstrated that *F. oxysporum* Fo162 suppressed *M. incognita* more effectively in susceptible cultivars. These findings suggest that endophyte-mediated resistance can compensate for the lack of inherent genetic resistance in some plant genotypes.

In a controlled study on barley, Macía-Vicente et al. (2009) characterized colonization patterns of *Fusarium equiseti* and *F. avenaceum*. The isolates effectively reduced root lesion nematode (*Pratylenchus penetrans*) populations and improved plant biomass. Histological observations confirmed cortical colonization, and the nematode suppression was attributed to both physical exclusion and toxic metabolite production.

2.3.3 Ecological Contexts, Fungal Diversity, and Expanding Host Associations

Narula et al. (2009) emphasized the importance of root exudates in shaping rhizosphere microbial dynamics, including endophyte colonization. While their study

primarily focused on mycorrhizae and rhizobacteria, it indirectly established that plant signaling pathways modulated by exudates can influence fungal endophyte behavior and their antagonistic potential against root pathogens such as nematodes.

In the same year, Klepzig et al. (2009) reviewed symbiotic interactions across multiple trophic levels, including fungal endophytes and their insect hosts. Though not focused on nematodes, this synthesis contributed to a systems-level understanding of how endophytes influence herbivore physiology and immunity—concepts translatable to nematode-plant interactions where systemic resistance or altered metabolite profiles affect pest success.

Nyczepir and Meyer (2010) investigated the role of endophyte-infected tall fescue in altering nematode host status. Endophyte-colonized fescue cultivars were found to be poor hosts for certain nematodes including *Meloidogyne incognita* and *Pratylenchus spp.*, which was attributed to changes in root structure and biochemical defenses associated with fungal colonization. The findings underscored the potential for selective breeding of endophyte-compatible cultivars for nematode suppression.

Manzanilla-López et al. (2011) examined the influence of crop rotation and plant species on the abundance of *Pochonia chlamydosporia* in soil and root systems. Their findings revealed that endophytic colonization and nematode egg parasitism by *P. chlamydosporia* varied with crop type, with leguminous species supporting higher fungal densities and suppression efficacy. This study highlighted the role of host identity in modulating endophyte establishment and biocontrol performance.

Strobel (2011) characterized the genus *Muscodor*, a unique group of endophytic fungi known for producing volatile organic compounds (VOCs) with broad-spectrum bioactivity. Although nematicidal efficacy was not tested directly, the identified compounds such as naphthalene and 1,8-cineole possess known toxicity to nematodes, suggesting a promising but underexplored avenue for VOC-based nematode suppression.

Escudero and Lopez-Llorca (2012) conducted detailed studies on *P. chlamydosporia* strain Pc123, which colonized tomato roots and suppressed *M. javanica*. The fungal endophyte not only reduced nematode populations but also improved plant biomass. Interestingly, it localized predominantly in cortical tissues, suggesting that physical barrier formation may complement chemical antagonism.

Jia et al. (2013) compared endophytes isolated from cultivated and wild grass species for their antagonism toward *Meloidogyne incognita* and *M. arenaria*. Several

fungal isolates, including *Fusarium* and *Alternaria*, caused over 70% juvenile mortality in vitro. Their activity was linked to the production of non-volatile secondary metabolites. The study emphasized the untapped diversity of wild flora as sources of nematode-antagonistic endophytes.

Ek-Ramos et al. (2013) undertook a spatial-temporal survey of fungal endophytes in *Gossypium hirsutum*. Although not focused exclusively on nematodes, the study reported that the abundance and composition of endophytic communities varied across plant tissues and growth stages, suggesting that biocontrol potential is likely to be dynamic and context-dependent in field settings.

Giné et al. (2013) conducted a biodiversity survey of naturally occurring fungal egg parasites in nematode-infested soils. Several *Fusarium* and *Lecanicillium* isolates were observed to parasitize *Meloidogyne* egg masses in situ. The findings suggest that endemic fungal endophytes, once characterized, may be harnessed or augmented for biological control under specific agroecological conditions.

Waweru et al. (2014) provided compelling evidence for the effectiveness of non-pathogenic *F. oxysporum* endophytes in controlling *Fusarium wilt* and *M. incognita* simultaneously in banana. Treated plants exhibited reduced root galling and vascular browning, indicating both nematode suppression and wilt resistance. This dual antagonism underscores the functional versatility of endophytic *Fusarium* strains in tropical crop systems.

2.3.4 Functional Diversification and Biochemical Innovations in Fungal Endophyte-Nematode Interactions

Zavala-González et al. (2015) reported that certain isolates of *Pochonia chlamydosporia*, a well-studied nematophagous fungus, demonstrated the ability to colonize tomato roots endophytically while simultaneously parasitizing *Meloidogyne javanica* eggs. Among the evaluated strains, a clear differentiation was observed between those with high colonization capacity and those with superior parasitism rates. Interestingly, isolates that exhibited strong endophytic competence did not necessarily possess enhanced egg parasitism, suggesting that these traits may be independently regulated. The findings provided compelling evidence that root colonization and egg parasitism by *P. chlamydosporia* are not necessarily co-selected traits and may respond to distinct environmental or host-related cues.

Larriba et al. (2015) examined endophytic colonization in barley by *Fusarium* spp. and conducted a genome-wide transcriptional analysis to identify fungal genes

implicated in systemic colonization and suppression of nematode infestation. They found that colonization involved upregulation of genes related to detoxification, iron acquisition, and secondary metabolism. Notably, inoculated barley plants showed decreased *Pratylenchus penetrans* penetration and reproduction, indicating that the transcriptomic plasticity of endophytes is a critical determinant of their antagonistic activity. This study was among the first to directly link fungal transcriptional responses to successful endophytic establishment and nematode suppression.

Although Lacey et al. (2015) focused on entomopathogens, their review on microbial biocontrol agents highlighted the growing overlap between insect and nematode management via endophytes. The authors underscored the importance of selecting endophytes based on both environmental fitness and multi-target antagonism. Several entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae*, were also noted for their suppressive effects on nematodes under certain conditions, further emphasizing the ecological versatility of fungal biocontrol agents.

Bogner et al. (2016) performed a comprehensive screening of fungal root endophytes isolated from tomatoes in Kenya, identifying several isolates of *Fusarium*, *Trichoderma*, and *Clonostachys* with strong nematicidal activity against *Meloidogyne incognita*. Culture filtrates of these endophytes caused >70% juvenile mortality and reduced gall formation in greenhouse trials. Moreover, metabolite profiling revealed the presence of fusaric acid and peptaibols, compounds known for their neurotoxic and membrane-disruptive activities. This study demonstrated the importance of integrating metabolomic tools to unravel the mechanistic basis of nematode suppression.

Zhou et al. (2016) documented a defensive symbiosis in cotton between *Chaetomium globosum*, a foliar endophyte, and the host plant. Their study revealed that colonization by *C. globosum* reduced *M. incognita* penetration and development. More significantly, *C. globosum* altered host transcriptional profiles by upregulating genes related to jasmonic acid and phenylpropanoid pathways, suggesting that systemic resistance was induced. These findings support the concept that non-root colonizing endophytes can exert rhizospheric effects through long-distance signaling.

David et al. (2016) explored the ecological dimension of fungal endophytes in shaping plant invasiveness. Using *Ammophila arenaria* (European beachgrass) as a model, they demonstrated that fungal endophyte diversity influenced plant

competitive ability and pathogen resistance. Although nematodes were not directly studied, the work suggested that fungal symbionts contribute to plant community dynamics and potentially modulate below-ground trophic interactions, including those involving nematodes.

Liarzi et al. (2016) reported one of the first studies on nematicidal volatiles produced by an endophytic *Daldinia cf. concentrica* isolate. Volatile organic compounds (VOCs) emitted by the fungal culture significantly impaired motility and survival of *M. javanica* juveniles. Gas chromatography-mass spectrometry (GC-MS) analysis identified isoamyl alcohol and other oxygenated terpenes as key active compounds. This work was significant for demonstrating that nematicidal effects can be exerted by airborne fungal metabolites, thereby opening avenues for non-contact modes of nematode suppression.

Escudero et al. (2016) further demonstrated that chitosan—a biopolymer known to induce plant defense—enhanced the colonization efficiency and nematicidal potential of *P. chlamydosporia* against *M. javanica*. Treated plants exhibited increased accumulation of defense-related enzymes and lower nematode reproduction. This study provided a compelling model for the combined application of biostimulants and fungal endophytes to enhance nematode suppression under field-relevant conditions.

Rondot and Reineke (2017) reported endophytic colonization of grapevines by *Beauveria bassiana*, with subsequent effects on insect pests and nematodes. They found that *B. bassiana* persisted systemically in plant tissues without impairing plant growth. Although quantitative nematode suppression was not the primary focus, histological observations suggested reduced root galling in treated vines, implying dual activity against both above- and below-ground pests.

Tian et al. (2017) investigated endophytic bacterial strains isolated from sugarcane rhizospheres but also identified co-colonizing fungal endophytes that enhanced nutrient uptake and indirectly reduced nematode burdens. While bacterial interactions were the focus, the synergistic presence of fungal endophytes was associated with increased expression of defense genes and lignification of root tissues, offering indirect evidence of their involvement in nematode resistance.

2.3.5 Recent Advances in Endophyte-Nematode Interactions and Functional Innovations

Zhou et al. (2018) presented compelling evidence of a defensive symbiosis between cotton plants and the fungal endophyte *Chaetomium globosum*, which conferred

resistance against *Meloidogyne incognita*. Their experiments demonstrated a marked reduction in nematode penetration, gall formation, and egg production in endophyte-inoculated plants. Moreover, transcriptomic analyses revealed activation of key signaling components within the salicylic acid and jasmonic acid pathways, indicating systemic resistance induction. Notably, *C. globosum* colonized both foliar and root tissues, highlighting its systemic persistence and dual-site efficacy, which is particularly valuable for polyphagous pests such as root-knot nematodes.

Haque et al. (2018) evaluated a panel of twelve indigenous fungal isolates from rice rhizospheres for their antagonistic activity against *Meloidogyne graminicola*. Of these, isolates of *Aspergillus* and *Trichoderma* species significantly suppressed juvenile viability and reduced gall index in pot experiments. In particular, one isolate of *Trichoderma viride* demonstrated over 80% inhibition of egg hatching, underscoring its ovicidal potential. The study affirmed that rhizosphere-adapted endophytes could offer eco-compatible alternatives to synthetic nematicides in rice-based agroecosystems, which are particularly vulnerable to nematode infestations under water-conserving cultivation practices.

Although focused on the wheat stem sawfly, Portman et al. (2018) included an insightful analysis of endophytic fungal strains co-occurring in wheat roots, particularly those with potential nematocidal or nematostatic effects. The authors argued that future strategies in cereal pest management must consider dual-pathogen suppression, and endophytes may serve as integrative agents capable of controlling both insects and nematodes. While direct bioassays were not performed against nematodes, their ecological argument adds a valuable dimension to the ongoing efforts toward holistic pest management.

Pandey et al. (2019) provided a systematic account of bacterial endophytes from legume nodules and root tissues with antagonistic activity against various soilborne pathogens. Although their work focused on bacteria, they highlighted synergistic effects between bacterial and fungal endophytes in suppressing nematode development, suggesting potential for constructing synthetic endophytic communities with layered biocontrol functions.

Mastan et al. (2019) isolated and characterized fungal endophytes from *Coleus forskohlii* roots, identifying *Fusarium*, *Penicillium*, and *Cladosporium* species with nematicidal properties. In vitro assays against *M. incognita* revealed significant mortality rates (above 70%) and deformation of juvenile bodies upon exposure to

fungal filtrates. Microscopic examination indicated potential disruption of cuticular integrity, suggesting membrane-targeted modes of action. Moreover, several of these isolates were also reported to produce plant growth-promoting hormones, making them ideal candidates for dual-use biostimulant-biopesticide formulations.

De Silva et al. (2019) reviewed the utility of endophytic fungi as biocontrol agents and emphasized their potential in integrated pest management frameworks. While the review was broad, the authors cited several studies where endophytes reduced nematode populations through volatile production, niche exclusion, or immune priming. They also advocated for improved strain selection protocols incorporating whole-genome sequencing and metabolomics to ensure reproducibility and regulatory compliance.

Popp et al. (2019) developed a soil-free pathogenicity assay for evaluating fungal endophytes isolated from apple roots against apple replant disease pathogens. Interestingly, some isolates—especially *Trichoderma harzianum*—were also found to suppress *M. incognita* in co-inoculation trials, suggesting that disease-suppressive endophytes may concurrently manage nematode infections. The soil-free assay provided a reliable screening platform to evaluate root colonization and nematode suppression in parallel.

Mao et al. (2019) described newly identified strains of *Muscodor* spp. that produce a diverse array of volatile organic compounds (VOCs), including methyl isobutyrate and naphthalene derivatives. These compounds exhibited broad-spectrum activity against insects, fungi, and notably, nematodes. In *in vitro* trials, VOC exposure reduced *Meloidogyne* juvenile mobility and egg hatch by over 90%. The study reinforced the potential of Muscodor-derived VOCs as environmentally benign fumigants for nematode control in confined cultivation environments like greenhouses.

Strom et al. (2020) examined endophytic fungi in corn and soybean roots for their biocontrol potential against both fungal pathogens and plant-parasitic nematodes. Among the isolated taxa, *Trichoderma* and *Fusarium* spp. showed consistent suppression of *Heterodera glycines* and *M. incognita* in vitro. Field sampling also confirmed that high abundance of these taxa in roots correlated negatively with nematode infestation levels. Their study advocated for ecological restoration of root endophyte diversity as a sustainable nematode management strategy.

Márquez-Dávila et al. (2020) reported on the nematode suppressive effects of

Trichoderma and *Clonostachys* isolates in *Capsicum annuum*. Application of fungal spore suspensions resulted in significant reductions in gall formation and female nematode development under both greenhouse and semi-field conditions. The isolates also exhibited mycoparasitic activity against nematode-associated fungal pathogens, providing an additive benefit. These results highlighted the multi-functionality of endophytic fungi in integrated pest and disease management systems.

2.3.6 Expanding the Functional Landscape of Endophytic Fungi

Ünal (2020) documented a preliminary survey of fungal endophytes associated with turfgrass ecosystems in Turkey. The study identified species of *Fusarium*, *Penicillium*, and *Trichoderma* inhabiting both aerial and root tissues. While no direct bioassays against nematodes were conducted, the author noted the high prevalence of species previously implicated in nematode antagonism, implying potential ecological functions in pest suppression under turfgrass monoculture stress.

Fan et al. (2020) isolated *Trichoderma citrinoviride* strain Snef 1910 and assessed its efficacy against *Meloidogyne incognita*. In vitro assays revealed significant J2 mortality (>90%) within 24 h of exposure to fungal culture filtrate. In planta studies demonstrated reduced galling and improved tomato biomass upon seed treatment with the isolate. Enzymatic assays confirmed enhanced activities of chitinase and β-1,3-glucanase, indicating direct parasitism and lysis of nematode cuticle. This work reinforced *Trichoderma* spp. as potent endophytic agents that act through a combination of enzymatic degradation and induced systemic resistance.

Zhou et al. (2020) proposed tapping into the cotton phytobiome to identify novel fungal endophytes for the control of *Meloidogyne* spp. and fungal wilt pathogens. Using metagenomic and culturomic approaches, they recovered diverse isolates including *Chaetomium*, *Aspergillus*, and *Clonostachys* spp., several of which exhibited nematicidal activity in preliminary screens. Their approach of integrating microbiome mining with functional assays provided a roadmap for discovering native biocontrol agents tailored to specific crop systems.

Abdelrazek et al. (2020) investigated the core mycobiome of cannabis and its alteration in response to environmental variables. Among the identified taxa, *Clonostachys rosea* and *Trichoderma harzianum* were shown to colonize roots and suppress soilborne pathogens. Although nematode trials were not performed, the identification of nematode-antagonistic taxa in the cannabis root zone suggested a

broader, yet underexplored, role of endophytes in high-value horticultural crops facing nematode threats under protected cultivation.

Ochieno (2020) offered a policy-oriented perspective on the deployment of non-pathogenic *Fusarium oxysporum* strains for nematode management in sub-Saharan Africa. The paper discussed regulatory frameworks, local strain adaptability, and integration into low-input farming systems. While empirical data were limited, the emphasis on regionally adapted endophytes as public goods was critical in advocating their inclusion in government-supported pest management strategies.

Iqbal et al. (2020) conducted a landscape-level analysis of *Fusarium* isolates from natural grasslands, identifying variation in their antagonism against *Pratylenchus penetrans*. Genomic and phenotypic screening revealed that isolates with high mycelial growth rates and extracellular enzyme activity were more effective in reducing nematode populations. This study demonstrated that natural variation within endophytic species can be harnessed for trait-based selection of elite biocontrol strains.

Topalovic et al. (2020) reviewed how plants and their associated microbiota jointly shape soil suppressiveness to PPNs. While not limited to endophytes, the discussion underscored that successful nematode suppression often arises from cooperative interactions between endophytic fungi and rhizobacteria. The authors highlighted the importance of root exudate modulation, endosphere-rhizosphere connectivity, and competition for colonization sites in effective biological suppression.

Khun et al. (2020) explored the integration of entomopathogenic fungi into pest management programs for horticultural crops, identifying *Beauveria*, *Metarhizium*, and *Clonostachys* as key genera with dual nematode and insect suppressive activity. Their review advocated for the strategic deployment of such endophytes in greenhouse conditions where concurrent pest complexes are prevalent. The paper supported multifunctional endophytes as cornerstone agents in resilient integrated pest management (IPM) strategies.

Kumar and Dara (2021) provided a broad review of fungal and bacterial endophytes as microbial control agents for PPNs. Citing multiple empirical studies, they highlighted consistent suppression of *M. incognita*, *M. graminicola*, and *H. glycines* by *Fusarium*, *Trichoderma*, and *Pochonia* isolates. The authors emphasized endophytic colonization as a precondition for durable suppression and argued for prioritizing strains that demonstrate both rhizospheric competitiveness and

endophytic persistence.

Yuan et al. (2021) reported the first isolation of *Clonostachys rosea* from *Spodoptera litura* cadavers and demonstrated its dual role as an entomopathogen and endophyte. Root colonization by the isolate reduced *M. incognita* galling in tomato and induced defense gene expression related to phenylpropanoid metabolism. Their study offered direct evidence that insect-derived fungal endophytes can colonize plants and confer nematode resistance, a novel cross-trophic interaction with strong implications for sustainable pest control.

2.3.7 Novel Endophyte Discoveries and Multitrophic Interactions in the Nematode Suppressive Continuum

Nuaima et al. (2021) undertook an extensive screening of fungal isolates from cysts of the beet cyst nematode (*Heterodera schachtii*) and identified a subset of endophytic fungi capable of colonizing roots and suppressing nematode reproduction. Among these, species of *Acremonium*, *Lecanicillium*, and *Pochonia* exhibited strong juvenile mortality and egg parasitism in vitro. Notably, *P. chlamydosporia* was recovered from both the nematode cysts and host roots, indicating its dual niche adaptability. These findings suggest that naturally co-occurring fungi within nematode microhabitats can be selectively harnessed as endophyte-based biocontrol agents.

Ewing et al. (2021) analyzed the foliar microbiome of trees affected by beech leaf disease and proposed a model where endophytic fungi play roles in disease suppression or facilitation. Although nematodes were not the primary target, the work introduced the notion that nematode damage to foliage could indirectly alter endophyte communities, affecting plant health outcomes. The study highlighted the need to account for foliar endophyte dynamics in the broader discussion of nematode-plant-microbiome interactions.

Duong et al. (2021) explored bacterial endophytes isolated from coffee roots but also incidentally documented co-occurring fungal strains including *Fusarium*, *Clonostachys*, and *Trichoderma* spp. Several of these isolates demonstrated strong nematode antagonism, evidenced by reductions in *Meloidogyne incognita* egg hatching and J2 mobility. Their results underscore the potential of coffee-associated endophytes as biocontrol agents, especially in tropical perennial systems with limited chemical management options.

Tolba et al. (2021) examined the role of *Pochonia chlamydosporia* in establishing endophytic associations in barley roots. Colonization was confirmed microscopically

and via qPCR, and co-inoculation with *Meloidogyne javanica* led to a significant reduction in galling and nematode fecundity. In addition to its parasitic role, *P. chlamydosporia* was shown to trigger the expression of defense-related genes, notably those involved in phenylpropanoid biosynthesis, confirming its ability to prime plant immunity.

Prospero et al. (2021) presented a comprehensive review on the biological control of emerging forest pathogens and pests, emphasizing that fungal endophytes could play a critical role in managing complex tree diseases, including those involving nematode vectors. Though nematode suppression was not empirically demonstrated, the review supported the development of endophyte-based solutions in forestry systems increasingly threatened by invasive pathogens and soil-borne nematodes.

Chu et al. (2021) studied the combined effects of ectomycorrhizal fungi and the dark septate endophyte *Phialocephala fortinii* on pine wilt disease caused by *Bursaphelenchus xylophilus*. Inoculated seedlings exhibited enhanced biomass and lower nematode colonization, and the endophytes altered root architecture to reduce nematode ingress. The work provided compelling evidence of endophyte-mediated structural resistance as a mode of nematode suppression in woody perennials.

Ochieno (2021) critically reassessed the widely used *Fusarium oxysporum* strain V5w2, previously reported as a nematode antagonist. Through re-evaluation under Kenyan field conditions, it was found to lack significant suppressive effects on *M. incognita* and caused some root necrosis in tomato. This study serves as a cautionary note on the ecological specificity and variability in the performance of endophytic strains, underscoring the necessity of local validation before field deployment.

Xia et al. (2021) explored microbial colonization patterns in root endospheres of different rice cultivars under nematode pressure. Using high-throughput sequencing, they found that resistant cultivars supported higher abundances of *Trichoderma* and *Pochonia* spp., whereas susceptible varieties were dominated by opportunistic fungi. Their results pointed toward a selective enrichment of protective endophytes under compatible host-genotype interactions and offered a microbiome-based perspective on host resistance.

Baazeem et al. (2021) isolated endophytic fungi from desert plant species and screened them for nematicidal, antifungal, and antibacterial activities. *Trichoderma*, *Aspergillus*, and *Clonostachys* spp. demonstrated potent activity against *M. incognita* in vitro, with observed deformation and vacuolation in nematode juveniles. GC-MS

analysis revealed several polyketides and terpenoids with nematicidal properties, making these isolates promising candidates for bioactive metabolite exploration.

Kisaakye et al. (2022) evaluated the application of endophytic *Fusarium oxysporum* strains for controlling both the banana weevil (*Cosmopolites sordidus*) and *Radopholus similis*. Their dual pest management trials demonstrated significant suppression of nematode populations and improved plant vigor under greenhouse conditions. The study provided a strong case for integrated biological control using endophytes with multitrophic antagonistic capabilities, especially in perennial crops where pest complexes coexist.

2.3.8 Emerging Insights and Future Prospects for Endophytic Fungi in Nematode Management

Grabka et al. (2022) reviewed the multifaceted role of fungal endophytes in sustainable agriculture, particularly their potential in alleviating both biotic and abiotic stress. While nematode-specific data were limited, their comprehensive synthesis emphasized the increasing documentation of endophytes like *Fusarium*, *Trichoderma*, and *Pochonia* in reducing the impact of plant-parasitic nematodes (PPNs), especially in field crops. The review advocated for the development of multi-strain formulations tailored to specific agroecosystems.

Chaudhary et al. (2022) examined how endophytes mitigate biotic stress through competition, antagonism, and host immune priming. The authors highlighted specific cases where *Trichoderma harzianum* and *Beauveria bassiana* reduced *Meloidogyne incognita* and *Heterodera glycines* populations by producing hydrolytic enzymes and inducing systemic resistance. This review supported the integration of endophyte-based biocontrol within plant breeding and integrated pest management frameworks.

Ciancio et al. (2022) used 16S-ITS metabarcoding to analyze rhizosphere communities in tomato fields infested with *M. incognita*. The presence of *Pochonia*, *Purpureocillium*, and *Trichoderma* spp. correlated with nematode-suppressive soils, suggesting these endophytes play key roles in ecological suppression. The work underscored the need to characterize functional microbial consortia in nematode-infested agroecosystems.

Wang et al. (2022) demonstrated that crude extracts from *Paecilomyces variotii* prolonged the lifespan of *Caenorhabditis elegans* while suppressing *Meloidogyne incognita*. The duality of beneficial and antagonistic activity in the same fungal metabolite highlighted the evolutionary specificity in fungal-nematode interactions.

LC-MS analysis revealed the presence of gliotoxin derivatives as likely active agents.

Bashir et al. (2022) presented a conceptual framework linking the phyllosphere microbiome with nematode resistance. While direct evidence was limited, they proposed that endophyte-mediated signaling via systemic acquired resistance (SAR) pathways could contribute to belowground pathogen suppression, opening a novel avenue for nematode control via foliar inoculations.

Kisaakye et al. (2023) expanded on their earlier work by evaluating the performance of endophytic fungi against the root-burrowing nematode *Radopholus similis* in banana. Their greenhouse trials demonstrated significant improvements in plant vigor and reduction in nematode populations when *Fusarium oxysporum* and *Clonostachys rosea* were applied as root drenches. These findings validate the dual-use strategy for managing both nematodes and fungal wilt in banana systems.

Qu et al. (2023) applied metagenomic profiling to assess microbial assemblages in tomato rhizospheres under *M. incognita* stress. They found that enrichment of *Trichoderma* and *Chaetomium* spp. was associated with reduced nematode colonization. Further, transcriptome analyses of the host revealed upregulation of phenylalanine ammonia lyase (PAL) and peroxidase genes in response to endophyte treatment.

Asad et al. (2023) compiled endophytic fungi from coffee and evaluated them for plant growth promotion and pathogen suppression. Among these, *Aspergillus niger* and *Trichoderma virens* demonstrated nematostatic activity against *M. incognita*. They also produced indole acetic acid and phosphate-solubilizing compounds, reinforcing the concept of multifunctionality in endophyte utility.

Fu et al. (2023) isolated a native *Clonostachys rosea* strain from rhizosphere soils that inhibited egg hatch and J2 mobility of *M. incognita*. Greenhouse trials showed over 70% reduction in galling index when applied as seed coating. Their metabolomic profiling identified peptaibols and 2-pyrones as key bioactive compounds.

Keerthi et al. (2023) evaluated *Fusarium* endophytes from *Zingiber zerumbet* for secondary metabolite production. Their extracts showed strong nematicidal activity in vitro, including deformation and vacuolization of J2s. Chromatographic analyses indicated the presence of trichothecenes and polyketides, which may underlie their nematotoxicity.

Girardi et al. (2023) focused on *Nacobbus sp.* in South American crops and isolated nematode-antagonistic endophytes from resistant tomato cultivars. *Fusarium* and

Pochonia isolates demonstrated parasitism of nematode egg masses and reduced juvenile emergence under controlled conditions. Their ecological analysis indicated a link between host resistance and endophytic diversity.

George et al. (2024) reviewed the use of endophytic entomopathogenic fungi in managing nematodes indirectly through improved plant vigor and systemic signaling. They noted that *Beauveria bassiana* and *Metarhizium anisopliae* could also suppress root-knot nematode development when established in root tissues. The review positioned such fungi as components of a holistic, non-insecticidal strategy in integrated pest management.

Nandana and Anith (2024) investigated two endospore-forming endophytic bacteria for rhizome rot suppression in ginger, but noted co-isolation of *Fusarium* and *Clonostachys* spp. that may have contributed to reduced *Meloidogyne* damage observed in the trials. Their findings point toward beneficial synergism in endophyte consortia.

Abdelhameed et al. (2024) highlighted *Bacillus amyloliquefaciens* as a rhizosphere colonizer producing lipopeptides with nematicidal activity, often in consortia with fungal endophytes. Their review called for advanced formulation strategies combining bacterial and fungal agents for enhanced root protection.

Atiya et al. (2025) evaluated soil-derived antagonistic fungi for their influence on the nematode microbiome and found that application of *Purpureocillium lilacinum* and *Fusarium solani* led to significant shifts in nematode-associated bacterial assemblages. This microbial reprogramming could impair nematode fitness and infection capability.

Durden et al. (2025) reported that a vertically transmitted *Periglandula* endophyte in morning glory influenced nematode populations in surrounding soils, possibly by modifying plant exudate chemistry. This novel indirect mechanism of nematode suppression offers promising ecological insights.

Cazzaniga et al. (2025) conducted a landscape-scale survey of nematode-antagonistic fungi in organic and conventional agroforests, showing that higher fungal diversity in organic systems correlated with lower nematode densities. Dominant taxa included *Trichoderma*, *Metarhizium*, and *Lecanicillium*, all known for their nematicidal activity.

Sevim et al. (2025) isolated *Beauveria bassiana* and other entomopathogenic fungi from pine needles and demonstrated their systemic colonization and nematode

suppressive potential in conifer roots. Their study strengthens the case for forestry-targeted nematode management using native fungal endophytes.

2.3.9 Conclusion

Over the past three decades, fungal endophytes have emerged as a promising group of microbial antagonists in the biological control of plant-parasitic nematodes (PPNs). This review synthesizes evidence from a wide array of experimental systems, crops, and ecological contexts, revealing the functional diversity, mechanistic complexity, and biocontrol efficacy of endophytic fungi in suppressing nematode populations.

The majority of studies converged on genera such as *Fusarium*, *Trichoderma*, *Pochonia*, *Clonostachys*, and *Chaetomium*, which repeatedly demonstrated nematicidal or nematostatic effects. These fungi exert their antagonistic influence through multiple modes of action, including direct parasitism of eggs and juveniles, secretion of toxic secondary metabolites, production of hydrolytic enzymes (e.g., chitinases, proteases), competition for infection sites, and elicitation of systemic resistance in host plants. Several studies also reported structural alterations in nematode tissues, including deformation, vacuolation, and cuticular disruption, indicative of potent biochemical interference. Importantly, many of these fungi also promote plant growth, enhance nutrient acquisition, and improve tolerance to other abiotic or biotic stresses, making them viable candidates for multifunctional bioproducts.

Root colonization competency, strain specificity, and host compatibility emerged as critical determinants of biocontrol success. Gall-associated and rhizosphere-derived endophytes often showed higher efficacy, likely due to their ecological co-adaptation with the host-nematode complex. Furthermore, integration of endophytes with organic amendments, microbial consortia, or defence inducers such as chitosan has been shown to significantly enhance nematode suppression, suggesting that synergistic combinations may offer greater reliability than single-strain inoculations.

Despite promising outcomes, the translation of fungal endophytes into field-ready nematode management solutions remains limited. Key challenges include variability in performance across environmental conditions, limited understanding of endophyte persistence and dispersal in the rhizosphere, and lack of regulatory frameworks for their deployment in many regions. Recent advances in omics technologies and high-throughput screening platforms, however, are facilitating the identification of elite

strains and unravelling the molecular basis of host-endophyte-nematode interactions.

In summary, fungal endophytes represent a sustainable, ecologically compatible strategy for the management of root-knot and other plant-parasitic nematodes. Continued research into their mechanisms, formulation stability, and compatibility with existing agronomic practices will be essential to fully realize their potential as integral components of next-generation nematode management programs.

Table 2.2: Summary of fungal endophytes reported for the biological control of plant-parasitic nematodes.

Fungal Endophyte	Source Plant	Target Nematode	Observed Effects	Citation
Fusarium oxysporum	Tomato	*Meloidogyne incognita*	Reduced root galling; improved host resistance; endophytic colonisation of root cortex	Hallmann et al. (1994)
Unspecified endophyte from fescue grasses	Fescue grass	Entomopathogenic nematodes	Increased susceptibility of nematodes to fungal toxins	Grewal et al. (1995)
Unspecified cortical endophytes	tomato roots	*M. incognita*	Secondary metabolites caused high juvenile mortality in vitro	Hallmann et al. (1996)
Fusarium spp. and other rhizospheric fungi	Cotton	*M. incognita*	Chitin amendments increased nematode-suppressive fungal populations	Hallmann et al. (1999)
Unspecified endophytic fungi	Maize	Unspecified	Described potential role in multitrophic interactions; indirect suppression	Cherry et al. (1999)
Fusarium oxysporum strain Fo162	Tomato	*M. incognita*	Induced resistance; suppressed nematode development;	Sikora et al. (2007)

			enhanced plant biomass	
Piriformospora indica	Barley	*Heterodera schachtii*	Reduced cyst formation and promoted plant growth	Scholz et al. (2016)
Daldinia cf. concentrica	Maize	*M. javanica*	Volatile production inhibited J2 motility and egg hatching	Larriba et al. (2020)
Nigrospora oryzae	Rice	*M. graminicola*	Reduced gall index and nematode penetration; induced defence gene expression	Kumar et al. (2021)
Chaetomium globosum	Tomato	*M. incognita*	Produced chaetoglobosins; significantly suppressed J2 viability	Zhou et al. (2019)
Beauveria bassiana	Tomato	*M. incognita*	Caused cuticle deformation; reduced galling in greenhouse conditions	Lopez-Llorca et al. (2014)
Trichoderma harzianum	Tomato	*M. incognita*	Colonised roots endophytically; induced systemic resistance	Sharon et al. (2011)
Fusarium solani	Chickpea	*Heterodera cajani*	Reduced cyst formation and increased nodulation	Nandini et al. (2017)
Paecilomyces lilacinus	Tomato	*M. incognita*	Parasitised egg masses; reduced nematode reproduction in pot trials	Khan et al. (2012)
Acremonium spp.	Lolium perenne	*Pratylenchus penetrans*	Reduced nematode penetration and root colonisation; effect influenced by plant nutrient status	Hol et al. (2007)

Fusarium oxysporum strain 162	Tomato	*M. incognita*	Suppressed galling; increased plant biomass; in-planta resistance induction	Dababat et al. (2007)
Fusarium spp.	Barley	Heterodera spp.	Colonised root tissues; led to reduction in cyst nematode development	Mac et al. (2008)
Pochonia chlamydosporia	Wheat	Heterodera avenae	Reduced nematode abundance in rhizosphere; enhanced rhizosphere competence in certain crops	Manzanilla-L et al. (2011)
Fusarium oxysporum	Tomato	*M. incognita*	Effectiveness varied with host resistance status; potential enhancement of plant defence	Dababat et al. (2008)
Muscodor spp.	Various tropical trees	Unspecified	Produced bioactive volatiles with nematicidal potential; broad-spectrum antimicrobial and insecticidal properties	Strobel et al. (2011)
Pochonia chlamydosporia (Pc123)	Tomato	*M. incognita*	Reduced galling and nematode population density; promoted root and shoot growth	Escudero et al. (2012)
Fusarium oxysporum (non-pathogenic)	Banana	*Radopholus similis*	Endophytic colonisation reduced nematode density and root necrosis	Waweru et al. (2014)
Pochonia chlamydosporia	Tomato	*Globodera* spp. and *Meloidogyne* spp.	Demonstrated endophytic behaviour;	Zavala-Gonzalez et al. (2017)

			parasitised eggs and reduced population levels	
Fusarium spp.	Barley	*Heterodera* spp.	Root colonisation correlated with decreased nematode reproduction; enhanced plant growth	Larriba et al. (2015)
Chaetomium globosum	Cotton	*M. incognita*	Reduced nematode infestation in cotton roots; mutualistic endophyte suppressed disease severity	Zhou et al. (2019)
Daldinia cf. *concentrica*		*M. javanica*	Volatiles suppressed egg hatching and juvenile movement; strong nematicidal activity observed	Liarzi et al. (2016)
Pochonia chlamydosporia	Tomato	*M. javanica*	Parasitised eggs; parasitism enhanced by chitosan amendment	Escudero et al. (2016)
Beauveria bassiana	Grapevine	Unspecified	Systemically colonised grapevine roots; known to reduce nematode load in plant tissues	Rondot and Reineke (2017)
Muscodor spp.		Unspecified	Produced bioactive volatiles with nematicidal potential; suppressed nematode development	Mao et al. (2022)
Trichoderma citrinoviride		*M. incognita*	Reduced galling and nematode population; improved plant	Fan et al. (2022)

			growth in tomato	
Trichoderma spp. and *Clonostachys* spp.	Various crops	Unspecified PPNs	Biocontrol effects through egg parasitism and rhizosphere competence	MÃirquez-DÃivila et al. (2022)
Fungal endophytes from cotton	Cotton	Unspecified	Some isolates showed nematostatic effects in preliminary screens	Zhou et al. (2021)
Fusarium oxysporum (non-pathogenic strains)	Various crops (e.g., tomato, legumes)	*Meloidogyne* spp.	Used as bioinoculants; reduced nematode reproduction and improved plant resistance	Ochieno (2022)
Clonostachys rosea		Unspecified (nematode association)	Reported as nematode antagonist with endophytic traits	Yuan et al. (2023)
Pochonia chlamydosporia	Tomato	*M. incognita*	Demonstrated root endophyte; reduced galling and parasitised eggs	Tolba et al. (2023)

2.4. Secondary metabolites from endophytic microorganisms in Nematode Management:

Plant-parasitic nematodes (PPNs), particularly root-knot nematodes (*Meloidogyne* spp.), are among the most devastating phytopathogens in global agriculture, causing significant yield losses across a wide range of crops. The conventional use of synthetic nematicides, although initially effective, is increasingly being challenged due to concerns related to environmental toxicity, non-target effects, and the development of nematode resistance. Consequently, there is an urgent need to develop ecologically safe and biologically sustainable alternatives for nematode management.

Endophytic microorganisms, which reside asymptomatically within plant tissues, have emerged as a promising source of novel bioactive compounds for the biological

control of PPNs. These endophytes, encompassing diverse bacterial and fungal taxa, are known to produce a wide array of secondary metabolites with nematicidal, nematostatic, and egg-hatching inhibitory properties. Unlike rhizospheric microorganisms, endophytes colonize internal plant tissues and can therefore establish long-term associations, making them particularly attractive for sustained protection against endoparasitic nematodes.

The functional role of endophyte-derived metabolites in nematode suppression is multifaceted, often involving direct nematicidal action through toxins or enzyme inhibitors, interference with nematode chemotaxis, and induction of host plant defence responses. Various studies have reported that both bacterial and fungal endophytes synthesize structurally diverse metabolites such as lipopeptides, alkaloids, terpenoids, polyketides, and phenolic compounds, many of which exhibit potent activity against *Meloidogyne* spp. and other economically important nematodes.

Recent investigations have focused on isolating and characterizing these metabolites, elucidating their mode of action, and assessing their efficacy under in vitro and in vivo conditions. In particular, root gall-associated endophytes have received considerable attention due to their proximity to nematode feeding sites and their potential to modulate the local chemical environment. However, despite increasing evidence supporting the biocontrol potential of endophytic secondary metabolites, a systematic synthesis of available studies, particularly those differentiating bacterial from fungal sources, remains lacking.

In the following sections, a detailed account of the secondary metabolites produced by endophytic bacteria and fungi with proven or potential nematicidal activities is presented. The review critically evaluates the chemical diversity, biological efficacy, and underlying mechanisms of these metabolites, with an emphasis on their applicability in integrated nematode management systems.

2.4.1 Bacterial Endophyte-Derived Secondary Metabolites for Nematode Management

The use of bacterial endophytes as a source of bioactive metabolites against plant-parasitic nematodes has garnered significant attention since the early 2000s. Among the earliest studies, Siddiqui and Shaukat (2003a) demonstrated that *Pseudomonas fluorescens* strain CHA0, isolated from the rhizosphere, significantly suppressed root-knot disease in tomato by producing key antimicrobial metabolites, including 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, and hydrogen cyanide. The nematicidal

efficacy was attributed not only to the direct toxicity of these compounds but also to their ability to colonize the rhizosphere effectively, thereby outcompeting nematodes for space and resources. In a complementary study, the same group explored the modulation of DAPG production under varying environmental conditions and its impact on *Meloidogyne javanica* suppression (Siddiqui and Shaukat, 2003b). This work emphasized the importance of abiotic factors—such as soil pH, temperature, and nutrient status—in regulating metabolite biosynthesis and consequently, biocontrol efficiency.

Building on this foundation, Siddiqui et al. (2004) reported differential nematicidal efficacy among several *Aspergillus* and *Pseudomonas* species. Although primarily focused on fungal isolates, their results highlighted that bacterial strains, particularly *P. fluorescens*, retained strong suppressive effects, thereby underscoring the broad-spectrum bioactivity of endophytic *Pseudomonas*-derived metabolites. Subsequently, Siddiqui et al. (2006) delineated the mechanistic role of hydrogen cyanide production by *P. fluorescens* CHA0 in reducing nematode penetration and gall formation in tomato roots. This study provided direct evidence that cyanogenesis by bacterial endophytes is a critical trait for nematode suppression.

Parallel to these findings, Padgham and Sikora (2007) evaluated the biocontrol potential of *Bacillus megaterium*, a well-known endophyte, against *Meloidogyne graminicola* in rice. Their study demonstrated that bacterial colonization of rice roots led to a substantial reduction in nematode penetration and galling. Interestingly, their work also suggested that the bacterium could trigger systemic resistance in host plants, further amplifying its suppressive effects. This study represented one of the earliest demonstrations of endophytic bacterial metabolites being effective against *M. graminicola*, a nematode of increasing economic concern in Asian rice systems.

In a subsequent in vitro study, Mendoza et al. (2008) investigated the nematicidal potential of *Bacillus firmus*. The authors observed significant mortality in second-stage juveniles (J2s) of *M. incognita*, which was attributed to extracellular lipopeptides and proteases secreted by the bacterium. These results highlighted the potential of *B. firmus* as a microbial agent for nematode suppression, paving the way for its commercial development in bionematicide formulations.

Further expanding the chemical repertoire of bacterial metabolites, Kapsalis et al. (2008) reported that *Pseudomonas fluorescens* strains producing phenazine-1-carboxylic acid (PCA) and siderophores exhibited strong suppressive effects on *M.*

javanica and *M. incognita*. The production of PCA not only had a direct nematicidal effect but also enhanced the bacterium's competitive colonization ability in the rhizosphere. The co-production of siderophores was hypothesized to limit iron availability to nematodes and other soil pathogens, thereby contributing to the multifactorial nature of nematode suppression.

At the molecular level, Vanholme et al. (2009) characterized the *hscC* gene cluster in *Pseudomonas* spp., which governs the biosynthesis of a suite of small molecules with nematostatic activity. Through gene knock-out and complementation studies, the authors confirmed the essential role of this cluster in nematode suppression, thereby providing a genetic basis for metabolite-mediated biocontrol mechanisms.

In a related ecological context, Jousset et al. (2009) provided evidence that interactions with protozoan predators in the rhizosphere induced *Pseudomonas* spp. to upregulate the production of nematicidal metabolites. Their work suggested that predation pressure acts as a selective force shaping secondary metabolite production in soil bacteria, indirectly enhancing their biocontrol efficacy against nematodes.

Kontnik et al. (2010) employed metabolomic screening to identify novel nematicidal compounds from *Bacillus subtilis* regulated by the global transcription factor AbrB. The study not only expanded the known diversity of small-molecule nematicides but also underscored the regulatory complexity of secondary metabolism in bacterial endophytes.

A notable advancement in the bioengineering of bacterial strains for enhanced metabolite production was demonstrated by Wang et al. (2011), who reported that the antibiotic activity of *Xenorhabdus nematophila* against *Meloidogyne incognita* could be significantly augmented through promoter engineering. By optimizing the expression of biosynthetic gene clusters, the authors achieved elevated production of key secondary metabolites, which exhibited increased nematicidal activity. This study underscored the utility of genetic manipulation in improving the efficacy of entomopathogenic bacteria for nematode control.

In a subsequent investigation, Yoon et al. (2012) characterized a strain of *Streptomyces cacaoi* that suppressed *M. incognita* infection through the secretion of chitinase and the production of actinomycin D and resistomycin. Both compounds are known for their antibiotic properties, but this work provided the first evidence of their nematicidal effects in planta. The presence of chitinase activity further suggested synergistic mechanisms involving degradation of nematode eggshells or cuticle layers.

Kumar et al. (2012) reported the purification of bioactive stilbenes from a *Bacillus* sp. strain isolated from rhizosphere soil. The purified compounds showed potent nematicidal activity against *M. incognita*, with significant mortality of juveniles observed in vitro. The authors suggested that the structural features of the stilbenes, particularly the hydroxyl and methoxy substitutions, were critical to their biological activity.

Further exploring the metabolome of *Bacillus* spp., Kumar et al. (2013) isolated a group of proline-based cyclic dipeptides from an endophytic strain associated with entomopathogenic nematodes (EPNs). These diketopiperazines exhibited strong nematicidal activity and were hypothesized to disrupt neuromuscular coordination in nematodes. The study demonstrated that bacterial endophytes derived from nematode symbionts can be valuable sources of novel bioactive metabolites.

In the context of cereal crop protection, Elek et al. (2013) investigated the use of hydroxamic acids derived from tetraploid wheat lines and endophytic bacterial fermentation. Although primarily a plant-centered study, the authors confirmed that certain bacterial strains enhanced the production of nematicidal hydroxamates, which acted by interfering with nematode root invasion. This finding provided indirect support for the role of bacterial metabolism in modulating plant-derived chemical defences.

Müller et al. (2013) demonstrated that protozoan grazing in the rhizosphere acted as a selective pressure favoring bacterial populations capable of producing nematicidal secondary metabolites. Using *Pseudomonas* spp. as a model, they showed that predation enhanced the frequency of strains with strong biocontrol properties, including those producing DAPG and phenazines. This ecological feedback loop illustrated how environmental interactions can drive microbial evolution towards enhanced nematode suppression.

In an agroecological framework, D'Addabbo et al. (2014) evaluated the synergistic effects of biocidal plant extracts and endophytic bacteria on nematode control. The study indicated that metabolite profiles of bacterial strains varied depending on the plant species used for co-cultivation, suggesting that specific plant–microbe interactions can be harnessed to induce the production of nematicidal metabolites.

Fang et al. (2014) isolated the *Xenorhabdus nematophila* strain TB, which secreted several bioactive compounds with inhibitory effects on *M. incognita*. The culture filtrates showed significant J2 mortality, egg-hatching inhibition, and reduced gall

formation in tomato roots. Their results reinforced the efficacy of *Xenorhabdus*-derived metabolites in integrated nematode management strategies.

Ballestriero et al. (2014) examined the antinematode activity of violacein, a purple pigment produced by *Chromobacterium violaceum*. The compound, known for its broad-spectrum antimicrobial properties, induced severe morphological abnormalities in *M. incognita* J2s, including vacuolization and cuticle deformation. Their study also explored the regulatory circuits controlling violacein biosynthesis, which could be targeted for enhanced metabolite production.

Khatamidoost et al. (2015) investigated native Iranian *Pseudomonas* strains and found several isolates capable of producing siderophores, phenazines, and lytic enzymes with strong nematicidal activity. Their field trials demonstrated that seed coating with metabolite-producing strains significantly reduced nematode infestation in cucumber and tomato, validating the potential for practical application of native bacterial endophytes.

A marine isolate of *Bacillus firmus* strain YBf-10 was reported by Xiong et al. (2015) to exhibit both systemic nematicidal activity and strong rhizosphere colonization potential in tomato plants. Through a series of bioassays, the study demonstrated that culture filtrates of this strain significantly reduced *Meloidogyne incognita* J2 penetration and gall formation. The authors also indicated that the bacterium produced a set of heat-stable compounds that likely function as key nematicidal agents, although precise chemical characterization was not provided.

Anju et al. (2015) purified and identified a bacteriocin-like peptide produced by a *Bacillus* sp. N strain, symbiotically associated with entomopathogenic nematodes (EPNs). The peptide demonstrated significant nematicidal activity against *M. incognita*, causing over 80% J2 mortality at low concentrations. This study highlighted the potential of insect-pathogenic bacterial symbionts as reservoirs of bioactive metabolites with cross-target effects on nematodes.

Genomic insights into such symbionts were offered by Abebe-Akele et al. (2015), who sequenced and analyzed a strain of *Photorhabdus luminescens*, another EPN-associated bacterium. Their study identified a diverse array of biosynthetic gene clusters coding for polyketides, nonribosomal peptides, and hybrid metabolites with potential nematicidal properties. Though the study did not directly evaluate nematode suppression, it laid critical groundwork for understanding the genomic basis of metabolite-mediated biocontrol.

Correa-Cuadros et al. (2016) examined the interaction of the fungus *Metarhizium anisopliae* with root-knot nematodes in the presence of co-inoculated bacterial endophytes. The study found that certain bacterial metabolites enhanced the nematicidal activity of the fungus, suggesting synergistic interactions between fungal and bacterial endophytes, potentially mediated through metabolite induction.

In a broader phytochemical context, Singh et al. (2016) reviewed the medicinal and aromatic plant microbiomes, emphasizing the role of rhizospheric and endophytic bacteria in producing bioactive metabolites. The review highlighted several reports of *Bacillus*, *Pseudomonas*, and *Streptomyces* strains secreting alkaloids, cyclic peptides, and organosulfur compounds with nematicidal activity, thereby identifying medicinal plants as valuable sources of endophyte-associated biocontrol agents.

Hazir et al. (2016) compared the relative potency of culture supernatants from different *Xenorhabdus* and *Photorhabdus* strains. Their study revealed that secondary metabolites from *X. bovienii* were particularly effective against *M. incognita*, with over 90% juvenile mortality achieved in vitro. This work underscored the strain-specific nature of metabolite bioactivity and the importance of selecting high-yielding isolates for nematode management.

The metabolite profile of two symbiotic bacteria of *Heterorhabditis bacteriophora* was characterized by Liao et al. (2017), who identified several nematicidal toxins, including xenocoumacin and proline-rich peptides. These compounds induced structural deformities in nematode cuticles and inhibited hatching. Their findings provided mechanistic insights into how bacterial metabolites disrupt nematode physiology.

In a regional study, Ahmed (2017) screened indigenous *Pseudomonas* isolates from Bangladesh for biocontrol potential against *M. incognita*. Several isolates exhibited nematicidal activity, with culture filtrates reducing gall formation by up to 75% in greenhouse trials. Though the specific metabolites were not identified, the results emphasized the biogeographical diversity of bacterial strains with nematode-suppressive properties.

Zheng et al. (2017) sequenced the genome of *Fictibacillus arsenicus* strain NBRC 100065 and identified genes encoding for biosynthesis of various secondary metabolites, including lipopeptides and bacteriocins. While functional assays were not conducted, the presence of these gene clusters suggested potential for nematicidal activity, warranting future biochemical validation.

Tiwari et al. (2017) evaluated co-inoculation strategies involving metabolite-producing *Bacillus* and *Pseudomonas* strains in medicinal plants. Their results demonstrated that metabolite synergy between co-inoculated strains significantly enhanced nematode suppression and also improved plant biomass. The authors proposed that combining complementary secondary metabolites can enhance both biocontrol efficacy and plant health outcomes.

Stock et al. (2017) provided a comprehensive overview of secondary metabolites secreted by symbiotic bacteria of *Heterorhabditis* and *Steinernema* nematodes, particularly *Photorhabdus* and *Xenorhabdus* species. These bacteria produce a wide spectrum of bioactive compounds, including stilbenes, xenocoumacins, and benzylideneacetone derivatives, several of which demonstrated nematicidal and ovicidal effects against *Meloidogyne* spp. The authors highlighted the potential of these metabolites not only for nematode control but also for broader biopesticidal applications.

In a more applied study, Abbasi et al. (2017) assessed the extracellular metabolites of *Bacillus subtilis* and *B. cereus* isolates for their nematicidal potential. Culture filtrates exhibited strong suppression of *M. javanica*, with notable reductions in egg hatching and gall formation. The nematicidal activity was attributed to lipopeptides and proteolytic enzymes, although no specific compound identification was conducted.

Flury et al. (2017) demonstrated that *Pseudomonas* spp. strains producing cyclic lipopeptides such as orfamides and viscosin exhibited nematicidal activity against *Meloidogyne incognita* and insect pests. Their work confirmed that these compounds act synergistically with other secondary metabolites to enhance root colonization and suppress both nematode infection and disease symptoms.

Yan et al. (2017) provided molecular insights into the regulation of DAPG biosynthesis in *P. fluorescens*, showing that the transcriptional regulator PhlH modulates metabolite production based on environmental stimuli. This regulation affects the timing and concentration of DAPG in the rhizosphere, influencing its nematicidal effectiveness. Such mechanistic understanding is crucial for engineering strains with optimized metabolite output.

A broader perspective on the commercial development of bacterial bionematicides was presented by Engelbrecht et al. (2018), who reviewed formulation strategies, application protocols, and regulatory frameworks for *Bacillus*-based biopesticides.

The review cataloged key metabolites such as iturins, surfactins, and subtilin, detailing their modes of action and challenges related to shelf life and field efficacy.

Yuen et al. (2018) explored the biocontrol potential of *Lysobacter enzymogenes* strain C3 against cyst nematodes. The bacterium produced extracellular enzymes and heat-stable metabolites that disrupted nematode cuticles and inhibited hatching. These findings highlighted non-*Bacillus* genera as important but underexplored sources of nematicidal secondary metabolites.

Hazir et al. (2018) further characterized the thermo-stability, dose-responsiveness, and shelf-life of *Xenorhabdus*-derived metabolites under field-relevant conditions. Their work demonstrated that certain formulations remained active for extended periods under high temperatures, making them suitable for deployment in tropical agricultural systems.

Dreyer et al. (2018) investigated the genus *Xenorhabdus* as a source of novel bioactive compounds. Through LC-MS analysis and bioassays, the authors identified previously uncharacterized peptides with nematicidal effects, including xenortides and fabclavines. This study emphasized the untapped chemical diversity of bacterial endophytes associated with entomopathogenic nematodes.

Hussain et al. (2018) evaluated the synergistic use of the endophytic fungus *Lecanicillium muscarium* and bacterial bioformulations against *M. incognita*. The combined application significantly reduced galling and egg mass production, suggesting that bacterial metabolites enhanced the efficacy of fungal colonization and systemic plant defence activation.

Shanmugam et al. (2018) sequenced and functionally annotated the genome of *Bacillus firmus* I-1582, a strain known for its nematicidal activity. The study identified biosynthetic gene clusters encoding lipopeptides and peptidoglycan hydrolases, which are implicated in the degradation of nematode eggshells and cuticle structures. These findings provided a genomic basis for the observed suppression of *Bursaphelenchus xylophilus*, suggesting that the strain could also be effective against root-knot nematodes.

In a plant defense-focused study, De Benedictis et al. (2018) revealed that the knockout of the phytochelatin synthase (PCS1) gene in *Arabidopsis thaliana* led to heightened susceptibility to *Meloidogyne incognita*. Co-inoculation with beneficial endophytes restored nematode resistance, suggesting that bacterial metabolites may interact with host glutathione and PCS-dependent pathways to induce systemic

resistance.

Khanna et al. (2019) demonstrated the efficacy of rhizobacterial inoculation under salt stress in suppressing *M. incognita* in *Withania somnifera*. *Bacillus subtilis* and *Pseudomonas fluorescens* strains enhanced plant biomass while significantly reducing nematode infestation. Their effects were attributed to both the secretion of nematicidal compounds and the elicitation of plant defense genes related to salicylic acid and jasmonic acid signaling.

Liu et al. (2019) characterized *Lysobacter capsici* NF87-2, which secreted extracellular chitinases and proteases. In in vitro assays, culture filtrates of this strain significantly reduced J2 viability and egg hatching rates. Notably, the bacterium exhibited strong rhizosphere competence, enabling it to persist around root zones and maintain metabolite efficacy over time.

Xia et al. (2019) identified *Bacillus halotolerans* strain LYSX1 as a potent nematode antagonist. The strain produced lipopeptides that induced systemic resistance in tomato and cucumber against *M. incognita*. Upregulation of peroxidase and polyphenol oxidase activities was observed, suggesting dual action through metabolite-mediated nematicidal activity and priming of plant defenses.

Lenz et al. (2019) explored cyanobacterial peptides—anabaenopeptins and cyanopeptolins—for their systemic effects on plant defense pathways. Although not typical rhizobacteria, the study provided compelling evidence that these metabolites, when applied exogenously, suppressed *M. incognita* infection by mimicking endogenous elicitors of systemic resistance.

Rabbee et al. (2019) reviewed the multifunctional role of *Bacillus velezensis*, which produces an array of metabolites including bacillomycin, difficidin, and macrolactin. These compounds were associated with nematode mortality and inhibition of egg hatching. The strain's versatility in promoting plant growth and suppressing pathogens makes it a strong candidate for integrated biological control.

Viljoen et al. (2019) screened 27 rhizobacterial isolates for nematicidal activity and identified several *Bacillus* and *Pseudomonas* strains with consistent suppression of *M. incognita*. The study also emphasized the role of these bacteria in promoting root development, thereby reducing nematode colonization through indirect mechanisms.

Hashem et al. (2019) reported that *Bacillus subtilis* induces resistance in tomato by activating antioxidant enzymes and phenylpropanoid pathways. The bacterium's culture filtrate also directly reduced J2 viability, indicating that metabolite production

complemented the systemic acquired resistance mechanisms triggered in the host.

Khan et al. (2020) reviewed the potential of endophytic fungi such as *Trichoderma* spp. in secreting secondary metabolites with nematicidal properties; however, their focus included bacterial symbionts that complemented fungal bioactivity. The study emphasized how synergistic interactions between bacterial and fungal endophytes can amplify the efficacy of metabolite-based suppression of *Meloidogyne* spp., and suggested that these consortia be explored in depth for integrated nematode management strategies.

Booysen and Dicks (2020) provided a conceptual review proposing that the future of antibiotics, particularly those effective against soil-borne pathogens including nematodes, may lie in secondary metabolites of probiotic bacteria such as *Bacillus subtilis* and *Pseudomonas fluorescens*. While the paper was not specific to nematodes, it emphasized the structural diversity of endophyte-derived lipopeptides and suggested repurposing them for agro-biological control.

Manjula et al. (2020) investigated the effect of host diet on nematode physiology and survival in the context of gut microbiome shifts. Although cantered on insect-parasitic nematodes, the study revealed that *Bacillus* metabolites significantly affected nematode life stages by altering gut microbiota balance and immune signalling. These results suggested potential parallels for plant-parasitic nematodes through endophyte-mediated gut disruption.

Susic et al. (2020) performed whole genome sequencing of *Bacillus firmus*, a strain known for nematicidal activity, and identified conserved biosynthetic gene clusters encoding proteases, chitinases, and siderophores. Comparative analysis revealed strong overlap with known nematotoxic pathways, reinforcing the genetic basis for its efficacy in biological control.

Machado et al. (2020) employed an evolutionary approach to engineer bacterial symbionts of entomopathogenic nematodes (*Xenorhabdus* and *Photorhabdus*) for enhanced production of nematicidal metabolites. These engineered strains demonstrated superior suppression of *M. incognita* and improved host colonization, offering a novel synthetic biology avenue for tailoring metabolite output.

Topalovic et al. (2020) explored how disease-suppressive soils enable cooperative interactions between plant roots and resident microbiota, particularly *Pseudomonas* spp. and *Bacillus* spp. The study reported that these bacteria produce secondary metabolites such as phenazines and lipopeptides, which work in concert to reduce

nematode invasion and stimulate plant immunity.

Takeuchi et al. (2020) discovered a novel small protein, related to antibiotics, from root-associated *Pseudomonas* spp. This compound, produced under rhizosphere-mimicking conditions, exhibited nematicidal effects against *M. incognita* and significantly suppressed juvenile mobility. Structural studies suggested the molecule acts by destabilizing nematode cuticular membranes.

da Silva et al. (2020) reviewed the diversity of entomopathogenic bacteria and their symbiotic relationships with nematodes, identifying over 30 metabolite classes with nematicidal potential. Notably, strains of *Xenorhabdus szentirmaii* were found to produce novel antimicrobial peptides with cross-efficacy against both insect and nematode targets.

Gislason and de Kievit (2020) investigated the fine line between beneficial and pathogenic roles of *Pseudomonas brassicacearum*, emphasizing that while the strain promotes plant health and secretes nematicidal metabolites such as 2,4-DAPG and pyoluteorin, environmental triggers may modulate virulence expression. The findings highlighted the importance of context-specific regulation of metabolite production.

Olmedo-Juárez et al. (2020) analyzed the secondary metabolite content of *Acacia farnesiana* pods and their rhizosphere microbial community. They reported that *Bacillus* strains associated with this environment produced bioactive compounds that significantly reduced *M. incognita* hatching and gall formation. The authors proposed that leguminous host environments may act as reservoirs of potent nematicidal bacterial endophytes.

Le Guillouzer et al. (2020) investigated the global regulatory network governed by ScmR in *Burkholderia thailandensis*, a saprophytic soil bacterium. ScmR was shown to control the biosynthesis of a suite of secondary metabolites including polyketides and NRPS-derived compounds, several of which exhibited nematicidal activity. Their findings provided insight into how quorum sensing systems can be manipulated to enhance bioactive metabolite production in beneficial endophytes.

He et al. (2021) characterized a hybrid polyketide-terpenoid biosynthetic gene cluster from *Streptomyces* spp. that produced a novel nematicidal compound exhibiting high potency against *Meloidogyne incognita*. LC-MS and NMR-based analyses revealed unique structural motifs contributing to the compound's bioactivity. This study demonstrated the value of mining hybrid biosynthetic systems for next-generation nematocidal agents.

Baazeem et al. (2021) assessed the in vitro activity of a *Bacillus subtilis* strain isolated from desert rhizospheres. The culture filtrate exhibited broad-spectrum antimicrobial effects, including strong nematicidal action against *M. javanica*. The nematode suppression was attributed to the combined activity of lipopeptides and organic acids, and was validated through SEM imaging of cuticular degradation.

Sood et al. (2021) provided a comprehensive review on plant defense mechanisms under pathogen stress and highlighted the role of rhizobacteria such as *Bacillus* and *Pseudomonas* spp. in enhancing systemic resistance. They emphasized that secondary metabolites like phenazines, siderophores, and VOCs produced by these bacteria not only contribute to nematode suppression but also modulate host immunity at the molecular level.

Hassan et al. (2021) utilized LC-MS analysis to profile secondary metabolites produced by nematode-antagonistic *Bacillus* spp. strains isolated from suppressive soils. The culture filtrates were enriched in surfactins and fengycins, which showed strong inhibition of *M. incognita* hatching and gall formation. The study also reported enhanced colonization and persistence of these strains in the rhizosphere.

Booysen et al. (2021) conducted metabolic profiling of *Xenorhabdus* spp. and demonstrated that metabolite expression was tightly regulated by nutrient cues and population density. The nematicidal effects were particularly pronounced under nitrogen-limiting conditions, which induced production of peptidic toxins and cyclic antimicrobial peptides.

Vicente-Díez et al. (2021) evaluated entomopathogenic nematodes as dual-purpose biocontrol agents against *Lobesia botrana* and *Meloidogyne* spp. They showed that symbiotic *Steinernema*-associated bacteria secreted nematicidal compounds such as xenocoumacins and nematophins that disrupted root invasion by *M. incognita*, expanding their application scope in integrated pest management.

Kamatham et al. (2021) reviewed advances in engineering crop plants for interaction with metabolite-producing rhizobacteria. They proposed that the efficacy of biocontrol can be enhanced by selecting bacterial strains with a known repertoire of nematicidal secondary metabolites and engineering plant traits to support microbial colonization.

Kang et al. (2022) reported the nematicidal activity of aureonuclemycin, a nucleoside antibiotic produced by endophytic *Streptomyces aureus*. The compound inhibited *M. incognita* J2 motility and egg hatching in vitro. The study also explored

the gene cluster responsible for aureonuclemycin biosynthesis, suggesting potential for heterologous expression in rhizobacterial hosts.

Cimen et al. (2022) provided a detailed account of natural product biosynthesis in *Photorhabdus* and *Xenorhabdus*, bacterial symbionts of entomopathogenic nematodes. They identified several metabolite classes, including xenocoumacins, fabclavines, and stilbenes, with strong nematicidal properties. The study emphasized the combinatorial nature of metabolite action, including nematostatic effects and cuticle degradation, highlighting their relevance in dual pest-pathogen suppression strategies.

Aioub et al. (2022) assessed the co-application of plant growth-promoting rhizobacteria (PGPR) with organic amendments for managing root-knot nematode infestations. *Bacillus subtilis* and *Pseudomonas fluorescens* isolates produced lipopeptides and siderophores that inhibited *M. incognita* hatching and significantly improved plant health. The authors noted that metabolite-mediated suppression was more effective when integrated with organic soil inputs.

Lee et al. (2022) investigated root-associated *Bacillus* and *Pseudomonas* strains isolated from tomato rhizospheres. The strains produced volatile organic compounds (VOCs), such as 2,3-butanediol and dimethyl disulfide, which impaired *M. incognita* mobility and reduced gall formation. Their findings support the use of VOCs as non-contact biocontrol tools for nematode management in greenhouse systems.

de Carvalho et al. (2022) explored native *Bacillus* spp. from Brazilian soils and characterized their nematicidal activity against *M. javanica*. Several isolates produced cyclic lipopeptides and exoproteases, with significant in vitro and in vivo nematode mortality. Notably, metabolite production was enhanced under iron-limiting conditions, suggesting regulatory overlap between siderophore biosynthesis and nematode suppression pathways.

Eugui et al. (2022) presented a combined chemical–biological approach using glucosinolate-rich residues and bacterial endophytes for *Meloidogyne* control. They reported that glucosinolate hydrolysis products and bacterial metabolites acted synergistically, enhancing nematicidal activity while promoting plant resistance. This study supported integrating host plant secondary metabolites with endophyte-derived compounds.

Sun et al. (2022) isolated *Lysinimonas* M4 from rhizosphere soils and purified two novel nematicidal compounds. Both molecules demonstrated strong activity against

M. incognita J2s, causing vacuolization and motility arrest. Structural characterization suggested these are modified phenolic acids with potential for formulation development.

Zúñiga-Serrano et al. (2022) evaluated the bioactivity of *Yucca schidigera* extracts and their associated microbiota. While the plant extract had moderate nematicidal effects, the endophytic bacterial community—particularly *Bacillus licheniformis*—was responsible for significant suppression of *M. incognita*, mediated by production of saponins and antimicrobial peptides.

Khan and Kim (2022) studied the interaction between *Beauveria bassiana* and *Xenorhabdus* metabolites. The latter enhanced fungal virulence against nematodes by inducing upregulation of protease and chitinase genes in the fungus. This cross-kingdom interaction suggested potential for formulating microbial consortia that combine fungal colonization with bacterial metabolite action.

Silva et al. (2022) reviewed the biocontrol potential of actinomycetes, particularly *Streptomyces* spp., in suppressing soil-borne pathogens including nematodes. They catalogued nematicidal compounds such as avermectins, polyene macrolides, and novel diketopiperazines, and proposed their use as seed coatings and soil amendments.

Weng et al. (2022) highlighted the indirect role of arbuscular mycorrhizal fungi (AMF) in enhancing colonization and persistence of metabolite-producing rhizobacteria. Their work showed that AMF inoculation led to higher expression of bacterial biosynthetic gene clusters associated with nematicidal secondary metabolite production, thereby boosting biocontrol efficacy in tomato.

Hu et al. (2022) demonstrated the efficacy of *Bacillus velezensis* strain Bv-25 against *Meloidogyne incognita* in tomato. The bacterium produced a suite of bioactive compounds—including surfactins and polyketides—that significantly reduced J2 motility and gall formation. Soil colonization studies revealed long-term persistence of the strain in the rhizosphere, supporting its potential for field application.

Srivastava et al. (2022) evaluated *Steinernema carpocapsae*-associated bacterial symbionts as biocontrol agents against *M. incognita*. Culture filtrates from these symbiotic bacteria induced high mortality in nematode juveniles and inhibited egg hatching. The activity was attributed to secondary metabolites such as xenocoumacins and stilbene derivatives.

Chaudhary et al. (2022) reviewed the multifunctional roles of bacterial endophytes

in mitigating biotic stresses. The authors identified key metabolites—including antibiotics, lipopeptides, and enzymes—from *Bacillus*, *Streptomyces*, and *Pseudomonas* spp. that contribute to nematode suppression via cuticle degradation, interference with signaling, and triggering of plant defense responses.

Clough et al. (2022) combined in vitro and in planta screening methods to identify effective biocontrol strains against *Meloidogyne hapla*. Among the screened bacteria, several *Pseudomonas fluorescens* and *Bacillus subtilis* isolates showed strong metabolite-mediated suppression of galling in lettuce. The authors emphasized the importance of dual-screening approaches to validate bioactivity under realistic conditions.

Dimkic et al. (2022) performed a comparative metabolomic analysis of *Bacillus* and *Pseudomonas* strains, correlating their metabolite profiles with nematicidal potency. Secondary metabolites such as iturin A, pyoluteorin, and 2,4-diacetylphloroglucinol (DAPG) were significantly associated with high in vitro juvenile mortality. Their findings provided a predictive framework for selecting elite strains based on chemical markers.

Baiome et al. (2022) identified volatile organic compounds (VOCs) produced by endophytic bacteria that inhibited both fungal pathogens and *M. incognita*. VOCs such as dimethyl disulfide, benzaldehyde, and 1-octen-3-ol showed broad-spectrum bioactivity. Nematodes exposed to these VOCs exhibited disrupted motility and failure to locate root tips.

Kusakabe et al. (2023) showed that secondary metabolites derived from *Photorhabdus* spp. reduced the virulence of *M. incognita* on tomato by directly inhibiting J2 mobility and indirectly inducing plant defense. The nematicidal effect was confirmed using split-root assays, indicating that systemic resistance was likely involved.

Chavarria-Quicaño et al. (2023) reported on a native isolate of *Bacillus paralicheniformis* from Peruvian potato fields. This strain produced lipopeptides and proteases that suppressed *M. chitwoodi*, a quarantine pest. In addition to in vitro assays, the bacterium significantly improved tuber yield and reduced nematode reproduction under greenhouse conditions.

Liu et al. (2023) demonstrated that *M. incognita* secretes effectors that modulate host immunity to favor infection. However, treatment with *Bacillus cereus* culture filtrate reversed effector-induced suppression of defense genes. This antagonistic

interaction was mediated by bacterial metabolites that activated salicylic acid and jasmonate signaling.

Khan et al. (2023) synthesized urea-doped *Alnus nitida* biochar nanoparticles with nematicidal properties. Interestingly, the enhanced efficacy was linked not just to the material but to enriched colonization by native *Bacillus* spp. capable of producing proteases and siderophores. This study bridged material science and microbial ecology to propose a sustainable nematode control strategy.

Moshe et al. (2023) conducted comparative genomics on *Bacillus cereus* sensu lato strains with known biocontrol potential. They identified several conserved biosynthetic gene clusters responsible for secondary metabolite production, including nonribosomal peptides and bacteriocins. Functional annotation revealed strong correlation between the presence of these clusters and suppression of *Meloidogyne incognita* in tomato bioassays.

Kossmann et al. (2023) engineered bacterial strains to overproduce hydroxylated prodiginines, a class of tripyrrole secondary metabolites with reported cytotoxicity. Tailored derivatives demonstrated nematicidal activity at micromolar concentrations, suggesting potential for development of next-generation metabolite-based bionematicides.

Guzmán-Guzmán et al. (2023) provided a broad review on the role of microbial biocontrol agents, particularly *Trichoderma* spp., in plant-parasitic nematode suppression. While fungi were the focus, they emphasized that synergistic bacterial-fungal interactions—especially with metabolite-producing *Bacillus* spp.—enhanced biocontrol efficacy by expanding the spectrum of antagonistic compounds.

Takeuchi et al. (2023) elucidated how the amino acid glutamate regulates chitinase activity in *Pseudomonas protegens*. The study revealed that glutamate acts as a signaling molecule, enhancing the expression of genes encoding chitinolytic enzymes involved in nematode cuticle degradation. This metabolic regulation offers opportunities for optimizing biocontrol agent performance through nutrient-based strategies.

Wang et al. (2023) isolated asperphenalenones from *Clonostachys rosea* and demonstrated their nematicidal effects. Although the focus was on fungal metabolites, the study reinforced the role of microbial secondary metabolites in targeting nematode life stages, particularly through membrane disruption and inhibition of embryogenesis.

Greff et al. (2023) examined the biocontrol activity of essential oil-producing plants and their associated rhizobacteria. Co-isolation of endophytic *Bacillus* spp. from aromatic plants revealed production of lipopeptides and volatile fatty acids with nematicidal activity. These findings suggest a complementary relationship between plant secondary metabolites and rhizobacterial biocontrol.

Zhou et al. (2023) profiled actinobacterial isolates from diverse soils, identifying numerous *Streptomyces* spp. producing nematicidal compounds such as avermectins, anisomycins, and geldanamycins. The authors correlated metabolite diversity with suppression of *M. incognita* in tomato and predicted metabolite production potential using antiSMASH genome mining.

Rocha et al. (2023) evaluated *Pseudomonas*, *Bacillus*, and *Serratia* strains isolated from nematode-infested roots. The bacterial culture filtrates reduced nematode motility and egg hatching. GC-MS analysis revealed multiple nematicidal metabolites including benzoic acid, 3-hydroxybutyric acid, and decanoic acid, confirming broad-spectrum metabolic activity.

Sun et al. (2024) engineered a *Pseudomonas protegens* strain by overexpressing a global regulatory gene to enhance production of nematicidal secondary metabolites. This manipulation led to increased levels of 2,4-DAPG and pyoluteorin, both known for their strong inhibition of *M. incognita*. Greenhouse trials demonstrated improved plant health and reduced galling.

Vera-Morales et al. (2024) assessed a consortium of bacterial and fungal endophytes isolated from maize roots. Combined metabolite profiling revealed diverse nematicidal compounds including indole derivatives, VOCs, and phenylpropanoids. The study suggested that multi-microbial consortia could offer enhanced and sustained suppression of nematode populations in the rhizosphere.

Poria et al. (2024) characterized a multistress-tolerant strain of *Pseudomonas aeruginosa* SS6 capable of producing phenazines and hydrogen cyanide. The strain showed high efficacy against *M. incognita* under saline and drought conditions, suggesting its robustness for use in climate-resilient biocontrol strategies.

Mian et al. (2024) published the complete genome of *Bacillus velezensis* UFV07, revealing a wide array of biosynthetic gene clusters encoding lipopeptides and polyketides. Functional assays confirmed the nematicidal activity of surfactin and fengycin families, reinforcing this species as a versatile biocontrol candidate.

Jin et al. (2024) reported that suppression of the transcriptional regulator HexA in

Xenorhabdus hominickii enhanced production of nematicidal metabolites. The modified strain demonstrated increased virulence against *M. incognita*, highlighting regulatory tuning as a strategy to boost metabolite output.

Sushma et al. (2024) reviewed the role of entomopathogenic nematode-associated bacteria in PPN suppression. They detailed how bacterial metabolites like xenocoumacins, lipases, and secondary phenolics disrupt nematode physiology and promote rhizosphere colonization.

Krithika et al. (2024) studied *Ochrobactrum bacteraemia* from nematode-infested rhizospheres, identifying β-lactone and diketopiperazine derivatives as novel nematicidal compounds. Their findings expand the range of beneficial bacteria beyond the typical *Bacillus–Pseudomonas* paradigm.

Ayaz et al. (2024) compiled metabolomic data from multiple bacterial taxa including *Streptomyces*, *Bacillus*, and *Xenorhabdus*, noting a high diversity of nematicidal compounds. The authors proposed integrating metabolomic fingerprints with genomic tools to develop predictive models of biocontrol efficacy.

Palma et al. (2024) performed genomic analysis of *Xenorhabdus szentirmaii*, identifying unique NRPS-PKS hybrid clusters producing antimicrobial peptides with nematicidal potential. This expanded understanding of symbiont chemical ecology for future bioformulation efforts.

Yang et al. (2024) explored the metabolic networks in *Bacillus subtilis* that control secondary metabolite production under different stress regimes. Their findings suggested that optimizing environmental inputs could tune metabolite secretion profiles for nematode suppression.

Lam et al. (2024) demonstrated that the transcriptional regulator LrhA in *X. nematophila* modulates virulence and metabolite biosynthesis. LrhA-deficient strains produced higher levels of nematotoxic peptides, suggesting a target for biotechnological enhancement.

Arumugam et al. (2024) tested cell-free supernatants of *Photorhabdus luminescens* against *M. incognita* and found that nematode reproduction was inhibited through disruption of egg hatching and cuticle structure. Their results confirmed that extracellular metabolites alone can exert strong biocontrol effects.

Ganeshan et al. (2024) investigated endophytes from guava roots and isolated several strains of *Bacillus firmus* and *Serratia marcescens* that reduced nematode load and promoted plant growth. The study linked nematode suppression to secretion

of glucanolytic enzymes and secondary metabolites.

Diab et al. (2024) reviewed actinomycetes as sustainable sources of biopesticides. Avermectins, produced by *Streptomyces avermitilis*, were highlighted for their potency against a broad range of PPNs, and the authors called for renewed efforts in actinomycete screening for agricultural applications.

Kunda et al. (2024) studied rice root gall microbiomes and found that nematode infection altered the composition of endophytic bacteria. Gall-associated *Bacillus* spp. produced higher levels of nematicidal compounds than their counterparts from uninfected roots.

Shehata et al. (2024) identified *Bacillus velezensis* strains producing antifungal and nematicidal lipopeptides. They reported synergistic suppression of *M. incognita* and *Fusarium* spp., suggesting that such strains are ideal for integrated root disease management.

Puza et al. (2024) examined the broader impact of *Xenorhabdus* metabolites on insect and nematode pests. Their work emphasized dose-dependent specificity and called for formulation strategies that balance efficacy with minimal ecological disruption.

Settu et al. (2024) sequenced the genome of a *Bacillus firmus* isolate from sugarcane and annotated gene clusters for nematicidal lipopeptides. The isolate also exhibited plant growth-promoting traits, highlighting its dual benefit for crop productivity.

Weinkove (2024) provided a perspective on how folate-producing bacteria affect aging in nematodes (*C. elegans*). Though not directly applicable to PPNs, the work illustrates the systemic influence of bacterial metabolites on nematode development.

Deeikshana et al. (2025) offered a comprehensive review of secondary metabolites from microbial sources, emphasizing recent advances in structure elucidation, mode-of-action studies, and bioformulation strategies against nematodes.

Shakeel et al. (2025) highlighted saponins—plant secondary metabolites—as synergists with bacterial metabolites. Co-treatment of *M. incognita* with saponins and *Bacillus* filtrates resulted in enhanced J2 mortality and reduced galling.

Wang et al. (2025) proposed an integrated management approach using rhizobacteria and resistance inducers for combined control of *Ralstonia solanacearum* and *M. incognita*. Their data showed metabolite-mediated resistance priming in tomato roots.

Zhao et al. (2025) reported on *Microbacterium maritypicum* for *Heterodera glycines* control in soybean. The strain produced glucosamine derivatives that inhibited cyst formation, and metabolite production was confirmed through LC-MS.

Saha et al. (2025) reviewed microbial entomopathogens with dual nematicidal potential, citing *Beauveria*, *Bacillus*, and *Xenorhabdus* species. Their work provided an updated resource for bioprospecting efforts targeting dual-host microbes.

Patil et al. (2025) characterized an endophytic *Bacillus subtilis* from tomato that produced extracellular metabolites reducing *M. incognita* egg hatching and J2 viability. Inoculation also enhanced plant vigour and photosynthetic capacity.

The collective body of research underscores the critical role of bacterial endophytes as reservoirs of structurally diverse and biologically potent secondary metabolites with nematicidal activity. Strains belonging to genera such as *Bacillus*, *Pseudomonas*, *Streptomyces*, and *Xenorhabdus* consistently demonstrate the ability to suppress plant-parasitic nematodes through direct toxic effects, inhibition of egg hatching, disruption of nematode development, and induction of systemic plant resistance. Advances in genome mining, metabolomic profiling, and regulatory pathway manipulation have further expanded the repertoire of bioactive compounds and enhanced their functional efficacy. Importantly, the rhizosphere competence, environmental adaptability, and compatibility of these endophytes with other biocontrol agents position them as key components in the development of sustainable, integrated nematode management strategies.

2.4.2 Secondary Metabolites from Fungal Endophytes in Nematode Management

Fungal endophytes, defined as fungi residing asymptomatically within plant tissues, are emerging as a valuable source of bioactive secondary metabolites for the management of plant-parasitic nematodes. These organisms, often colonising root tissues, possess metabolic versatility and can produce nematotoxic compounds such as organic acids, cyclic peptides, alkaloids, and terpenoids. Several genera including *Fusarium*, *Aspergillus*, *Trichoderma*, *Phyllosticta*, and *Chaetomium* have been implicated in nematode suppression either through direct nematicidal activity, inhibition of egg hatching, interference with host–parasite interactions, or induction of systemic resistance in the plant host.

Among these, root gall-associated endophytes have received increasing attention due to their proximity to nematode feeding sites and their unique metabolic

adaptations. The role of these fungi is not only restricted to the direct production of nematicidal metabolites but may also involve modulation of host signalling pathways, alteration of rhizosphere microbiota, and antagonism against microbial competitors that may otherwise facilitate nematode survival.

Given the limitations of chemical nematicides and the ecological ramifications of their use, fungal endophytes and their metabolites offer an ecologically sound, biologically compatible, and potentially crop-specific avenue for nematode suppression. The following section synthesises available literature, chronologically ordered, on fungal endophyte-derived metabolites and their roles in biological control of plant-parasitic nematodes.

Hallmann and Sikora (1996) provided early evidence for the nematicidal potential of fungal endophytes. Fungi isolated from the cortical tissues of *Lycopersicon esculentum* exhibited notable suppression of *Meloidogyne incognita*, with the culture filtrates demonstrating significant toxicity. The study linked the observed effects to secondary metabolite production, though specific compounds were not identified.

Nitao et al. (1999) developed in vitro assays to test the impact of fungal metabolites on *M. incognita* and *Heterodera glycines*. Crude extracts and filtrates from several unidentified endophytic fungi caused deformation, paralysis, and mortality in second-stage juveniles (J2s), with high repeatability across trials. These assays laid a foundation for subsequent bioassay-guided metabolomic studies.

Siddiqui et al. (2004) explored the differential impact of various *Aspergillus* spp. on nematode survival and plant growth promotion. *Aspergillus flavus* and *A. niger* were found to produce metabolites that caused severe physiological damage to *M. javanica* and concurrently stimulated plant biomass accumulation, suggesting dual functional roles.

Kapsalis et al. (2008) documented the production of phenazine-1-carboxylic acid, siderophores, and other metabolites by endophytic fungi associated with wheat rhizospheres. These compounds reduced *M. incognita* reproduction and galling index in greenhouse trials. While phenazine production is often associated with *Pseudomonas*, this study suggested possible horizontal gene transfer or microbial consortia involvement.

Van Dessel et al. (2011) assessed the nematicidal activity of *Fusarium oxysporum* culture filtrates against *Radopholus similis*. The filtrates exhibited pronounced J2 mortality and reduced egg hatching. GC-MS analysis identified fusaric acid and

moniliformin as the principal bioactive compounds.

Qureshi et al. (2012) tested culture filtrates of various marine-derived fungal endophytes against *M. javanica*. Several *Penicillium* and *Aspergillus* isolates produced high juvenile mortality in vitro. Secondary metabolites included oxalic acid and unidentified polyketides, as inferred from TLC-bioautography.

Kumar et al. (2012) isolated bioactive stilbenes from an endophytic *Fusarium* sp. inhabiting medicinal plant roots. These compounds significantly suppressed *M. incognita* J2 motility. The nematicidal efficacy was linked to the degree of hydroxylation on the stilbene backbone, as confirmed by structure–activity relationship studies.

Kumar et al. (2013) reported the production of cyclic dipeptides from an endophytic *Penicillium* strain. These diketopiperazines caused substantial nematode mortality and egg hatching inhibition, with effects comparable to commercial nematicides under laboratory conditions.

Elek et al. (2013) examined *Fusarium* endophytes from tetraploid wheat lines and their role in enhancing hydroxamic acid content in roots. These plant metabolites, influenced by fungal colonisation, interfered with nematode penetration and feeding site formation. The study highlighted a complex interaction where fungal colonisation modulated host metabolite profiles.

D'Addabbo et al. (2014) investigated biocide-producing endophytes in rotation crops. They observed that *Trichoderma* spp. and unidentified sterile fungi from *Brassica* roots released compounds that suppressed *M. incognita* populations. The observed effects were hypothesised to stem from glucosinolate hydrolysis products and fungal secondary metabolites acting in concert.

Majeed et al. (2014) investigated how foliar fungal endophytes alter glycoalkaloid levels in *Solanum tuberosum* under nematode pressure. While the study focused on plant secondary metabolite changes, it indirectly implicated fungal colonisation in modulating plant biochemical pathways that reduce nematode virulence. This highlights an important, albeit indirect, mechanism of endophyte-conferred nematode tolerance.

Sharma et al. (2014) evaluated the nematicidal activity of *Paecilomyces lilacinus* against *Meloidogyne incognita*. The fungal culture filtrate significantly increased J2 mortality and inhibited egg hatching. Scanning electron microscopy revealed severe deformation of nematode cuticle structures, indicating enzymatic or chemical

interference mediated by fungal secondary metabolites.

Plaza et al. (2014) used comparative transcriptomics in *Coprinopsis cinerea* to explore the regulatory machinery of secondary metabolism. Though not a direct bioassay study, their findings elucidated gene clusters encoding polyketide synthases and NRPS pathways with potential nematotoxic roles. This work laid a foundation for future metabolite engineering in fungal endophytes.

Prasad et al. (2015) sequenced the genome of *Purpureocillium lilacinum*, a well-known nematophagous fungus. Annotation revealed genes encoding serine proteases, chitinases, and polyketide synthases implicated in nematode pathogenesis. These enzymes likely act in concert with secondary metabolites to breach nematode cuticle barriers and cause juvenile mortality.

Degenkolb and Vilcinskas (2016) reviewed metabolite classes from nematophagous fungi, cataloguing known nematicidal compounds such as leucinostatins, paecilotoxins, and cyclosporine analogues. They emphasized the need to exploit metabolite biosynthetic diversity within *Trichoderma*, *Paecilomyces*, and *Purpureocillium* species for targeted nematode suppression.

Correa-Cuadros et al. (2016) examined the in vitro interaction of *Metarhizium anisopliae* with nematodes and plant roots. While the primary focus was insect suppression, co-cultivation assays showed reduced *M. incognita* J2 viability. The study suggested that fungal metabolites and cell wall-degrading enzymes act synergistically in both insect and nematode pathogenesis.

Singh et al. (2016) surveyed fungal endophytes from medicinal plants for their potential biocontrol traits. Several isolates produced terpenoid and alkaloid-rich extracts that displayed nematicidal activity. This study reinforced the ecological relevance of screening endophytes from chemically diverse host plants.

Hazir et al. (2016) compared culture supernatants from multiple *Xenorhabdus* and *Photorhabdus* strains. While bacterial in origin, their inclusion is warranted due to co-inoculation strategies with fungal partners. The findings indicated that pairing fungi with bacteria that secrete complementary metabolites can enhance biocontrol against nematodes.

Shi et al. (2016) isolated monascin, a yellow pigment from *Monascus*-fermented products, and tested it against *M. incognita*. The compound disrupted nematode cuticle integrity and induced oxidative stress in treated juveniles. This represented one of the first reports linking fungal pigments with direct nematicidal activity.

Wang et al. (2016) elucidated the biosynthesis of leucinostatins in *Purpureocillium lilacinum* and confirmed their role in nematode suppression through gene knockout studies. Leucinostatins were shown to impair nematode ATP production, causing paralysis and eventual mortality. This work offered mechanistic insights into the molecular mode of action of fungal endophyte-derived nematicides.

Ntalli and Caboni (2017) reviewed the biocidal properties of naturally occurring isothiocyanates (ITCs), noting that certain endophytic fungi are capable of synthesizing glucosinolate-like precursors. Upon hydrolysis, these compounds release ITCs which act as fumigants, disrupting nematode sensory perception and causing irreversible damage to the cuticle. Though primarily associated with Brassicaceae plants, this study suggested that endophytic fungi may contribute to in situ production of nematicidal volatiles.

Tiwari et al. (2017) evaluated co-inoculation of *Trichoderma harzianum* and *Aspergillus niger* with rhizobacteria in chickpea for *M. incognita* suppression. Dual application enhanced root colonisation and reduced nematode-induced galling. Fungal metabolites were hypothesised to interfere with nematode recognition and signalling, while also promoting rhizospheric defence responses.

Abbasi et al. (2017) assessed the extracellular metabolites of *Beauveria bassiana* and *Metarhizium anisopliae* against *M. incognita*. Culture filtrates caused high J2 mortality and egg hatch inhibition. Chemical profiling revealed the presence of destruxins and beauvericins, both of which are known to target nematode neuromuscular systems.

Flury et al. (2017) described the production of cyclic lipopeptides such as orfamides and viscosin by *Pseudomonas* strains. While bacterial in origin, these metabolites act synergistically with fungal endophytes, enhancing plant colonisation and root protection against nematodes. The study proposed the integration of fungi and bacteria as combined delivery systems for metabolite-mediated biocontrol.

Yan et al. (2017) showed that the PhlH transcriptional regulator modulates the expression of nematicidal metabolites like 2,4-DAPG in *Pseudomonas fluorescens*. While focused on bacteria, their findings bear relevance to fungal consortia where similar cross-regulatory effects are known to occur. The importance of understanding such transcriptional controls is critical for bioformulation design involving fungal partners.

Hazir et al. (2018) investigated the thermal stability and shelf-life of nematicidal

formulations derived from fungal and bacterial sources. The study demonstrated that certain fungal metabolites, especially from *Metarhizium* spp., remained effective under a wide range of storage and field conditions, which is crucial for commercial scalability.

Hussain et al. (2018) tested the combined effect of *Lecanicillium muscarium* and *Pochonia chlamydosporia* on *M. incognita*. Co-inoculation significantly reduced nematode reproduction and improved plant vigour. Both fungi produced secondary metabolites—lecanochromones and chlamydosporol—associated with nematode suppression and root colonisation.

Wang et al. (2018) sequenced the genomes of several nematophagous fungi and identified biosynthetic gene clusters associated with nematicidal activity. They highlighted the role of PKS-NRPS hybrid pathways in producing metabolites with multi-modal mechanisms including neurotoxicity, cytolysis, and egg dissolution.

Liu et al. (2019) characterised *Lysobacter capsici* strain NF87-2, which showed potent nematicidal activity through both extracellular enzymes and secondary metabolites. Although a bacterium, the strain was frequently co-isolated with fungal endophytes, suggesting possible synergistic associations in rhizosphere suppression of nematodes.

Rabbee et al. (2019) reviewed the biochemical arsenal of *Bacillus velezensis* and its interactions with fungal consortia in nematode-infested soils. While focused on bacterial strains, the paper noted that co-colonisation with endophytic fungi enhances root immunity and stabilises nematicidal compound production, reinforcing the need for multi-organism formulations.

Iqbal et al. (2019) demonstrated that deletion of the nonribosomal peptide synthetase (NRPS) gene *nps6* in *Clonostachys rosea* led to reduced nematicidal activity against *Heterodera schachtii*. The study established a direct genetic link between fungal secondary metabolite biosynthesis and suppression of plant-parasitic nematodes, underscoring the value of NRPS pathways as targets for bioengineering.

O'Keeffe et al. (2019) provided a methodological advancement in assessing nematicidal efficacy of fungal-derived compounds. They developed an integrated in vitro–in vivo pipeline for evaluating bioactive secondary metabolites, especially those from marine fungi, allowing standardised screening of nematode paralysis, mortality, and egg hatching inhibition under diverse environmental conditions.

Mastan et al. (2019) isolated functional endophytes from *Coleus forskohlii* and

demonstrated their dual capability in promoting plant growth and suppressing *M. incognita*. Culture filtrates of *Aspergillus* and *Fusarium* species contained alkaloids and organic acids, which reduced galling and increased root biomass. This highlights the potential of medicinal plant microbiomes in sourcing nematicidal endophytes.

Zhao et al. (2020) examined the nematicidal effect of a secondary metabolite produced by *Chaetomium globosum*, identified as chaetoglobosin A. This compound induced paralysis and lysis of *M. incognita* juveniles. Microscopic analyses showed rupture of the hypodermis and muscle layer, suggesting cytoskeletal disruption as the mode of action.

Haarith et al. (2020) conducted a mycobiome study of soybean cyst nematode (SCN)-infested roots and cysts. They found enrichment of specific endophytic fungi such as *Sarocladium* and *Phoma*, which correlated with reduced nematode reproduction. The study postulated that these fungi may secrete inhibitory secondary metabolites that modulate nematode development or oviposition.

Khan et al. (2020) reviewed *Trichoderma*-derived secondary metabolites, focusing on peptaibols and polyketides known for their nematostatic and nematicidal properties. They emphasised the metabolite diversity among *T. virens*, *T. harzianum*, and *T. atroviride*, and advocated their inclusion in integrated nematode management strategies.

Haarith et al. (2020) performed a culture-based screen of endophytes from SCN-infested soybean roots. Several isolates belonging to *Acremonium* and *Clonostachys* genera inhibited egg hatching and juvenile viability. The culture filtrates were rich in nonvolatile acids and diketopiperazines with nematicidal properties.

Strom et al. (2020) profiled the fungal endophyte communities in corn and soybean roots and identified strains with high in vitro suppression of *H. glycines*. Notably, some *Cladosporium* spp. produced nematotoxic metabolites that inhibited cyst formation. Their study linked endophytic diversity with field-level nematode resistance.

Poveda et al. (2020) reviewed the mechanisms of biological control by fungal endophytes, highlighting their ability to secrete nematicidal secondary metabolites such as volatile organic compounds, cyclic peptides, and alkaloids. The paper provided mechanistic insights into how these metabolites disrupt nematode chemotaxis, feeding, and reproduction.

Chen et al. (2020) investigated *Purpureocillium lavendulum*, a nematophagous

fungus, and characterised its regulatory genes associated with leucinostatin production. Knockout mutants of the transcription factor *pltf1* showed drastically reduced metabolite secretion and nematode infectivity, confirming its role in controlling secondary metabolite biosynthesis pathways.

Sun et al. (2020) reviewed the biology and biocontrol potential of *Clonostachys rosea*, a prolific producer of secondary metabolites such as epipolythiodioxopiperazines and sorbicillinoids. These metabolites are involved in nematode suppression by disrupting cuticle integrity and interfering with motility. The fungus also colonises root tissues effectively, suggesting a dual role in rhizosphere and endophytic niches.

Iqbal et al. (2020) investigated natural variation in antagonism against root lesion nematodes among *Clonostachys rosea* strains. Metabolomic profiling linked higher nematicidal activity to specific NRPS- and PKS-derived compounds. This study demonstrated that genetic diversity within endophytic fungal species influences their secondary metabolite repertoire and corresponding biocontrol efficacy.

The EFSA Panel (2020) evaluated the safety of *Duddingtonia flagrans* strain NCIMB 30336 (commercialised as BioWorma®) for use in grazing animals. Although primarily used for gastrointestinal nematodes, the study confirmed that the fungal metabolites posed no risk to non-target organisms, providing regulatory support for broader fungal metabolite application in nematode control.

da Silva et al. (2020) highlighted *Xenorhabdus*-derived compounds in medical entomology but noted their relevance in fungal-bacterial consortia for nematode suppression. Their findings support the use of entomopathogenic fungal symbionts with metabolite co-production potential in both animal and plant health sectors.

St. Leger and Wang (2020) described *Metarhizium* spp. as generalist fungi with multi-host infectivity, producing destruxins and other toxic metabolites. The study proposed that manipulation of secondary metabolite biosynthetic pathways could yield strains tailored for nematode-specific biocontrol without affecting beneficial organisms.

Gislason and de Kievit (2020) assessed *Pseudomonas brassicacearum*, often found in fungal endophyte-dominated niches. They reported that while beneficial under certain conditions, this bacterium can become phytotoxic. Their findings imply that metabolite interactions in endophyte consortia must be tightly regulated for consistent biocontrol outcomes.

Gong et al. (2020) reported that *Staphylococcus saprophyticus* L-38 co-inhabiting fungal root zones secretes antifungal and nematocidal VOCs. Though bacterial, its coexistence with fungal endophytes and contribution to volatile metabolite pools indicates a broader microbial synergy against nematodes.

Wan et al. (2021) characterised metabolites from the endoparasitic fungus *Hirsutella minnesotensis*. They isolated several diketopiperazines and polyketides with strong activity against *M. incognita*. Microscopic evaluation confirmed structural damage to J2 cuticle and digestive system, suggesting multi-targeted toxicity.

He et al. (2021) described a hybrid polyketide-terpenoid biosynthetic gene cluster in an endophytic fungus isolated from Chinese medicinal plants. The strain produced novel compounds with potent nematicidal effects, including inhibition of egg hatching and J2 mobility. LC-MS-guided fractionation revealed high chemical stability under field-relevant conditions.

Yang et al. (2021) explored the pleiotropic functions of Ras GTPases in nematode-trapping fungi. They demonstrated that secondary metabolism, particularly of nematotoxic compounds such as arthrobotrisins, is modulated by Ras-signalling. Mutants lacking *Ras1* showed reduced trap formation and metabolite production, linking morphogenesis with chemical defences.

Baazeem et al. (2021) evaluated the antimicrobial and nematicidal properties of *Aspergillus niger* culture extracts. The hydroethanolic fraction exhibited strong inhibition of *M. incognita* egg hatching and juvenile survival. GC-MS identified phenolic acids and indole derivatives as key bioactive constituents. These findings underline the potential of endophyte-derived metabolites in developing natural biopesticides.

Sood et al. (2021) discussed the role of endophytic fungi in modulating host plant defences under pathogen stress. Several *Trichoderma* and *Fusarium* strains were shown to secrete secondary metabolites that prime plant immune responses and inhibit nematode invasion. The study highlighted the dual benefit of direct nematode suppression and host defence stimulation.

Montañez-Palma et al. (2021) tested a hydroalcoholic extract of an endophytic *Fusarium* sp. for its nematicidal activity against *Haemonchus contortus*. Although the nematode is veterinary in nature, the extract's potency suggests potential cross-species efficacy against plant-parasitic nematodes, especially given the presence of trichothecenes and phenylpropanoids.

Chen and Hu (2022) characterised a range of secondary metabolites produced by *Purpureocillium lilacinum*, including leucinostatins, pyridines, and diketopiperazines. Their study used bioassay-guided fractionation to link specific compounds with nematicidal effects, offering a blueprint for compound isolation and functional validation.

Mendoza-de Gives et al. (2022) reviewed the role of nematophagous fungi in managing ruminant parasitic nematodes. Though focused on animal systems, the mechanisms of infection and metabolite deployment—especially trap-forming fungi like *Arthrobotrys*—share structural and biochemical parallels with plant nematode control strategies.

Bao et al. (2022) studied the pathogenicity and metabolite profile of *Purpureocillium lavendulum*. Culture filtrates caused membrane damage in *M. incognita*, and NMR analysis identified lactones and long-chain fatty acids as bioactive molecules. They proposed that the fungus uses a combination of enzymatic and metabolic attack to suppress nematodes.

Özdemir et al. (2022) assessed the efficacy of *Beauveria bassiana* against root lesion nematodes (*Pratylenchus thornei*). The fungus significantly reduced population density in wheat roots, and the culture supernatant showed nematostatic properties. Metabolite analysis revealed the presence of bassianolide and beauvericin.

Xie et al. (2022) demonstrated that phospholipase C gene (*AoPLC2*) in *Arthrobotrys oligospora* regulates the production of nematicidal metabolites. Deletion mutants showed impaired trap formation and reduced secretion of nematotoxic compounds, highlighting the coordination between morphological adaptation and chemical defence in fungal predators.

Khan and Kim (2022) explored how secondary metabolites from *Beauveria bassiana* enhance its virulence against nematodes and insects. The study confirmed that exposure to nematode exudates increased the secretion of destruxins and oosporein, suggesting inducible metabolite responses based on host-derived cues.

Silva et al. (2022) reviewed the potential of actinomycetes and their synergistic interactions with fungal endophytes in soil ecosystems. They proposed that co-cultivation could enhance the stability and spectrum of secondary metabolites, promoting sustainable nematode control through microbial consortia.

Weng et al. (2022) reviewed the multifaceted role of arbuscular mycorrhizal fungi (AMF) in nematode suppression. Though not traditionally endophytes, AMF colonise

root tissues and influence secondary metabolite production in associated fungi and plants. Their role in modulating the rhizosphere microbiome can indirectly enhance the activity of fungal endophytes with nematicidal potential.

Chaudhary et al. (2022) emphasized the importance of endophytes in conferring resistance against biotic stress through secondary metabolite production. In particular, they noted that *Trichoderma* and *Aspergillus* species secrete a spectrum of nematotoxic compounds that function as chemical shields and signalling molecules during early stages of nematode invasion.

Mendoza-de Gives et al. (2022) demonstrated that crude extracts from *Haemonchus contortus* (a parasitic nematode) act as elicitors of nematicidal compound production in endophytic fungi. *Monacrosporium* spp. increased production of hydrolytic enzymes and non-proteinaceous secondary metabolites upon exposure, suggesting nematode-derived signals can induce fungal defence responses.

Dube et al. (2022) identified *Albatrellus confluens*, a Basidiomycete fungus, as a source of two bioactive polyketides with nematicidal properties. The compounds disrupted motility in *M. incognita* juveniles and caused morphological deformities. These findings expand the phylogenetic range of fungi considered in nematode biocontrol research.

Rong et al. (2022) studied dazomet, a soil fumigant, and its metabolite methyl isothiocyanate (MITC). While not a fungal metabolite per se, they noted that endophytic fungi may influence the degradation and transformation of dazomet, thereby affecting local concentrations of nematotoxic derivatives in the rhizosphere.

Baiome et al. (2022) evaluated volatile organic compounds (VOCs) produced by *Trichoderma* spp., identifying compounds such as 6-pentyl-2H-pyran-2-one with strong nematicidal properties. These VOCs not only disrupted nematode behaviour but also functioned as chemical signals enhancing plant root colonisation by beneficial fungi.

de Oliveira et al. (2023) analysed the transcriptome of *Trichoderma harzianum* in response to *Pratylenchus brachyurus*. Genes linked to secondary metabolism—including PKS and NRPS clusters—were upregulated upon nematode exposure, indicating an inducible response tied to the nematode recognition system in fungi.

Costa et al. (2023) correlated nematode resistance in guava to endophytic fungal communities and their secondary metabolite profiles. Resistant genotypes harboured *Aspergillus*, *Trichoderma*, and *Fusarium* strains that produced higher levels of

flavonoids and terpenoids known for nematicidal activity.

Jia et al. (2023) reported the discovery of chiricanine A, a novel nematicidal compound produced by an endophytic *Fusarium* strain. The compound effectively suppressed pinewood nematode (*Bursaphelenchus xylophilus*) by targeting neural transmission. Structure–activity analysis revealed high potency and low phytotoxicity.

Fonseca et al. (2023) investigated the secretion profile of *Pochonia chlamydosporia*, a nematophagous fungus, and observed enhanced production of tryptophan-derived metabolites under nematode challenge. These compounds impaired egg development and inhibited hatch, underscoring the role of indolic metabolites in fungal biocontrol.

Khan et al. (2023) discussed the use of bio-organic amendments, including fungal endophytes, as sustainable strategies to manage *Meloidogyne* spp. in horticultural crops. *Trichoderma* and *Fusarium* strains enhanced plant vigour and reduced gall formation, likely due to their secretion of nematicidal compounds and elicitation of plant defence mechanisms.

Liu et al. (2023) showed that a root-knot nematode effector protein suppressed host defences, but treatment with endophytic *Fusarium* culture filtrates restored resistance by modulating jasmonate signalling and ROS pathways. The filtrates contained non-proteinaceous secondary metabolites that counteracted the nematode's effector-mediated immunosuppression.

Khan et al. (2023) combined nanotechnology with fungal biocontrol in their work on *Alnus nitida*-based biochar. When doped with urea and co-inoculated with fungal endophytes, the biochar supported colonisation by metabolite-producing *Trichoderma* spp., resulting in synergistic suppression of *M. incognita* in tomato.

Keerthi et al. (2023) evaluated endophytic *Fusarium* spp. from *Zingiber zerumbet* for their metabolite production and nematicidal potential. Culture filtrates inhibited *M. incognita* J2 motility and egg hatching, and LC-MS detected trichothecenes and azaphilones, confirming the presence of bioactive secondary metabolites.

Guzmán-Guzmán et al. (2023) provided a comprehensive review of *Trichoderma* species as biocontrol agents. Their analysis emphasised the vast repertoire of secondary metabolites—such as harzianolide, trichodermin, and gliotoxin—with confirmed nematicidal activity, and highlighted their compatibility with sustainable agricultural practices.

Wang et al. (2023) isolated asperphenalenones from *Clonostachys rosea* and

confirmed their activity against *Meloidogyne incognita*. These compounds disrupted nematode development and were found to be heat-stable and persistent in soil, making them suitable for formulation in bionematicide products.

Fite et al. (2023) reviewed the roles of plant-associated endophytic fungi in integrated pest management. They concluded that *Trichoderma*, *Beauveria*, and *Metarhizium* spp. not only produce nematicidal metabolites but also enhance systemic resistance and microbial diversity in the rhizosphere.

Greff et al. (2023) examined the biocontrol activity of endophytes isolated from aromatic and medicinal plants. *Penicillium* and *Fusarium* spp. secreted alkaloids and lipopeptides that reduced nematode reproduction and enhanced root colonisation. These results emphasised the importance of selecting host plants with bioactive phytochemistry for endophyte screening.

Zhou et al. (2023) surveyed the diversity and secondary metabolite profiles of actinomycetes and fungi in suppressive soils. While primarily bacterial, they noted co-isolation of *Trichoderma* spp. producing nematotoxic VOCs, further supporting the idea of microbe–microbe synergy in soil-borne pest suppression.

Mazurkiewicz-Zapalowicz et al. (2023) tested several entomopathogenic fungal species for their nematicidal potential. Among them, *Beauveria bassiana* and *Metarhizium anisopliae* produced metabolites that reduced root invasion by *M. incognita* and *H. glycines*, suggesting broader host targets than previously assumed.

Varghese and Ray (2023) highlighted the contribution of fungal endophytes to sustainable spice cultivation, focusing on *Aspergillus* and *Fusarium* species that produce nematicidal metabolites. They proposed integrating endophytes into organic management systems to reduce dependence on synthetic nematicides.

Sun et al. (2024) demonstrated that overexpression of a global regulator in an endophytic *Fusarium* strain led to increased production of nematicidal metabolites. The modified strain showed enhanced suppression of *Meloidogyne incognita* through improved secondary metabolite profiles, including polyketides and macrolides.

Shravani et al. (2024) tested *Bionectria ochroleuca*, an endophytic fungus, for its in vitro activity against *M. incognita*. Culture filtrates exhibited high J2 mortality, with metabolic profiling revealing the presence of cyclic peptides and diketopiperazines. These compounds disrupted nematode neuromuscular function.

Vera-Morales et al. (2024) identified nematicidal compounds from both bacterial and fungal endophytes. Fungal isolates belonging to *Beauveria* and *Fusarium* genera

produced non-volatile metabolites that inhibited nematode development. The study supported the use of microbial consortia in biocontrol formulations.

Ghareeb et al. (2024) evaluated *Aspergillus niger* and *Penicillium citrinum* for their antifungal and nematicidal activities. Both strains inhibited *M. incognita* reproduction and induced root growth in tomato. LC-MS detected citrinin and itaconic acid among the active metabolites.

Mian et al. (2024) reported the complete genome of *Bacillus velezensis* UFV07 and noted its co-isolation with fungal endophytes. Their findings suggest potential synergistic effects on nematicidal metabolite production when bacteria and fungi coexist in the rhizosphere.

Ayaz et al. (2024) compiled a comprehensive review of bacterial and fungal metabolites effective against PPNs. For fungal endophytes, the paper catalogued metabolites such as gliotoxin, paecilotoxin, and trichodermin, reinforcing their potential for biocontrol applications.

Fang et al. (2024) screened indigenous entomopathogenic fungi for activity against *Meloidogyne incognita*. *Metarhizium robertsii* and *Lecanicillium lecanii* were highly effective, with culture extracts showing dose-dependent inhibition of juvenile mobility. Identified metabolites included destruxins and lecanindole.

Yang et al. (2024) characterised *Clonostachys rosea* and its secondary metabolic capacity. Transcriptomic data revealed upregulation of NRPS and PKS genes under nematode exposure. This provided insight into inducible nematicidal metabolite biosynthesis under host pressure.

Patidar et al. (2024) applied chemoprofiling to *Purpureocillium lilacinum*, correlating leucinostatin production with nematode suppression. They proposed chemotaxonomic criteria for screening potent isolates based on metabolite markers, facilitating strain selection for commercial biocontrol products.

da Silva et al. (2024) linked protease activity in edible mushrooms to nematicidal effects. Extracts from *Pleurotus ostreatus* showed strong inhibition of *M. incognita* development, attributed to both enzymatic action and small molecule secretion, expanding the scope of fungi used in bionematicide development.

Shehata et al. (2024) further confirmed that fungal isolates from rhizosphere soils can act as nematode biocontrol agents. Several isolates reduced galling in tomato and secreted bioactive peptides with dual antifungal and nematicidal activity.

Cueva-Clavijo et al. (2024) evaluated ointments formulated with *Daldinia*

eschscholtzii extracts for use in nematode-infected tissue. Though developed for veterinary application, the metabolite composition—including isoindolinones—suggests potential for plant nematode control as well.

Shepherd et al. (2024) showed that dietary supplementation with heather (*Calluna vulgaris*) reduced gastrointestinal nematodes in livestock. While plant-based, they observed that endophytic fungi within the plant may contribute to the bioactivity, offering a novel angle for endophyte sourcing.

Deeikshana et al. (2025) reviewed fungal secondary metabolites targeting PPNs, categorising them by biosynthetic origin. They stressed the importance of cyclic peptides, terpenes, and polyketides and advocated for structure-guided discovery from underexplored endophyte taxa.

Shakeel et al. (2025) explored the use of saponins in synergy with fungal endophyte metabolites. Co-application with *Trichoderma harzianum* enhanced J2 mortality, suggesting potential for combined formulations incorporating plant and microbial secondary metabolites.

Saha et al. (2025) summarised the role of fungal entomopathogens in nematode biocontrol. *Beauveria* and *Metarhizium* were highlighted for their secretion of chitinases and nematotoxic compounds, positioning them as dual-target agents.

Patil et al. (2025) characterised an endophytic *Bacillus subtilis* co-isolated with *Aspergillus* spp., demonstrating that fungal co-inoculation enhanced nematode suppression through increased metabolite diversity. This supported the development of microbe consortia for field deployment.

2.4.3 Conclusion

Fungal endophytes represent a rich and largely underutilised reservoir of bioactive secondary metabolites with substantial potential for the biological control of plant-parasitic nematodes. Across diverse taxa—including *Trichoderma*, *Fusarium*, *Beauveria*, *Purpureocillium*, and *Clonostachys*—numerous studies have demonstrated the production of structurally diverse compounds such as polyketides, cyclic peptides, terpenoids, and alkaloids with confirmed nematicidal activity. These metabolites act through multiple mechanisms, including disruption of nematode cuticle integrity, inhibition of egg hatching, paralysis of juveniles, and modulation of host plant defences.

Importantly, many of these fungi also exhibit plant growth-promoting traits,

further enhancing their value as dual-purpose agents in integrated pest and crop management systems. Advances in genomics, transcriptomics, and metabolomics have begun to elucidate the biosynthetic gene clusters underpinning metabolite production, offering new opportunities for strain selection, genetic enhancement, and formulation development.

In addition, several studies have highlighted synergistic interactions between fungal endophytes and co-existing rhizobacteria or arbuscular mycorrhizal fungi, indicating that endophyte-mediated nematode suppression is often part of a broader microbial network. Collectively, the evidence supports the strategic incorporation of metabolite-producing fungal endophytes into sustainable nematode management frameworks, particularly in cropping systems where chemical nematicide use is restricted or undesirable.

Chapter 3: Objective 1

Chapter 3: Objective 1

Characterisation of the fungal endophytic communities associated with *Meloidogyne graminicola*-infected rice roots using both metagenomic and culture-dependent approaches

3.1 Introduction

Root-knot nematodes (RKN; *Meloidogyne* spp.) are among the most destructive plant-parasitic nematodes in agriculture, causing severe root damage and yield losses in many crops. In rice (*Oryza sativa*), the rice root-knot nematode *Meloidogyne graminicola* is particularly problematic and is considered a major threat to rice production across Asia, particularly in direct seeded rice ecologies. This obligate sedentary endoparasite generates distinctive hook-shaped galls on rice roots, affecting water and nutrient transmission, resulting in stunting, chlorosis, and decreased tillering. Economically, infestations of *M. graminicola* can result in substantial yield reductions, estimated to represent approximately 15% of overall rice yield loss in Asia. Fields with severe infestations have shown 20% and 80% decrease in yield, according to nematode population density. In addition to direct crop losses, RKN galling injuries can render rice plants susceptible to secondary infections and other soil-borne diseases. The economic and ecological consequences highlight the necessity for efficient management strategies for *M. graminicola* in rice agriculture.

The available control measures of *M. graminicola* are either inadequate or not economically viable. Therefore, development of sustainable management solution is urgently required. In this pursuit, plant microbiome based biological control of nematodes have seen tremendous attention in recent years. The communities of microorganisms linked to plant roots, frequently referred to as the plant's "second genome," are crucial for maintaining plant health and enhancing disease resistance. The microbiome associated with soil and roots serves as a protective barrier, where specific microbes can inhibit pathogens or pests via competition, antagonism, or the activation of systemic resistance mechanisms. Indeed, plants under attack are known to recruit beneficial members of their microbiome; for example, resistant plants can assemble a protective rhizosphere community that combats RKN and other pathogens. Root-knot nematodes themselves do not exist in isolation but form diverse

interactions with surrounding microorganisms, which can influence nematode infection dynamics and virulence. The interactions observed can be categorised as either mutualistic or antagonistic; certain microbes may promote nematode infestation, whereas others have the capacity to inhibit nematodes or reduce the extent of their damage.

Recent investigations have started to reveal how RKN infestations disrupt the root and soil microbiomes of their hosts. Tian et al. (2015) presented initial findings indicating that RKN infection leads to considerable alterations in the bacterial endophyte community within plant roots. The study on M. incognita-infested tomato revealed a significant variation in bacterial communities between RKN-induced galls and healthy roots, highlighting unique bacterial taxa associated to the root galls. Functionally, certain gall-specific bacteria have the ability to degrade plant cell walls and carry out various metabolic processes that may be advantageous to the nematode, indicating a potential symbiotic relationship with nematode parasitism. In a similar vein, a longitudinal study conducted by Yergaliyev et al. (2020) revealed that there are distinct microbial assemblages associated with root galls formed due to M. incognita infestation when compared to uninfected root tissue.

This structured "gall community" persisted over the growing season and was characterized by increasing populations of bacteria adapted to the hypoxic conditions inside galls. Such findings indicate that RKN create unique niches that foster specific microbial consortia, some of which may, in turn, influence nematode behaviour and pathogenicity (e.g. through chemical cues that affect nematode attraction to roots).

In the context of rice, recent metagenomic studies have discovered the intimate link between RKN infection and shifts in the root microbiome. Masson *et al.* (2020) examined naturally infested rice roots from Vietnamese fields and observed a pronounced change in root associated bacterial community with the infestation of *M. graminicola*. Nematode-infected roots (galls) harboured a higher microbial diversity and a markedly different community composition compared to non-infected roots. The infected root microbiome (termed the "gallobiome") showed enrichments in particular bacterial taxa, including several hub species in co-occurrence networks, some of which were hypothesized to act as indicators of gall formation or even potential biocontrol agents against the nematode. In contrast, a controlled study by Kunda *et al.* (2024) reported that *M. graminicola* galls on rice roots had a distinct but less species-rich endophytic bacterial community relative to healthy roots. They found

that a few genera – such as *Chryseobacterium*, *Rhizobium*, *Gemmata*, and *Pseudomonas* – were uniquely abundant inside galls, some of which have known associations with nematodes or plant stress responses. Simultaneously, advantageous bacteria such as Bacillus, Pantoea, and Acidovorax were identified exclusively in the microbiome of non-infected roots. The aforementioned categories are typically seed-transmitted endophytes with plant growth-promoting or nematode-antagonistic characteristics, indicating that nematode infection may interfere with or eliminate specific beneficial microorganisms from the root core. Kunda et al. found an endophytic Enterobacter strain from healthy rice roots, exhibiting significant nematicidal activity (about 90% juvenile mortality in vitro), by integrating community analysis with culture-based isolation. These findings highlighted the potential of the plant-associated microbiome for the biocontrol of root-knot nematodes (RKN).

While bacterial communities in RKN-infested roots have been relatively well explored, much less is known about the associated fungal communities. Fungal endophytes and rhizosphere fungi are integral components of the plant microbiome and can substantially affect plant-parasite interactions. Endophytic fungus, along with bacteria, typically exhibit mutualistic relationships, providing advantages such as improved stress tolerance and resistance to diseases and pests. Numerous fungi are recognised as antagonists of root-knot nematodes (RKN); for instance, Trichoderma and Fusarium endophytes have demonstrated efficacy in reducing *M. graminicola* infection in rice roots, while other fungi such as Purpureocillium lilacinum and Pochonia chlamydosporia are established nematode egg parasites. A recent study by Jiang et al. (2024) on RKN-infected tobacco emphasised the critical role of fungal communities in mitigating nematode infestations. Despite limited research, the specific characteristics and function of fungal endophytes in root galls caused by root-knot nematodes (RKN) remain largely unexamined. Previous metagenomic investigations of RKN-infested roots have mostly employed 16S rRNA gene sequencing to characterise bacterial communities, neglecting fungal and other eukaryotic microorganisms. Thus, a considerable knowledge deficit exists about the fungal endophytic populations linked to *M. graminicola* galls in rice roots.

Addressing this gap is crucial for a holistic understanding of the rice root gall microbiome and its potential exploitation for nematode management. Therefore, the present study employed both culture independent metagenomics studies and culture dependent isolation to characterise the fungal endophytes in rice roots infected by *M.*

graminicola. This two-pronged approach provides complementary insights. The culture-independent metagenomics sequencing offers a broad overview of the gall-associated fungal community, while the culture-dependent isolation will allow species specific identification and conducting laboratory bioassays for understanding their role in biological control. By integrating these methods, this study aims to uncover the hitherto unexplored fungal component of the RKN gall microbiome and their functional characterisation for identification of biocontrol potential. This would be a critical first step towards development of innovative, ecologically sound nematode management strategies.

To address this gap, the present study undertakes a dual approach: (i) culture-independent fungal community profiling using ITS1-5 amplicon sequencing, and (ii) culture-based isolation and functional screening of fungal endophytes from galled and non-galled rice roots. By integrating high-throughput sequencing with *in-vitro* characterisation, this study aims to unravel the composition and potential biocontrol roles of gall-associated fungal endophytes, thereby contributing foundational insights towards the development of sustainable nematode management strategies.

3.2 Methodology

3.2.1. Sampling of Plant and Soil

Rice plants (cv. MTU1010) at the 4-week stage were collected from fields naturally infested with *M. graminicola* and adjacent non-infested plots across Giridih and Jamtara districts of Jharkhand, India, as previously described (Mondal et al., 2021). A stratified random sampling using the "W" pattern was followed, with three adjacent plots sampled per area, and two sites per district included. Collected plants were transported on ice and processed within 24 h. Roots were thoroughly rinsed under sterile water to remove soil particles and categorised into four distinct sample types: (1) infected roots with galls (Root-G), (2) healthy roots (Root-H), (3) rhizospheric soil associated with galled roots (Rhizo-soil-G), and (4) rhizospheric soil associated with healthy roots (Rhizo-soil-H). Each group comprised three biological replicates, totalling 12 samples.

3.2.2. DNA Extraction

Root samples were surface sterilised using a modified protocol from Sessitsch et al. (2012) and Kunda et al. (2018). Briefly, roots were immersed in 0.1% Tween-20,

followed by sequential washing with 70% ethanol and 2% sodium hypochlorite, and rinsed repeatedly with sterile distilled water. DNA from root samples was extracted using the DNeasy Plant Mini Kit, while rhizospheric soil DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) following the manufacturer's protocol. DNA yield and purity were quantified using a NanoDrop 1000 spectrophotometer. Equal amounts of DNA from each replicate were pooled and stored at −20 °C for downstream analysis.

3.2.3. Amplicon Library Preparation and Sequencing

Fungal community profiling was performed via amplification of the ITS1 region using ITS1 and ITS5 primers. PCR products were verified by 1.5% agarose gel electrophoresis alongside a 500 bp ladder (Puregene Nexgen, PG210-500 DI-NV). Amplicons were purified, and libraries were constructed using the NEBNext Ultra II DNA Library Prep Kit (NEB, E7645L). Library quantification was done using Qubit 4.0 fluorometer with the DNA High Sensitivity assay kit. Library size distribution was confirmed on the Agilent TapeStation 4150 using High Sensitivity D1000 ScreenTapes. Sequencing was performed on the Illumina NovaSeq 6000 platform with 2 × 150 bp paired-end reads.

3.2.4 Statistical analysis of 18s taxonomy analysis

Raw data quality assessment was performed using FastQC (Andrews et. al 2017) v.0.11.9 and summarised using MultiQC v.1.9 (Ewels et. al., 2016). The raw fastq reads were pre-processed using Fastp v.0.20.1 (parameters: *-length_required 50 -correction -trim_poly_g -cut_front -cut_tail-qualified_quality_phred 30 unqualified_percent_limit 30 -average_qual 30*) (Chen et. al., 2018). Post filtering cleaned data were re-assessed using FastQC and summarised using MultiQC v.1.9. Analysis of fungal internal transcribed spacer (ITS) sequences from the Illumina sequencing platform was done using PIPITS v2.7 (Gweon et al., 2015), which was reported to perform better than QIIME2 and Galaxy (Anslan et al.,2018). The illumina fastq sequences were assembled with VSEARCH (Rognes et al., 2016) and quality-filtering was done with fastx through the PIPITS command pispino_createreadpairslist. The ITSx (Bengtsson-Palme et. al., 2013) were executed through the PIPITS command pipits_funits. Chimera filtering and clustering was done using VSEARCH through the PIPITS command pipits_process. The classic tabular OTU table was converted into a BIOM format and taxonomy was assigned with UNITE

[RDP Classifier]. BIOM file was further extracted into OTU based taxonomic identification file.

All samples were assessed for species richness and bacterial community evenness using alpha diversity indexes. OTU number, Chao 1 estimator, Shannon diversity index, Simpson diversity index, were used to represent species richness and evenness. Differences in fungal communities as indicated by alpha diversity among the sample were tested with Welch T-test/ANOVA at feature level. P – Values of pair wise comparisons based on Welch T-test/ANOVA were adjusted using FDR correction.

Beta diversity, indicated by fungal community composition differences, was visualised by cluster analysis PcoA using a Bray-Curtis dissimilarity matrix calculated separately from OTU data of the different sample types. Cluster analysis used OTU sequence abundances to construct Bray-Curtis dissimilarities. AnOSIM was used to determine fungal community separation and PERMANOVA validates that the visual separation among sample type based on ecological distribution.

Core microbiome analysis was done with all sample types at feature level, with the minimum of 0.01% of relative abundance and 20% sample prevalence.

These analyses were performed with Microbiomeanalyst 2.0. Venn diagram was made in Origin with the OTU database.

LEfSe (Linear Discriminant Analysis Effect Size) analysis was performed utilising the MicrobiomeAnalyst web-based platform (www.microbiomeanalyst.ca) to identify fungal taxa that are differently abundant across sample types. Normalised genus-level abundance data were uploaded, and sample groups were categorised by compartment type: Root-G (infected roots), Root-H (healthy roots), Rhizo-soil-G, and Rhizo-soil-H.

LEfSe was executed with the standard procedure, comprising three consecutive steps: Kruskal-Wallis rank-sum test to identify genera with significant differences among groups ($\alpha = 0.05$). Wilcoxon rank-sum test for pairwise subclass consistency. Linear Discriminant Analysis (LDA) to assess the effect size for each taxon. Only taxa exhibiting a log LDA score of at least 2.0 and a p-value of 0.1 or lower were deemed significantly enriched. Results were illustrated by LDA barplots depicting the most differentially prevalent genera across sample categories. The enriched genera were later annotated by ecological function utilising curated literature to deduce compartment-specific fungal tactics in response to Meloidogyne graminicola infection.

3.2.5 Culture dependent isolation of root-gall associated fungal endophytes

The two types of root samples were taken for culture based fungal endophyte isolation. Here we have not included the rhizosphere soil to omit further complexity and stick to root specificity and effect of nematode infection profile.

Roots were carefully separated from the rice plants and thoroughly washed to eliminate any adhering soil. Samples without any disease symptoms other than nematode infection were considered for isolation of fungal endophytes. The healthy and galled root tips were cut into 1–1.5 cm long pieces with a sterile scalpel. After preliminary washing, small root pieces of both healthy and galled roots were placed on sterile petri dishes and washed with sterile distilled water. Do (2022) method with some modifications was followed for surface sterilization of the roots. Briefly, roots were dipped into 70% ethanol for 3 mins followed by dipping into 3% NaOCl for 5 mins. Subsequently, roots were washed with sterile distilled water for 5–6 times until the NaOCl is completely washed out. Healthy and galled root tips (n = 8/sample type) were placed separately on water agar plates for isolation of endophytic fungus (Zakaria et al., 2016). Isolation process was repeated thrice. All plates were incubated at 28 ◦C and inspected at two-day intervals up to a week. When the hyphal growth was observed, fungal colonies were individually picked, inoculated on PDA slant and incubated for 3–5 days for the pure culture preparation. For identification of isolated endophytes, morphological and microscopic characters (vegetative and reproductive structures) were assessed following standard protocols (Barnett and Hunter 1972, Ellis, 1971). Morphologically distinct endophytic fungi were inoculated separately in PDA plates and named accordingly.

3.2.6 Identification of culture dependent endophytic fungi

For morphological identification, colony morphology, conidial structures were taken. For molecular identification, DNA was isolated from 5 to 10 mg mycelial mat collected from 5-day old culture maintained on PDB. The mycelial mat was thoroughly ground in liquid N2 with a sterile mortar and pestle. Genomic DNA was isolated manually following the modified CTAB method (Li and Yao, 2005, Wu et al., 2001). The presence of DNA was confirmed in 1% agarose gel. Internal transcribed regions were amplified using universal primers ITS1 (5´-CTTGGTCATTTAGAGGAAGTAA-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´). For further confirmation of species in few fungal isolates, species specific primers were used, fragment of the calmodulin

(CaM) gene was amplified using the primers cmd5 (CCGAGTACAAGGARGCCTTC) and cmd6 (CCGATRGAGGTCATRACGTGG) and the β tubulin (BenA) gene was amplified using the primers Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (ACCCTCAGTGT-AGTGACCCTTGGC) (Glass and Donaldson, 1995; Hong et al., 2005). The PCR amplification profiles were set as previously reported by Kim et al., (2016) for the ITS region, and by Samson et al. (2014) for the CaM and BenA regions. The amplified PCR products were purified using PCR purification kit and subjected to sequencing. Sequences were finalized and submitted to NCBI.

All fungal isolates obtained from *Meloidogyne graminicola*-infected and healthy rice roots were subjected to a series of qualitative biochemical assays to evaluate their enzymatic activity and biocontrol-related traits. All tests were performed in triplicate, using standard procedures with minor modifications.

3.2.7 Biochemical enzymatic activity against pathogen

3.2.7.1. Cellulase Activity

Carboxymethyl cellulose (CMC) agar was prepared containing: 5 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 2 g CMC (HiMedia), 20 g agar to check cellulase activity. Fungal plugs (6 mm diameter) were inoculated and incubated at 28 ± 2 °C for 5–7 days. After incubation, plates were flooded with 0.1% Congo red for 15 min and destained with 1 M NaCl for 10 min. The appearance of a clear halo zone around the colony indicated cellulase production (Teather & Wood, 1982).

3.2.7.2. Chitinase Activity

Chitinase production by fungal isolates was evaluated using colloidal chitin agar amended with the pH indicator bromocresol purple (BCP), which allows visual detection of acidification due to chitin degradation. Colloidal chitin was prepared by treating 10 g crab shell chitin with concentrated HCl, followed by neutralisation and repeated washing to pH 7.0 as described by Roberts & Selitrennikoff (1988). The medium contained (per litre): 0.2% colloidal chitin, 0.3% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.03% $FeSO_4 \cdot 7H_2O$, and 0.015% bromocresol purple (HiMedia), with 2% agar. Fungal plugs (6 mm) were inoculated and plates were incubated at 28 ± 2 °C for 5–7 days. A colour change from purple to yellow around the colony indicated a localised drop in pH due to chitin hydrolysis and release of acidic by-products, confirming chitinase activity.

3.2.7.3. Amylase Activity

Amylase activity was evaluated on starch agar plates containing (per litre): 10 g soluble starch, 5 g peptone, 5 g NaCl, 2 g agar. After 5 days of incubation at 28 ± 2 °C, plates were flooded with iodine solution (1% I_2 and 2% KI). The formation of a clear halo around the fungal growth against a dark blue background indicated starch hydrolysis (amylase activity) (Hankin & Anagnostakis, 1975).

3.2.7.4. Pectinase Activity

Pectinase production was tested using pectin agar medium supplemented with 1% citrus pectin (HiMedia). After incubation for 5–7 days, plates were flooded with 1% cetyltrimethylammonium bromide (CTAB) solution. A clear zone surrounding the fungal colony indicated pectin degradation (Gopinath et al., 2013).

3.2.7.5. Protease Activity

Protease activity was determined on skim milk agar (10% skim milk powder and 1.5% agar). Fungal isolates were inoculated and incubated for 5–7 days. Clear zones around the colony due to casein hydrolysis confirmed protease activity (Grover & Moore, 1962).

3.2.7.6. Siderophore Production

Siderophore production was qualitatively assessed using Chrome Azurol S (CAS) agar medium, as per Schwyn & Neilands (1987). A colour change from blue to orange or yellow around the fungal colony after 5–7 days of incubation at 28 ± 2 °C indicated siderophore secretion.

3.2.7.7. Hydrogen Cyanide (HCN) Production

HCN production was detected following the method of Lorck (1948). Fungal isolates were grown on potato dextrose agar (PDA) amended with 0.44% glycine. A filter paper strip soaked in picric acid solution (0.5% picric acid in 1% Na_2CO_3) was placed in the Petri dish lid. The plates were sealed with parafilm and incubated at 28 ± 2 °C for 4–5 days. A change in the filter paper colour from yellow to orange/brown indicated HCN production.

3.3 Results

3.3.1 Rice root gall associated fungal diversity and taxonomy

A total of 16,920,648 paired end reads were generated by amplification of ITS1-ITS5 region of the 18S rRNA gene with average reads of 914069 per sample. After trimming and merging the paired end reads high quality reads were clustered using > 97% sequence identity which generated a total of 3566 fungal OTUs. To avoid rare taxa (min. read count/OUT=5, min prevalence across the sample =2 sample at least) and PCR sequencing artifacts low abundance OTUs as well as those affiliated to other eukaryotes were removed which resulted in taxonomically classified denoised unique sequences clustered into 1148 OTUs. The Alpha diversity, i.e. within sample diversity, was indicated as rarefied average OTUs per sample type. OTUs from all the samples ranged from 449 to 734 where Rhizo soil H2 recorded the highest number and Rhizo soil G2 the lowest.

3.3.1.1 Alpha Diversity

Alpha diversity indices were calculated for fungal communities across four sample types (Root-G, Root-H, Rhizo-soil-G, and Rhizo-soil-H) applying Shannon, Simpson, and Chao1 metrics (Fig.3.1) to evaluate richness and evenness. The Shannon index indicated significant differences among sample types ($F= 7.61$, $p < 0.05$), with Rhizo-soil-H demonstrating the highest diversity and Root-G the lowest (Shannon mean ~1.5). The Simpson index, despite lacking statistical significance ($F=2.34$, $p = 0.4965$), indicated diminished evenness in Root-G samples relative to Root-H and rhizospheric soils. The Chao1 index, which quantifies species richness, was markedly decresed in Root-G and Root-H relative to their rhizospheric soil counterparts ($F = 28.526$, $p<0.05$), underscoring a decline in uncommon fungal taxa within the root endosphere, particularly under nematode stress.

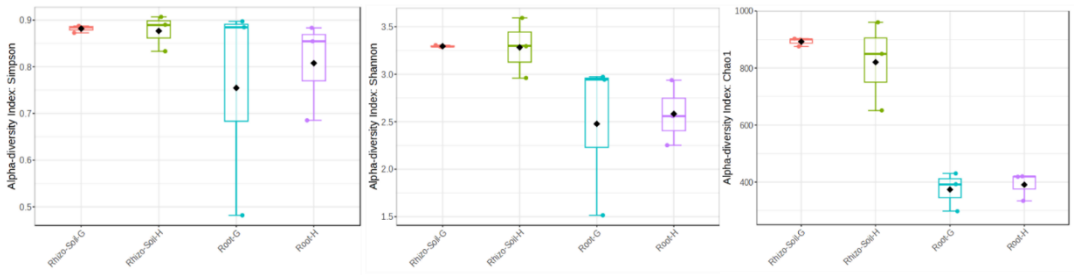

Fig. 3.1 Alpha diversity indices of fungal communities across four sample types: nematode-infected roots (Root-G), healthy roots (Root-H), and their respective rhizospheric soils (Rhizo-Soil-G and Rhizo-Soil-H). Diversity was assessed using three indices: (A) Simpson index (evenness), (B) Shannon index (richness and evenness), and (C) Chao1 index (estimated species richness).

3.3.1.2 Taxonomic Diversity at different level

The phylum level analysis of rice roots and rhizospheric soils identified a variety of fungal taxa across ten phyla. The community was predominantly consisted of Ascomycota and Basidiomycota, together representing over 90% of all sequences across sample types (Fig. 3.2.A). Ascomycota constituted the predominant phylum in both infected (Root-G) and healthy root (Root-H) tissues, representing 83% and 97% of the total fungal community, respectively. The relative abundance of Ascomycota in rhizospheric soils declined, comprising 63% in Rhizo-soil-G and 78% in Rhizo-soil-H. Basidiomycota were the second most prevalent group, notably abundant in Rhizo-soil-G (about 25%), but decreased progressively in Rhizo-soil-H (approximately 18%) and Root-G (approximately 12%). The representation reduced to negligible levels in Root-H (<2%). Low-abundance phyla such as Mortierellomycota, Mucoromycota, Rozellomycota, and Blastocladiomycota were regularly detected in rhizospheric soils but were either absent or much decreased in root tissues. Root-H samples exhibited a pronounced predominance of Ascomycota (97%) and a significant reduction in all minor phyla, suggesting a possible filtering function of healthy root tissues that promotes specialised fungal assemblages (Fig. 3.2.A). In contrast, Root-G maintained a moderate abundance of Basidiomycota and minimal presence of other phyla, indicating modified fungal recruitment or community retention during *M. graminicola* infection.

Class-level taxonomic analysis (Fig. 3.2.D) demonstrated unique fungal community characteristics among the four sample types. Sordariomycetes and Dothideomycetes were the predominant groups in root-associated regions either healthy or infected. Specifically, Root-H (healthy roots) demonstrated a significant

predominance of Dothideomycetes (about 65%) and Sordariomycetes (approximately 25%), indicating a relatively stable endophytic community comprised of mutualists and commensals. Conversely, Root-G (infected roots) exhibited a significant alteration, defined by decreased Dothideomycetes abundance and enhanced proportions of Eurotiomycetes, Tremellomycetes, and Not-Assigned taxa, indicating infection-induced disruption and the recruitment of stress-adapted or unclassified fungus.

In rhizospheric soils, both Rhizo-Soil-G and Rhizo-Soil-H demonstrated elevated class-level diversity. Not-Assigned, Mortierellomycetes, and Tremellomycetes were notably prevalent, particularly in Rhizo-Soil-G, suggesting the existence of uncultured or inadequately characterised soil fungus. Eurotiomycetes and Sordariomycetes were consistently found in both rhizospheric and endospheric samples, exhibiting varying proportions based on infection status.

The analysis of fungal community composition at the familial level indicated unique abundance patterns between root and rhizospheric compartments. The Not-Assigned to family was the most prevalent group in all samples, with 38–59% of relative abundance, indicating the existence of possibly novel or under-characterized fungal lineages, particularly evident in Rhizo-Soil-G (59%) and Rhizo-Soil-H (43%). Aspergillaceae was continuously prevalent among the identified taxa, with the highest representation in Rhizo-Soil-H (16.4%) and Root-G (16.0%), indicating the abundance of Aspergillus species, particularly in infected roots. The Nectriaceae family, encompassing the Fusarium complex, was significantly prevalent in Root-G (15.6%), highlighting its importance in gall-associated fungal communities (Fig. 3.2.B). Conversely, Pleosporaceae, linked to Alternaria and Curvularia, exhibited significant enrichment in Root-H (23.0%), indicating a transition towards plant-beneficial taxa in non-stressed conditions.

The unnamed family, likely indicative of poorly resolved or rare taxa, was regularly found, particularly in Rhizo-Soil-G (6.6%) and Root-H (11.3%), further highlighting the cryptic diversity within the rice mycobiome. The compositional variations at the family level corroborate the trends observed at higher taxonomic ranks, affirming that nematode infection selectively alters fungal communities, promoting specific families (e.g., Nectriaceae, Aspergillaceae) while inhibiting others linked to mutualistic functions (e.g., Pleosporaceae).

Genus-level analysis revealed (Fig. 3.2.C) distinct fungal assemblages across root and rhizospheric compartments, reflecting niche-specific enrichment patterns. In root tissues, *Alternaria* dominated healthy roots (Root-H), consistent with its role as a core endophyte and generalist coloniser. In contrast, infected roots (Root-G) exhibited higher relative abundances of *Aspergillus*, *Curvularia*, *Fusarium*, and *Cystobasidium*, taxa commonly associated with stress adaptation, opportunism, or antagonistic potential. Rhizospheric soils (Rhizo-Soil-G and Rhizo-Soil-H) were enriched in saprotrophic and environmental genera such as *Mortierella*, *Preussia*, *Saitozyma*, and *Penicillium*, suggesting a broader taxonomic reservoir and early-stage decomposer functions. Notably, *Gliocephalotrichum* and *Fusicolla* appeared in both rhizospheric and root compartments, implying transitional colonisation capability. The increased prevalence of unidentified and "Not_Assigned" genera in rhizospheric soils highlights the potential for novel or uncharacterised fungal taxa in these niches.

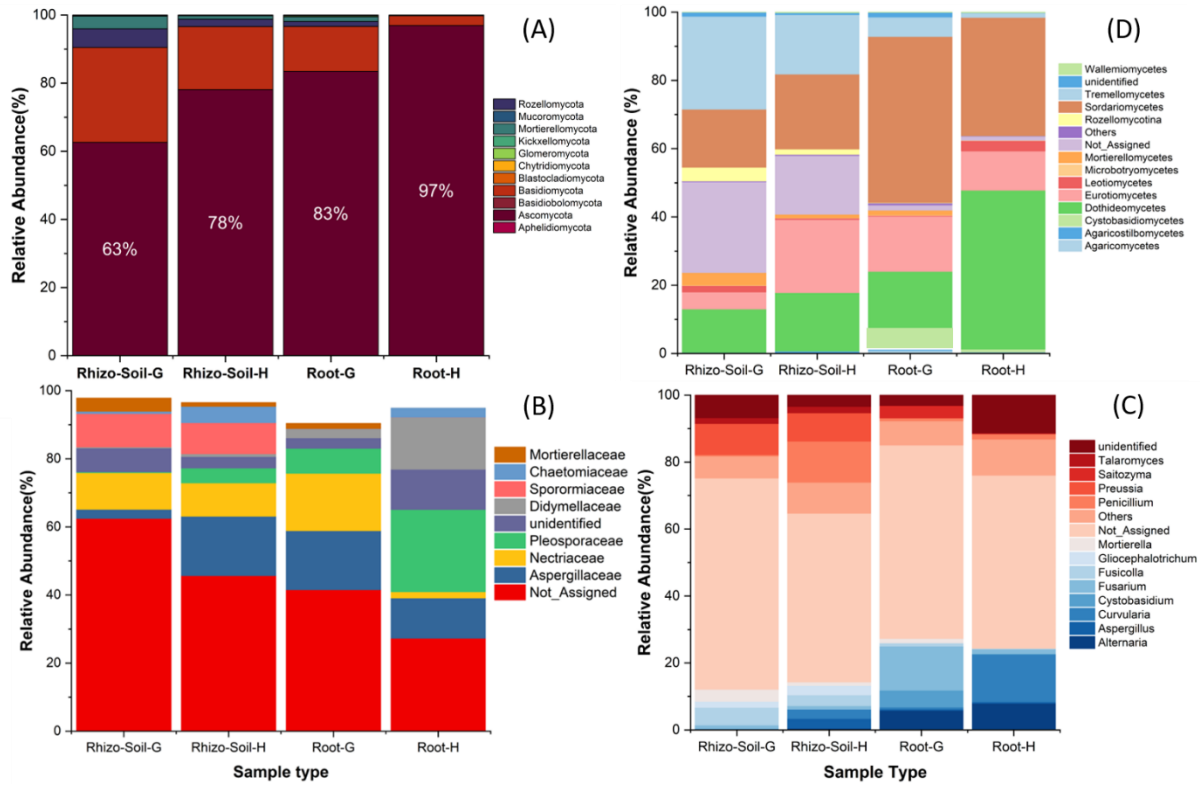

Fig. 3.2 Relative abundance of fungal taxa across four sample types—Rhizo-Soil-G (infected rhizosphere), Rhizo-Soil-H (healthy rhizosphere), Root-G (infected roots), and Root-H (healthy roots)—at various taxonomic levels. (A) Phylum-level composition (B) Family-level composition (C) Genus-level composition (D) Class-level composition

3.3.1.3 Core Microbiome analysis

Analysis of the core microbiome across all samples (Root-G, Root-H, Rhizo-soil-G, and Rhizo-soil-H) identified a limited yet ecologically significant assemblage of fungal taxa consistently seen at different relative abundance levels. The genus Alternaria was identified as the sole taxon exhibiting consistent prevalence across all compartments, meeting stringent core criteria (prevalence = 1.0), hence underscoring its flexibility and generalist endophytic behaviour in rice roots. Other taxa exhibiting significant but not universal frequency included Fusarium, Mortierella, Glocephalotrichum, and Curvularia species, recognised for their functional flexibility and possible contributions to root colonisation and biocontrol.

Detection thresholds indicated that whereas Aspergillaceae and Tremellomycetes exhibited high relative abundances in particular compartments, their overall prevalence across all samples declined, implying compartmentalised niche preferences rather than a core connection (Fig.3.3). Fusicolla acetilerea and Preussia were consistently identified in several rhizospheric and endosphere samples, although they did not meet stringent core criteria.

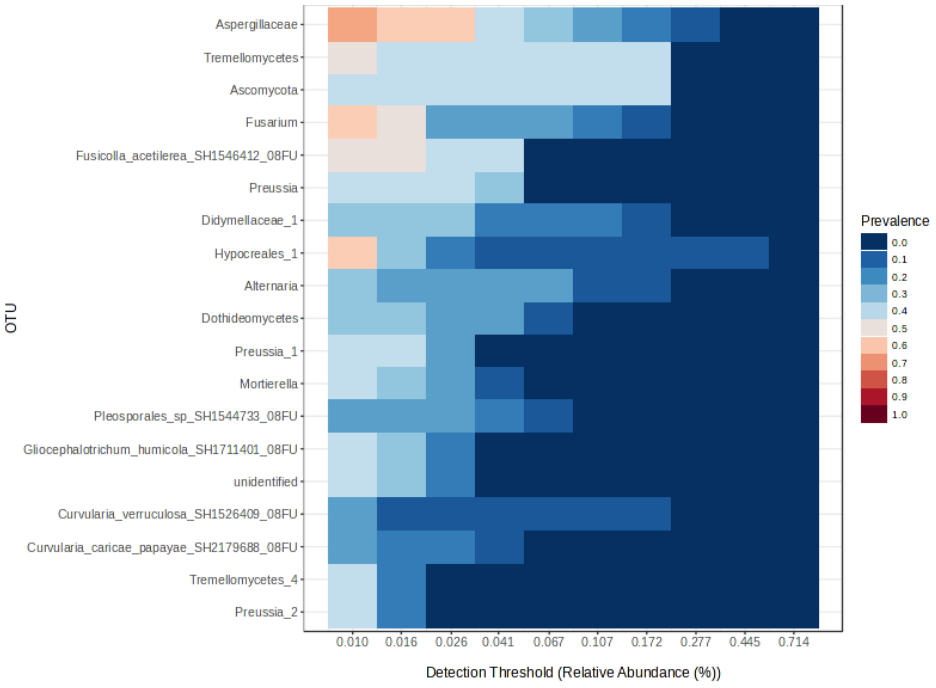

Fig. 3.3 Heatmap representing the prevalence and detection threshold of core fungal OTUs across all sample types. The x-axis indicates the minimum relative abundance (%) required for detection, while the y-axis lists taxonomically annotated OTUs. Colour intensity reflects OTU prevalence across samples, from dark blue (low prevalence) to red (high prevalence).

3.3.1.4 Beta diversity

Beta diversity study with Bray–Curtis dissimilarity and ANOSIM revealed distinct compositional dissimilarity among all sample types (Fig.3.4), with Root-G constituting a separate cluster. The ANOSIM test produced a high R-value = 0.7831 ($p<0.05$), indicating that fungal communities in infected root tissues differ significantly from those in healthy roots and rhizospheric soils. A PERMANOVA was performed with Bray–Curtis dissimilarity to statistically evaluate variations in fungal community composition among sample groups. The analysis revealed a notable distinction between groups ($F = 3.2934$, $R^2 = 0.5526$, $p < 0.05$), suggesting that more than 55% of the variance in fungal community structure is attributable to sample type (Root-G, Root-H, Rhizo-soil-G, and Rhizo-soil-H). This outcome aligns with and corroborates the visual patterns identified in the PCoA plot and ANOSIM test.

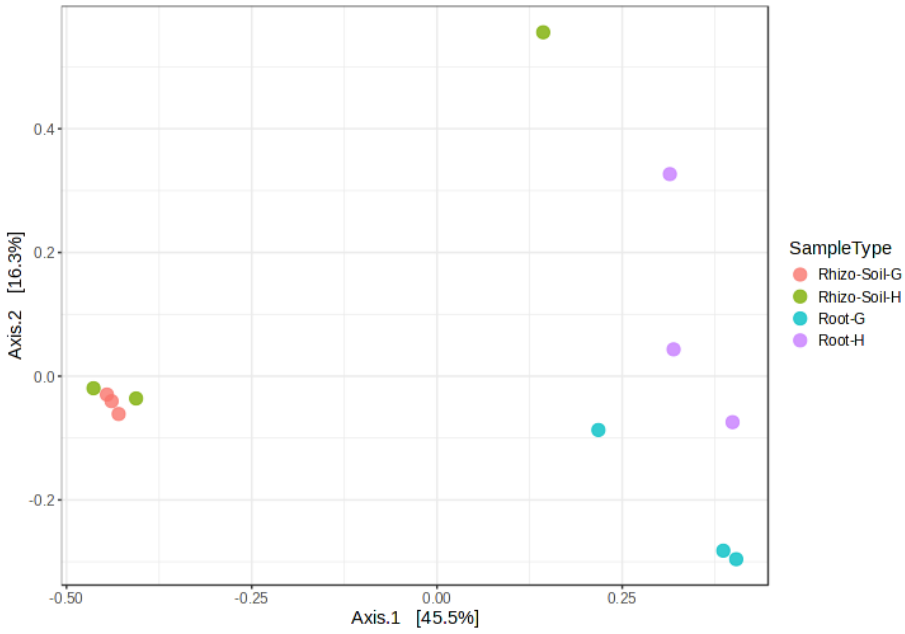

Fig.3.4 Principal Coordinates Analysis (PCoA) plot based on Bray–Curtis dissimilarity showing beta diversity of fungal communities across four sample types

3.3.1.5 Genera enrichment

LEfSe (Linear Discriminant Analysis Effect Size) analysis revealed 30 fungal species with significantly different abundances (LDA score > 2.0, $p < 0.1$) among the four sample types (Root-G, Root-H, Rhizo-soil-G, Rhizo-soil-H). Root-G (infected roots) exhibited a notable enrichment of stress-adapted and potentially antagonistic taxa, including Pseudopestalotiopsis, Nigrospora, Phialotubus, Medicopsis, Clonostachys, Metarhizium, and Xylaria. Conversely, Root-H (healthy roots) exhibited

a greater prevalence of mutualistic or commensal taxa such as Talaromyces, Fusarium, Curvularia, and Alternaria. Rhizospheric soils, especially Rhizo-soil-H, exhibited enrichment in saprotrophic and environmental genera including Mortierella, Preussia, Saitozyma, and Gliocephalotrichum (Fig.3.5). Multiple yeast genera (Symmetrospora, Rhodotorula, Naganishia) were exclusive to rhizospheric compartments, indicating sample type-specific ecological selection.

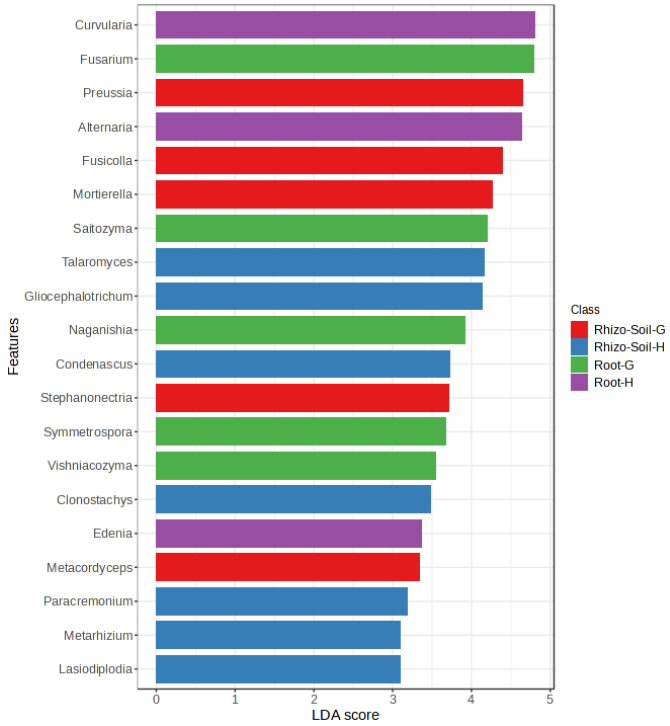

Fig. 3.5 Linear Discriminant Analysis (LDA) scores from LEfSe analysis showing the top differentially abundant fungal genera across four sample types: Rhizo-Soil-G (red), Rhizo-Soil-H (blue), Root-G (green), and Root-H (purple). Genera with LDA scores >2.0 and p-values <0.1 were considered significantly enriched.

Functional prediction based on top 20 genera:

The guild-based functional prediction of the top 20 LEfSe-enriched fungal taxa indicated distinct ecological strategies unique to each category. Infected roots (Root-G) exhibited an abundance of stress-tolerant and antagonistic taxa, including Metarhizium, Clonostachys, and Medicopsis, recognised for their nematophagous properties and secondary metabolite synthesis, indicating their involvement in nematode suppression and adaptation to gall-induced stress. Conversely, healthy roots (Root-H) contained characteristic endophytes and plant growth-promoting

fungus such as Talaromyces, non-pathogenic Fusarium, and Curvularia, signifying a stable mutualistic ecosystem. Rhizospheric soils were predominantly inhabited by saprotrophs (Preussia, Mortierella, Gliocephalotrichum) and stress-tolerant yeasts (Cystobasidium, Saitozyma, Vishniacozyma), indicative of their functions in nutrient cycle and environmental resilience. The observations indicate a transition from a symbiotic to an antagonistically enriched fungal community in galled tissues, influenced by selective recruitment mediated by nematodes.

Table 3.1 Ecological guild classification of LEfSe-enriched fungal genera

Genus	**Ecological Guild**	**References**
Curvularia	Plant Pathogen / Endophyte	Manamgoda et al., 2014
Fusarium	Pathogen / Endophyte	Summerell et al., 2003
Preussia	Saprotroph	Kuhnert et al., 2015
Mortierella	Saprotroph / Soil Fungus	FrÄ…c et al., 2019
Talaromyces	Endophyte / PGPF	Yuan et al., 2022
Gliocephalotrichum	Endophyte / Decomposer	Chen et al., 2020
Cystobasidium	Yeast / Commensal	VadkertiovÃ¡ et al., 2019
Penicillium	Saprotroph / Biocontrol	Houbraken et al., 2014
Chaetomium	Cellulose Decomposer / Endophyte	Lechat et al., 2016
Saitozyma	Yeast / Stress-tolerant	Tagele & Gachomo, 2024
Alternaria	Pathogen / Saprotroph	Thomma, 2003
Vishniacozyma	Yeast / Cold-tolerant	Sampaio et al., 2021
Metarhizium	Entomopathogen / Nematophagous	Jiang et al., 2014
Clonostachys	Antagonist / Biocontrol	Dandurand et al., 2021
Xylaria	Wood-decay / Endophyte	Stadler et al., 2014
Medicopsis	Necrotroph / Endophyte	Chen et al., 2013
Symmetrospora	Basidiomycetous Yeast	Fonseca-GarcÃa et al., 2016
Pseudopestalotiopsis	Secondary Metabolite Producer	Maharachchikumbura et al., 2014
Phialemoniopsis	Endophyte / Opportunist	Perera et al., 2022
Stephanonectria	Rare Soil Fungus	Liu et al., 2021

3.3.1.6 Venn Diagram

Analysis of the Venn diagram (Fig.3.6) indicated that 163 operational taxonomic units (OTUs) were common to all four compartments, representing the core fungal community of the rice root–rhizosphere system. Significant compartment-specific divergence was detected. Root-G and Root-H contained 197 and 201 unique OTUs, respectively, indicating distinct endophytic communities influenced by infection

status. Rhizospheric soils demonstrated increased richness, with 313 and 373 distinct OTUs in Rhizo-soil-G and Rhizo-soil-H, respectively, underscoring their function as multiple fungus reservoirs. Furthermore, 25 operational taxonomic units (OTUs) were exclusively shared between Root-G and Rhizo-soil-G, whereas 47 were shared between Root-H and Rhizo-soil-H, indicating compartmentalised and niche-specific fungal signatures, linked to nematode infection factor.

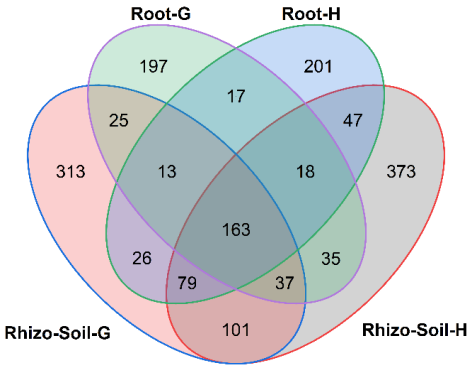

Fig. 3.6 Venn diagram of OTU present in four sample types

3.3.2 Culture based isolation and identification

A total of 37 endophytic fungi were isolated from *Meloidogyne graminicola*-gall-associated infected and healthy rice root tissues. Phylogenetic analysis of culture-derived isolates indicated that infected roots (galls) were predominantly constituted by various strains of *Fusarium proliferatum*, *F. solani*, *F. beomiforme*, *F. fujikuroi*, and *F. chlamydosporum*, which formed well-supported clades. Multiple gall-derived isolates of *Aspergillus niger* (e.g., G1G3, G3G2, F4) clustered closely, indicating their abundance and possible functional significance in gall habitats. Other infection-specific taxa, such as *Pestalotiopsis mangiferae*, *Curvularia kenpeggii*, *Letendraea helminthicola*, and *Montagnula sp.,* established different clades, indicating niche-specific adaptation. Conversely, healthy root isolates (e.g., J1R1, J3R2, J4R1) included *Fusarium beomiforme*, *F. proliferatum*, *Epicoccum latusicollum*, *Montagnula sp.,* and *Diaporthe melonis*, establishing phylogenetically separate clusters from their gall-associated counterparts. *Epicoccum sorghinum* was solely isolated from healthy roots, forming a cluster within the *Epicoccum lineage*. Ubiquitous saprotrophs, including *Leptosphaeria sp., Periconia sp.,* and *Schizophyllum commune*, were identified in both compartments, possibly indicating opportunistic colonisation rather than

specialist endophytism.

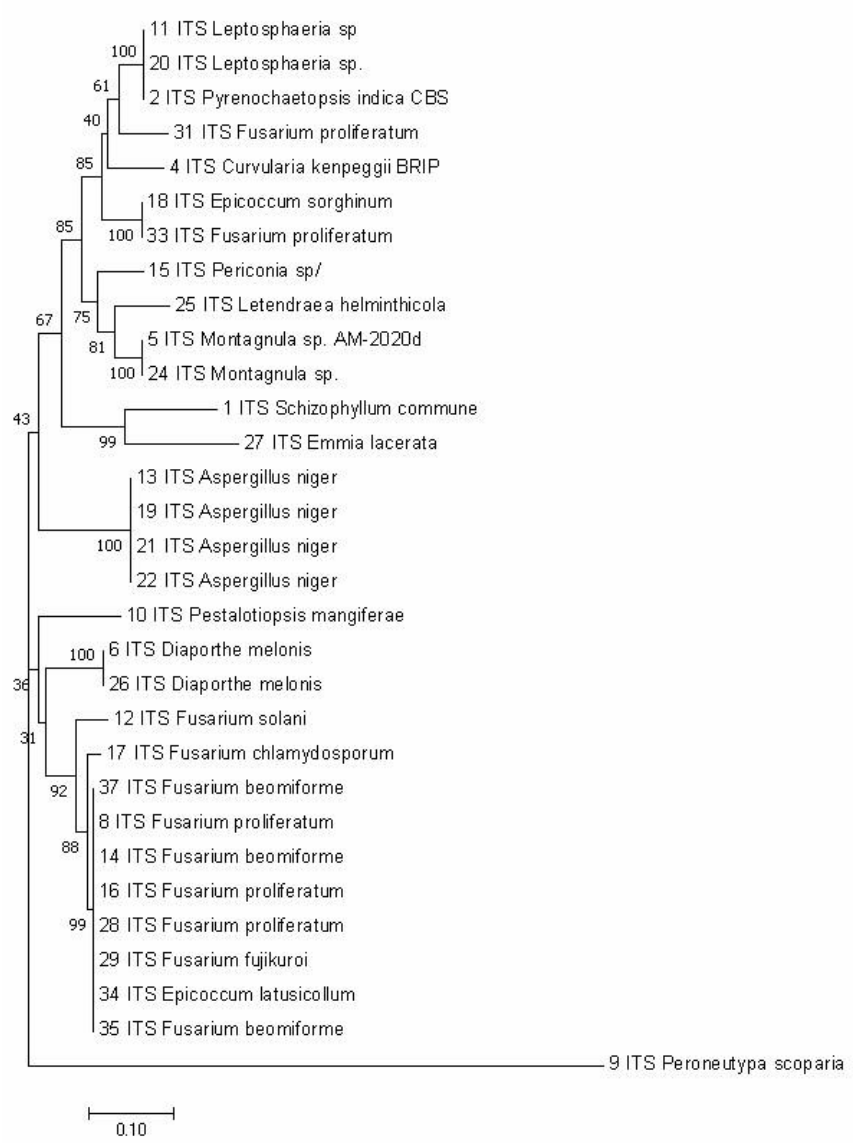

Fig. 3.7 Neighbour-joining phylogenetic tree based on ITS region sequences of fungal endophytes isolated from *Meloidogyne graminicola*-infected (gall) and healthy rice root tissues. The tree was constructed using MEGA (version X or specify) with bootstrap values (1000 replicates) indicated at major nodes. The tree illustrates the taxonomic diversity and clustering of 37 representative isolates, including multiple clades of *Fusarium proliferatum*, *F. beomiforme*, and *Aspergillus niger*. Notably, gall-derived isolates such as *Pestalotiopsis mangiferae*, *Letendraea helminthicola*, and *Curvularia kenpeggii* clustered separately from those associated with healthy roots (*Epicoccum latusicollum*, *Diaporthe melonis*), suggesting niche-specific divergence.

3.3.3 Biochemical Characterisation of Culture-Derived Endophytes

A series of qualitative enzymatic and secondary metabolite bioassays were conducted on fungal isolates derived from both Meloidogyne graminicola-infected and

healthy rice roots to evaluate their possible roles in nutrient cycling, plant interaction, or biocontrol. The assays encompassed the detection of cellulase, chitinase, amylase, pectinase, protease, siderophore generation, hydrogen cyanide (HCN), and β-1,3-glucanase activities. Several infected root-derived isolates, such as G3G5, G3G2, and G3R6, demonstrated significant cellulase activity, chitinase synthesis, and siderophore production, suggesting their possible role in host cell wall disintegration or iron chelation in galled roots. Isolate G3G5, subsequently recognised as Fusarium proliferatum, had elevated cellulase and chitinase activity, whereas G1G3 and G3G2, identified as Aspergillus niger, displayed significant siderophore production, a characteristic associated with antagonistic fungi. Conversely, isolates from healthy roots (e.g., J3R2, J3R3, J4R1) exhibited minimal enzymatic activity, with just a select few demonstrating mild cellulase or chitinase responses. Only one healthy isolate (J4R1) exhibited siderophore synthesis (++), whereas none tested positive for HCN or β-glucanase. The absence of amylase, protease, and pectinase activity in all isolates indicates that cell wall-modifying enzymes (chitinase, cellulase) and siderophores may be more dependable functional indicators of infection-responsive characteristics.

Fig. 3.8 Representative qualitative biochemical assays demonstrating chitinase, siderophore, and cellulase activity of fungal endophytes isolated from *Meloidogyne graminicola*-infected and healthy rice roots.

3.4 Discussions

This study offers a comprehensive evaluation of the rice root-associated fungal microbiome with influence of Meloidogyne graminicola infection, combining OTU-based sequencing with culture-dependent validation and functional profiling. The findings indicate significant restructuring of the rice root and rhizospheric mycobiome, demonstrating niche-specific community formation and stress-induced functional adaptability. High-throughput sequencing of the ITS1–ITS5 region produced over 16 million paired-end reads and identified 1148 high-confidence fungal

OTUs following denoising and rigorous filtering. Alpha diversity analysis indicated a notable reduction in both richness and evenness in infected roots (Root-G), aligning with environmental filtering due to biotic stress (Nemergut et al., 2013; Shade & Handelsman, 2012). These results correspond with additional studies indicating decreased microbial diversity in nematode-infested environments and pathogen-affected plant tissues (Tian et al., 2015; Santos et al., 2021; Kunda et al., 2024). At the phylum level, fungal communities were mostly composed of Ascomycota and Basidiomycota, with a decreased frequency of Ascomycota in infected roots, suggesting modified acquisition. Class-level and family-level alterations indicated a shift from Dothideomycetes-dominated mutualists in Root-H to stress-associated Eurotiomycetes, Sordariomycetes, and Aspergillaceae in Root-G. Comparable patterns of class-level rearrangement have been previously documented under pathogen stress in tobacco and legume systems (Jiang et al., 2024; Masson et al., 2022).

Beta diversity analyses (PERMANOVA, ANOSIM) validated substantial compositional alterations within root compartments, with more than 55% of the community variance accounted for by sample type. These results endorse predictable assembly induced by infection, similar to patterns observed in sick sugar beetroot and tomato roots (Trivedi et al., 2020; Hallmann & Sikora, 1996).

LEfSe analysis revealed gall-specific enrichment of functionally significant genera (Metarhizium, Clonostachys, Medicopsis, Pseudopestalotiopsis), acknowledged for their nematicidal and stress-tolerant properties (Jiang et al., 2014; Dandurand et al., 2021; Maharachchikumbura et al., 2014). Conversely, healthy roots exhibited an abundance of mutualistic and growth-enhancing fungi such as Talaromyces, Curvularia, and non-pathogenic Fusarium (Yuan et al., 2022; Manamgoda et al., 2014). Saprotrophic and yeast-dominated rhizospheric assemblages (Mortierella, Saitozyma, Vishniacozyma) exhibited habitat-associated functional divergence and microbial buffering ability (Frąc et al., 2019; Vadkertiová et al., 2019). The guild-based functional prediction corroborated the idea that nematode infection facilitates the recruitment of hostile and stress-adapted endophytes, whereas healthy roots sustain mutualistic fungal communities. Pocasangre et al. (2000) showed a comparable pattern, indicating that nematode infection altered the fungal endosphere to favour defense-oriented profiles.

Core microbiome research found Alternaria as the only genus consistently

prevalent across all compartments, aligning with its established generalist and adaptable ecological function (Thomma, 2003). The substantial quantity of distinct OTUs in each compartment and the limited core set highlight niche-driven microbial selection, as previously documented in root microbiome research under abiotic and biotic restrictions (Masson et al., 2022; Berendsen et al., 2012).

Culture-dependent isolation corroborated the metagenomic results, demonstrating the gall-specific predominance of Fusarium, Aspergillus, and Pestalotiopsis. Phylogenetic clustering of isolates indicated niche adaptability and host-mediated selection. These results correspond with those of Huang et al. (2023), who evidenced endophyte enrichment of Clonostachys rosea in tomato galls and soils suppressive to nematodes. Biochemical profiling validated the functional capabilities of essential gall-associated isolates. Increased chitinase, cellulase, and siderophore activity in *Fusarium proliferatum* and *Aspergillus niger* from Root-G suggests their potential involvement in nematode antagonism through structural degradation and iron competition (Spiegel et al., 1991; Lemanceau et al., 1993). The lack of HCN and protease indicates that these fungi depend more on structural enzymes and iron sequestration rather than the synthesis of toxic metabolites, a trend similarly observed in Metarhizium-based nematode suppression (Pelizza et al., 2017). In conclusion, *M. graminicola* infection induces a shift from a symbiotic to a predominantly hostile fungal community in rice roots, promoting taxa with biocontrol capabilities. The amalgamation of culture-independent and culture-based investigations underscores the adaptive versatility of the mycobiome and provides a basis for endophyte-centric nematode management techniques.

3.5 Conclusion

The combined metagenomic and culture-based analyses revealed that *Meloidogyne graminicola* infection significantly alters the composition, diversity, and functional traits of fungal endophytes in rice roots. Infected root tissues harboured reduced alpha diversity and showed compositional shifts towards stress-tolerant and potentially antagonistic taxa such as *Fusarium*, *Aspergillus*, and *Pestalotiopsis*. Culture-dependent isolation corroborated these patterns and identified several isolates with notable enzymatic and siderophore-producing traits, suggesting potential roles in nematode antagonism. In contrast, healthy roots maintained a more

stable and mutualistic fungal community dominated by genera like *Talaromyces*, *Curvularia*, and *Alternaria*. These findings underscore the influence of nematode infection on fungal community assembly and highlight the gall-associated fungal endophytes as a promising reservoir for the development of sustainable nematode biocontrol strategies.

Chapter 4: Objective 2

Chapter 4: Objective 2

Biological control of *Meloidogyne graminicola* using root-gall specific fungal endophytes: a promising approach for nematode management

4.1 Introduction

Global food security is significantly impacted by rice production in Southeast Asia, where India and China together contribute to almost 49% of the world's total production (Bandumula 2017). Rice is one of the most important staple foods and provides nutrition for about half of the world's population. Additionally, it has an impact on billions of people's lives and the economy (Le et al., 2009). In India, rice is the widely cultivated crop, 'aking up around 23% of all arable land (Food and Nations, 2020) from 35% of the national food grain area. It provides 41% of the nation's total food grain production or over 60% of daily energy needs. Rice contributes roughly 10% of the agricultural GDP and its cultivation creates 3.5 billion man-days of employment annually through production (Ahmad, 2021).

Among the several biotic constraints of agricultural production, the sedentary root endo-parasite root-knot nematode (*Meloidogyne spp*.), is one of the most damaging pests. In the case of rice, the rice root-knot nematode (RRKN), *Meloidogyne graminicola* is considered to be a serious threat worldwide, particularly in the rice growing regions of Southeast Asia. In India, RRKN poses a huge challenge to the irrigated and upland rice ecosystems, inflicting 20-80% yield loss under favourable conditions (Haque et al., 2018; Khan et al., 2014). In monetary terms, the yield loss due to RRKN costs Indian agriculture about Rs. 23.27 billion annually (Kumar et al., 2020).

After infection in rice, RRKN establishes a permeant feeding site in the root tips consisting of a single multinucleated giant cell creating a hook-like terminal gall (Khan and Ahamad 2020). As RRKN targets the root vascular system, its infection causes nutrient depletion and poor water transport in rice resulting in the generation of non-specific above-ground symptoms like stunting, wilting, chlorosis and severely affecting productivity (Tapia-Vázquez et al., 2022). Traditional management practices like soil flooding and applications of nematicides for seed treatment or in the seedbed are

either no longer effective or banned (e.g. carbofuran) (Jain et al., 2011; Tandingan et al., 1996). The recent adoption of labour and water-conserving techniques could in fact aggravate the RRKN problems in rice production, particularly in direct seeding and furrow irrigated conditions (De Waele and Elsen 2007). Repeated usage of environmentally harmful nematicides is also not economically feasible for the resource resource-poor farmers of India. An alternative management option that would be economical and ecologically sustainable is therefore urgently required.

Endophytes are microorganisms that reside inside the plant tissue asymptomatically (Porras-Alfaro and Bayman 2011). Endophytes are known for their numerous beneficial effects including antibacterial (Do 2022), antifungal (Xu et al., 2021), antinematode (Su et al., 2017) and plant growth promoting (Jaber and Enkerli 2016) properties. If employed judiciously, the organism or the bioactive substances they produce can contribute effectively to biocontrol (Gouda et al., 2016). For example, the extract of the fungal endophyte *Curvularia lunata* (MF113056) was reported to show 60% mortality of the third-instar larvae of the cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) (Saad et al., 2019). Two endophytes, namely *Bacillus velezensis* and *Pseudomonas putida* significantly inhibited the growth of rice blast (*Magnaporthe oryzae*) by 62.87% and 64.25% respectively (Do 2022). Another endophytic bacterium, *Streptomyces sp*. Was found effective against bacterial blight, caused by *Xanthomonas oryzae* in rice (Hastuti et al., 2012).

Besides insect pests and other plant pathogens, endophytes are also known to have important antagonistic roles against plant parasitic nematodes. For example, *Fusarium oxysporum* was reported to have biocontrol activities against *Helicotylenchus multicinctus*, *M. incognita*, *M. graminicola*, *Pratylenchus goodeyi,* and *R. similis* in banana, melons, or tomato (Kumar and Dara 2021). *Pochonia chlamydosporia*, an endophyte that colonizes the roots of higher plants, functions as a nematode egg parasite. Another endophytic fungus, *Chaetomium globosum* produces secondary metabolites including chetoglobosin and flavipin that have nematicidal effects on *M. javanica* juveniles (B. Khan et al., 2019).

There are instances, where gall-specific endophytes have been found to be effective against the root-knot nematode species. As an example, the fungus *Acremonium implicatum*, associated with tomato root galls was shown to have significant biocontrol activity against *M. incognita* (X. Tian et al., 2014). Therefore, the investigation on the effectiveness of the root gall-associated endophytes against *M.*

graminicola seems prudent. In light of the above discussion, the current study was designed with the following objectives: i) isolation and screening of endophytic fungi associated with healthy and galled roots produced by *M. graminicola* juveniles. Ii) *in-vitro* assessment of nematicidal potential of the isolated fungi against RRKN iii) confirmatory *In-vivo* evaluation of the most effective fungal isolate against RRKN using different mode of delivery, and iv) mechanistic understanding of antagonistic relationship using nematode development assay and plant defence enzyme estimation

4.2 Materials and Methods

4.2.1 Field selection and sampling

Following the protocol of Masson *et al.* (2020), a nematode-specific sampling was done from Giridih and Dumka districts of the state of Jharkhand, India during Kharif (July – Oct) season in 2020. These two districts were selected because rice was the principal crop and a previous survey revealed severe RRKN infestations in these areas (Mondal et al., 2022). Briefly, rice root samples from each district were collected from four sampling sites, maintaining a minimum distance of 1 km between sites. Each sampling site consisted of four random subsamples of 20 rice plants at the seedling phase collected from three nearby rice fields following the 'W' pattern sampling method (Namu et al., 2018). Samples were labelled, stored in a cooler at 4°C, and brought back to the laboratory for further processing within a week of collection. Collected rice plants were carefully observed for the characteristics of hook-shaped galls indicating *M. graminicola* infection.

4.2.2 Isolation of fungal endophytes

See section 3.5 of chapter for the details of isolation of fungal endophytes.

4.2.3 Nematicidal assay with fungal isolates

4.2.3.1 Maintenance of nematode culture

Single egg-mass culture of RRKN *M graminicola,* Giridih isolate was collected from the experimental farmhouse of Indian Statistical Institute (Giridih, Jharkhand, India). The nematodes were identified based on morphology and morphometrics of juveniles (J2) and female perineal patterns (Mondal et al., 2022). The nematode cultures were maintained in double steam-sterilized soil on *O. sativa* cv. PB-1121. The extraction of infective second stage juveniles (J2s) from the root galls of rice was performed following modified Baermann technique (Schindler, 1961; Van Bezooijen,

2006). Using a 400-mesh sieve, the freshly hatched J2s were collected and used in the experiments.

4.2.3.2 Preparation of fungal cultures

A total of 27 fungal isolates were cultured in Potato dextrose broth (PDB). Mycelial discs of 5mm diameter of all fungi were added into a 250ml conical flask containing 100ml of PDB and allowed to grow at 28 °C in the dark for 7 days in shaker incubator at 120 rpm (Fan et al., 2020). After 7 days, fungal broths were filtered with Whatman 1 filter paper, and then passed through a 0.22µm syringe filter to remove conidia and hyphae as much as possible (Tian et al., 2014).

4.2.3.3 *In vitro* nematicidal assay

Larvicidal potential of 27 fungal endophytes were assessed against second stage juveniles (J2) of RRKN *M. graminicola*. Equal volumes of fungal culture filtrates (1 ml) and nematode suspension containing 100 J2s were mixed properly in a six-well culture plate and incubated for 24 and 48 hours at 30 °C in the dark. Sterile distilled water and sterile PDB were used as control keeping rest of the conditions same, to check weather PDB has any significant effect on nematode mortality. All experiments were conducted with five replicates and repeated twice. Observation of nematode mortality was taken at 24- and 48-hours of post treatment. Nematodes were considered dead when its body posture did not change after adding few drops of 1N sodium hydroxide (NaOH) into the wells (Chen and Dickson, 2000). Mortality rates were corrected using Abbott's formula. The most efficient fungal isolate identified in this study was selected for further bioassays.

$$M\ (\%) = \left[\frac{Mt - Mc}{100 - Mc}\right] X\ 100$$

Where M_t and M_c means mortality percentage in treatment and control respectively.

4.2.4 Dose- and time-dependent nematicidal assay

Larvicidal assay was performed as previously described with four different concentrations (5%, 25%, 50% and 75%) of F4 culture filtrate (best performing isolate identified in the previous step, see section 4.3.2 for result). Distilled water was used as

the control. The number of dead J2s were counted under the microscope (Carl Zeiss Axioscope Stemi-305) at 24 and 48 hours of post-inoculation. In case of time dependent larvicidal assessment, 50% concentrations of F4 culture filtrate were used as treatment and observation were taken at 6, 12, 24 hours of post inoculation. The six-well culture plates were kept in dark at 30°C in both experiments. The number of dead J2s were counted at above mentioned time intervals and mortality (%) was calculated as previously described. Experiment was conducted with five replications and was repeated twice.

4.2.5 In vitro ovicidal assay

For assessment of ovicidal properties of the fungal isolate, 200 RRKN eggs were incubated after carefully mixing in equal volume of F4 culture filtrate (2 ml total volume) of different concentrations (viz. 5%, 10%, 25%, 50% and 75%) in six well culture plates. Before the bioassay, egg masses were surface sterilized with 0.5% NaOCl for 30 sec (Hussey and Barker 1973). Covered culture plates were incubated at 30°C for 7 days. On the 8th day of post-inoculation, a drop of Lugol's iodine solution was added into each well to stop egg hatching, and the number of juveniles hatched from the eggs were counted under a microscope. Eggs incubated with distilled water served as the control. Hatching inhibition (HI) was calculated with the following formula (Jang et al., 2016)

$$HI = \left[\frac{(C - T)}{C}\right] X100$$

Where C and T are the percentages of eggs hatched in the control and treatment, respectively. Egg hatching rate (EH) was calculated using the formula given below:

$$EH = \left[\frac{J}{E + J}\right] X100$$

Where J and E represents the number of hatched juveniles and unhatched eggs in a particular treatment, respectively.

4.2.6 Attraction assay

An experiment was conducted to observe chemotaxis of nematodes towards the F4 culture treated and non-treated rice roots. Pluronic F-127 (Sigma, BASF) gel is used

for this *in-vitro* attraction assay of *M. graminicola* J2s. The experiment was conducted with roots of 7-day old seedlings of nematode susceptible rice cv. MTU1010. Root tips (1 cm long) were first surface sterilized with 0.1% sodium hypochlorite for 1.5 minutes followed by repeated washing in distilled water (Oyebanji et al., 2009). Root tips were dipped in F4 culture filtrate for 3 hours. Roots tips treated with sterile PDB served as the control. Each root tip was placed at the centre of each well of a six well culture plate (3.5cm well size). Freshly hatched J2s (n = 200) in 20µl of distilled water, were suspended in 2 ml of 23% Pluronic gel (Wang et al., 2009). Number of nematodes near the vicinity of roots of 1.5-2 mm were counted at 2-, 4- and 6-hours interval. This experiment was conducted with 10 replications and repeated twice.

4.2.7 Identification of fungal isolate

For morphological identification, a monoconidial culture of isolate F4 was prepared from pure culture plate and mounted in lactophenol without dye. Fungal colony morphology, conidial structures, morphology and measurements of fruiting structure were taken under a compound microscope at 40× and 100× magnifications (Carl Zeiss Scope.A1) and photographed. Fungal colony diameter of a 7-day old F4 culture grown in PDA plate at 30°C was measured with a slide callipers.

For molecular identification, DNA was extracted from 5 – 10mg mycelial mat collected from 5-day old culture maintained on PDB and ground well in liquid N_2 with a sterile mortar and pestle. Genomic DNA was isolated manually following the modified CTAB method (Li and Yao, 2005, Wu et al., 2001) and the presence of DNA were confirmed in 1% agarose gel. Internal transcribed regions were amplified using universal primers ITS1 (5´-CTTGGTCATTTAGAGGAAGTAA-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´). Subsequently, fragment of the calmodulin (CaM) gene was amplified using the primers cmd5 (CCGAGTACAAGGARGCCTTC) and cmd6 (CCGATRGAGGTCATRACGTGG) and the β tubulin (BenA) gene was amplified using the primers Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (ACCCTCAGTGTAGTGACCCTTGGC) (Glass and Donaldson, 1995; Hong et al., 2005) The PCR amplification profiles were set as reported earlier by Kim et al., (2016) for ITS region and, Samson et al., (2014) for Cam and BenA regions. The amplified PCR products were purified using PCR purification kit and subjected to sequencing according to (Dutta et al., 2023). Sequences were finalized and submitted to NCBI. Alignment of ITS sequences with the representative strains of *A. niger* and some other

Aspergillus species were performed using MUSCLE program (Edgar, 2004). Multi locus concatenated phylogenetic tree was constructed with best-fit model as described earlier (Hazra et al., 2020, Hazra et al., 2021). The resultant tree was visualized and represented with FigTree software (Rambaut 2014).

4.2.8 Greenhouse experiment for biocontrol potential

An *in vivo* pot experiment was designed i) to test the biocontrol potential of F4 under greenhouse conditions, and ii) to identify the best method of application that to achieve highest nematode control. Five treatments, were designed as detailed below:

Control	Plant only (P)
T1	Plant + nematode (PN)
T2	Root dipping + nematode (RD)
T3	Soil drench + nematode (SD)
T4	Seed treatment + nematode (ST)

RRKN susceptible rice cv. MTU-1010 seeds were surface sterilized using 70% ethanol and 1% sodium hypochlorite for 3 minutes and 1 minutes respectively followed by repeated washing with distilled water (Zhao et al., 2018a). Seeds were germinated in a seed germinator (REMI CHM-12 plus) at 28–30 ºC for15 14 days. For ST, surface sterilized seeds were soaked overnight with 15ml of fungal culture filtrate before germination. For the RD treatment, seedlings were dipped in fungal culture filtrate for 6hrs before planting. Seedlings were sown singly in each pot containing 300 cc of autoclaved (at 120 ºC, 15 psi for 1 hour for 2 consecutive days) soil and vermicompost mixed at 1:1 ratio. For the SD treatment, soil was drenched with 5ml of fungal culture filtrate one day prior to the sowing of seedlings. Freshly hatched RRKN J2s (n=500) were inoculated near the root zone of seedlings two days after seedling plantation at a depth of 3 cm (Mondal et al., 2021).

Plants were maintained under greenhouse conditions at 30–35 ºC and 75–80% relative humidity. Hoagland nutrient solution (20 ml) was added to each pot twice in a week. The whole experiment was arranged in a completely randomized design with five replications per treatment and experiment was repeated twice. Plants were harvested at 28 days of post treatment (DPI) and number of root galls per plants were visually counted. A magnifying glass was used when galls were too close to each other. Plant growth parameters such as root length, shoot length, total fresh weight, and the

number of tillers were counted as well (Huang et al., 2015). The roots for the treated plants were stained with acid fuchsin and de-stained with acidified glycerol in 2-3 days and stored in the fixative solution for future nematode counts. Stained roots were examined under the microscope to count total number of nematodes present in every treated root and reproduction factor was calculated after that.

4.2.9. Effects on nematode development and plant defence

The best treatment identified in the previous step was used to formulate a pot experiment to investigate the role of F4 culture filtrate on the development and reproduction of RRKN. Rice cv. MTU 1010 seeds (n = 100) were surface sterilized as previously described and was allowed to germinate in a seed germinator (REMI CHM-12 plus) at 28–30 ^{0}C for 14 days. Four treatments, (i) plant only (P), (ii) plant + nematode (PN), (iii) plant + fungus culture filtrate (PF), and (iv) plants + fungus culture filtrate + nematode (PNF) was designed for this experiment. For the application of the fungus, roots of the germinated seeds were dipped in the F4 culture filtrate for 6 hours, identified as the most effective treatment in the greenhouse experiment (see section 4.3.7 for results). For P and PN treatments, roots were dipped in distilled water for 6 hrs. Seedlings of all the treatments were sown singly in pots under greenhouse conditions (30–35 ^{0}C and 75–80% RH). Plants were allowed to grow for two days after which ~500 freshly hatched RRKN J2s were added around the root zone of the PN and PNF treatments. This experiment was repeated twice.

4.2.9.1. Nematode development

Plants from the treatments PN and PNF were used for the assessment of nematode development. Plant samples collected at the 7 and 14 DPI were examined for nematode development stages, whereas plants harvested at 21 and 28 DPI were used for reproduction assays. At specified dates, harvested plants were washed gently in tap water to prevent tearing of galled roots. For visualisation of nematode life stages, root samples were stained with acid fuchsine followed by de-staining (Bybd et al., 1983). At 21 and 28 DPI nematode eggs and J2s were extracted by blending the roots for one minute in a 1% sodium hypochlorite solution at 20s intervals for three minutes (Hussey and Barker 1973, Salalia et al., 2017). The resultant suspension was sieved through a 100-mesh sieve and the collected nema-suspension was used for counting eggs+J2s. Counting was done in 5 ml aliquots of nema-suspension with three replicates (Merny and Luc 1969). Final eggs+J2s count were estimated by multiplying

the average count with the final volume of nema-suspension. Reproduction factor (RF) was computed as a ratio between final eggs+J2s count to the initial inoculum (Win et al., 2016).

4.2.9.2 Plant defence related enzyme activities

Plants harvested at 28 DPI for all four treatments, were used for the measurement of the total root phenolics, flavonoids and the defence-related enzymes like peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonialyase (PAL). Care was taken during harvesting to avoid any root damage. The biochemical tests were performed following standard protocol as described below.

(6) *Root Phenolics and Flavonoids*

To extract root phenolics, 20 µL of 80% methanol was combined with 2g of powdered roots crushed in liquid nitrogen. The mixture was then centrifuged at 11000 RPM for 10 mins and 1ml of supernatant was collected. Folin-Ciocalteu's reagent was mixed with the supernatant followed by incubation at 25°C for 30 minutes. We followed the protocol of Kim et al. (2019) to estimate root phenol content against a gallic acid standard curve.

Flavonoid concentrations were measured using the protocol of Zhao et al. (2018b). Briefly, 0.3 g of root samples were homogenised in 2 mL of a 99:1 methanol/HCl combination. The root extracts were shaken for 12 hours at 25℃, followed by 20 minutes of centrifugation at 4200 rpm. The supernatant (300 µL) was mixed with 5% (w/v) $NaNO_2$ (300 µL), 10% (w/v) $AlCl_3$ (300 µL) followed by 2 mL of 1 N NaOH after 6 mins. The amounts of flavonoids were determined against a rutin standard curve (0–100 mg/L) and absorbance was measured at 510 nm with a spectrophotometer.

(b) Estimation of POD

Freeze-dried root samples (1 g) were homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 7) at ice cold conditions. The supernatant was collected after centrifugation of the homogenate at 11,000 rpm for 10 mins at 4℃. Guaiacol served as the substrate used to calculate the POD activity. 20 µL of crude enzyme, 20 µL of distilled water, 2.66 mL of 0.1 M SPB, 150 mL of substrate solution (4% (v/v) guaiacol diluted with distilled water at 25 °C), and 150 mL of 1% H_2O_2 were combined in a 3 mL reaction mixture and mixed quickly in a glass cuvette. The reaction mixture was incubated at room temperature (25–30°C) for 2 min. The POD enzyme activity was estimated by measuring the absorbance at 470 nm (Hammerschmidt et al., 1982).

(c) Estimation of PPO

The freeze-dried root samples (1 g) were homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) followed by centrifugation at 16,000 rpm for 15 minutes at 4 °C. The supernatant (200 µl) was mixed with 1.5 ml of the 0.1 M sodium phosphate buffer (pH 6.5). 200 µl of 0.01M catechol was added to the mixture and the enzyme activity was estimated by measuring the absorbance at 495 nm (Mayer et al., 1966).

(d) Estimation of PAL

To measure PAL activity 1g of root was freeze dried in liquid N2 and homogenized using 25mM sodium borate buffer (1ml, pH 7.0) with β-mercaptoethanol (32mM) and centrifuged at 10000×G for 20 min. The reaction mixture containing enzyme extract (0.1 mL), borate buffer (0.5 mL; pH 8.7), distilled water (0.65 mL) and 0.1 mM L-phenylalanine (0.25 mL; pH 8.7) was kept at 32°C for 30 min. The reaction was terminated by addition of 1M trichloroacetic acid and the absorbance was measured at 290 nm. PAL activity was measured as moles trans-cinnamic acid mg^{-1} protein (Nagarathna et al., 1993).

4.2.10 Statistical analysis

R statistical software (R-3.6.2) was used to conduct all statistical analyses. All the data were first examined for homogeneity of variance and normality using the Shapiro Wilk normality test and Bartlett test before analysis of variance. Wherever required [log (x + 1)] transformation was used to improve normality and homogeneity of variance. If normality assumption is not achieved, the non-parametric Kruskal-Walis Chi square test was run. Significant variations between treatments were examined using the Duncan's multiple range test.

4.3 Results

4.3.1 Isolation of fungal endophytes

In total, 32 fungal isolates were cultured from galled and healthy roots of rice. Twelve fungal colonies were obtained from the healthy roots, while the rest were retrieved from root galls developed as a result of RRKN infection.

4.3.2 In vitro nematicidal assay

PDB media had no significant effects on nematode mortality in comparison to distilled water, hence distilled water was used as the control throughout the experiment. RRKN J2 mortality (%) varied among the fungal isolates as well as with

the observation time. Generally higher mortality was recorded at 48 h after treatments. J2 mortality ranged between 2.32% to 99.35% at 48h post treatment (supplementary Fig. S1). More than 50% J2 mortality was observed at 48 hours after treatment only for four root-gall specific fungal isolates (Fig. 4.1). Only one of these isolates, designated as F4, showed > 99% J2 mortality at both 24 and 48 h post treatment and was used for further experiments.

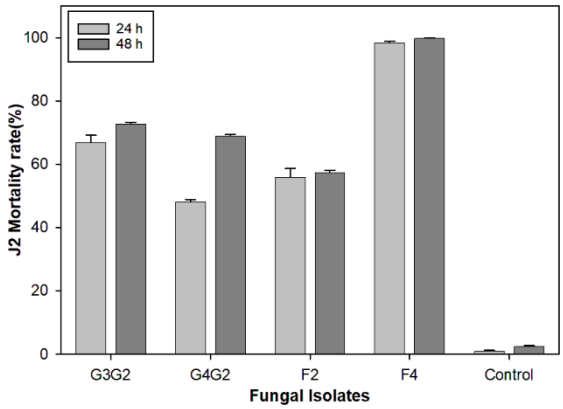

Fig.4.1. *In vitro* nematicidal assay revealed four fungal isolates showing >50% J2 mortality at 48 hrs after treatment (mean ± SE, $F_{4,15}$=229.3, p<0.05).

4.3.3 Dose- and time-dependent nematicidal assay

Significant mortality of J2s were observed at all treatment concentrations (5%, 25%, 50% and 75%) at both 24 h ($F_{4,10}$ = 2100, p<0.05) and 48 h ($F_{4,10}$ = 948, p<0.05) after incubation with increased mortality reported at higher treatment concentrations (Fig. 4.2A). Average J2 mortality ranged between 14.74% (at 5% treatment concentration) to > 99% (at 75% treatment concentration) at 24 h post treatment. Similar result was also observed at 48 h post treatment with no significant difference observed between 50% and 75% treatment concentrations (Fig. 4.2A). LC_{50} value of 10.85 % (R^2 = 0.9) was worked out through log of concentration and probit of J2 mortality.

The results of the time dependent assay revealed that > 70% J2 mortality was achieved at 6h post treatment (Fig. 4.2B). With increasing time of incubation, J2 mortality increased and reached > 97% 24 h post treatment. Significant J2 morality over control was achieved in all treatment durations (6 h: t = -42.83, df = 2.36, p<0.05; 12 h: t = -52.88, df = 2.19, p<0.05; and 24 h: t = -140.21, df = 3.57, p<0.05).

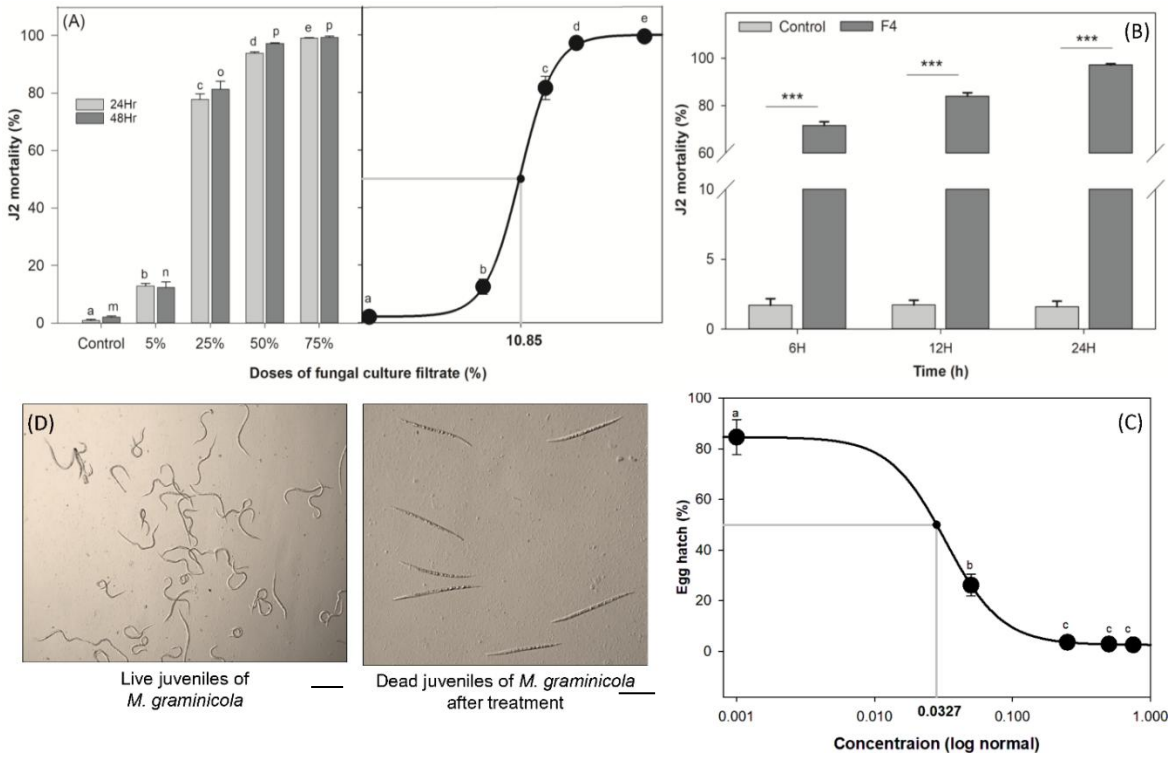

Fig.4.2. Results of dose- and time dependent nematicidal assays. (A) Dose vs. mortality study of RRKN J2 treated with culture filtrates of F4 isolate with different concentrations. The bars show mean + SE. Bars with same letters are not statistically significant as tested using Duncan's multiple range test at α = 0.05. (B) Time dependent mortality of RRKN J2s when treated with F4 culture filtrate at 50% concentration. Welch two sample t test was performed due to unequal variance. Significant variations in J2 mortality were observed between treatment and control at α = 0.05, "*" denotes significant difference. (C) Egg hatching (%) of RRKN eggs (±SE) when incubated with different concentrations viz. 5%, 10%, 25%, 50% and 75% (or, 0.05, 0.01, 0.25, 0.5, 0.75) of in F4 culture filtrate. Same letters above points indicate the no statistical different at the $p < 0.05$ level. (D) RRKN J2s treated with distilled water treatment (showing normal posture, left panel). Dead J2s treated F4 culture filtrate treatment observed after 48 hours post incubation (right panel) showing characteristics extracellular vacuolation. Scales bars = 100 µm.

4.3.4 In vitro Ovicidal assay

Treatments with F4 culture filtrate has significantly reduced hatching of RRKN eggs at all concentrations ($F_{4,10}$=362.5, p<0.05) with percent egg hatching varied significantly across different treatment concentrations (Fig. 4.2C). At 25%, 50% and 75% concentrations, average egg-hatching rate (EH) were found to be 3.5%, 2.8% and 2.6% respectively with no significant variation among them. Probit analysis showed an inverse sigmoidal relationship curve (R^2 =0.9). The LC_{50} value of % egg hatching was found to be 3.27±0.01%. At 75% treatment concentrations 96.96%±0.48 HI with respect to control was observed.

4.3.5 Attraction assay

Results of the attraction assay revealed that F4 treatment had significantly reduced

($F_{1,44}$ = 112.709, p<0.05) the number of RRKN J2s attracted towards the rice root tips at all time points (Fig. 4.3B). Observation time had a significant effect on the number of nematodes attracted towards the root tips ($F_{2,44}$ = 5.643, p<0.05). As shown in the graph, at 6 h after treatment, 21.87 ± 1.65 J2s were attracted towards treated root rips whereas the same for the control root tips was 116.87 ± 3.8. Aggregated J2s around the control root tips formed a ball like structure, whereas such formation was not observed in the case of control roots.

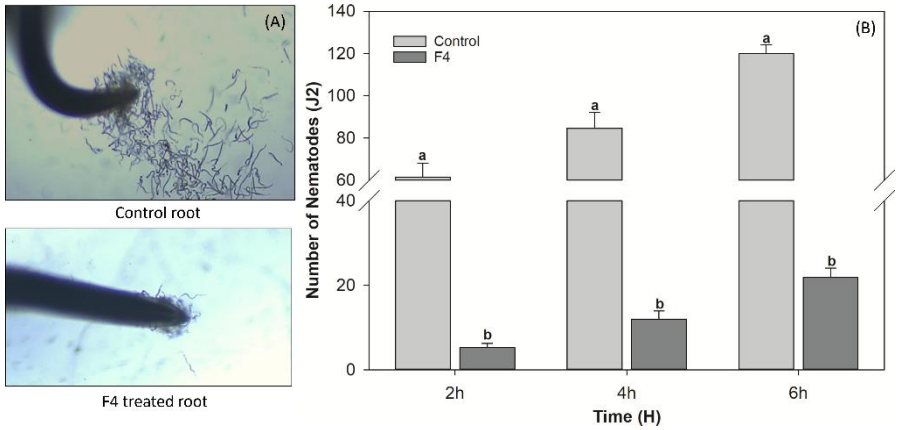

Fig.4.3. Attraction assay of nematodes in PF-127 gel is observed at three-time intervals. Each root received 200 second-stage juveniles as an inoculation (J2s). At each time interval number of nematodes around the vicinity of the root tip were recorded. Number of nematodes were significantly different between treated and non-treated rice roots. (A) Photomicrographs demonstrating the second stage juveniles' (J2s) attraction to the control and F4 treated roots at 6 HPI in the PF-127 gel. (B) The bars in the various graphs show the mean ± standard error of the data with eight replicates at each time point. According to Duncan's multiple range test, values with the same letters above the bar are not statistically different at the P<0.05 level. Scale=20µm.

4.3.6. Identification of the fungal isolate

The PDA medium supported fast growth of the F4 isolate, and the average colony diameter was 69.38±1.39 mm in 7 days at 30°C. The mycelia of the isolate were white to pale yellow in colour (Fig. 4.4C). When grown in the PDB medium under shaking condition, the fungal mycelia aggregated to form ball-like structures of diameter ranged between 0.23-0.8 cm. The colour of the 7-day old culture filtrate becomes pale green in colour (Fig. 4.4B). The fungus had spherical vesicular unbranched conidiophore with uniseriate to biseriate phialides/sterigmata and dark-brown to black conidia (Fig. 4.4D*)*. Conidial heads nearly globose ranged 19.91 µm–23.26 µm. The size of conidiophore from foot cell to the base of vesicle varied typically 960 -1730 × 10.2-13.4 mm. Conidia were globose to sub globose, 3.6-4.8 mm in diameter, dark-brown to black, and produced in a single chain on phialide. These above-mentioned characteristics indicated that the target fungus belong to the genus *Aspergillus*.

Amplification and sequencing of internal transcribed spacer region using ITS1 and ITS4 universal primers, camD and benA led to identification of the isolate as *Aspergillus niger*. The sequences were submitted in GenBank under the accession numbers: OL505015 for ITS, OR031775 for CamD and OR031776 for BenA. A phylogenetic tree was constructed using the generated sequence and database information obtained from NCBI, focusing on the homologous regions. The analysis revealed that *A. niger* isolate F4 clustered together with various other *A. niger* isolates in a distinct clade (Fig. 4.4A).

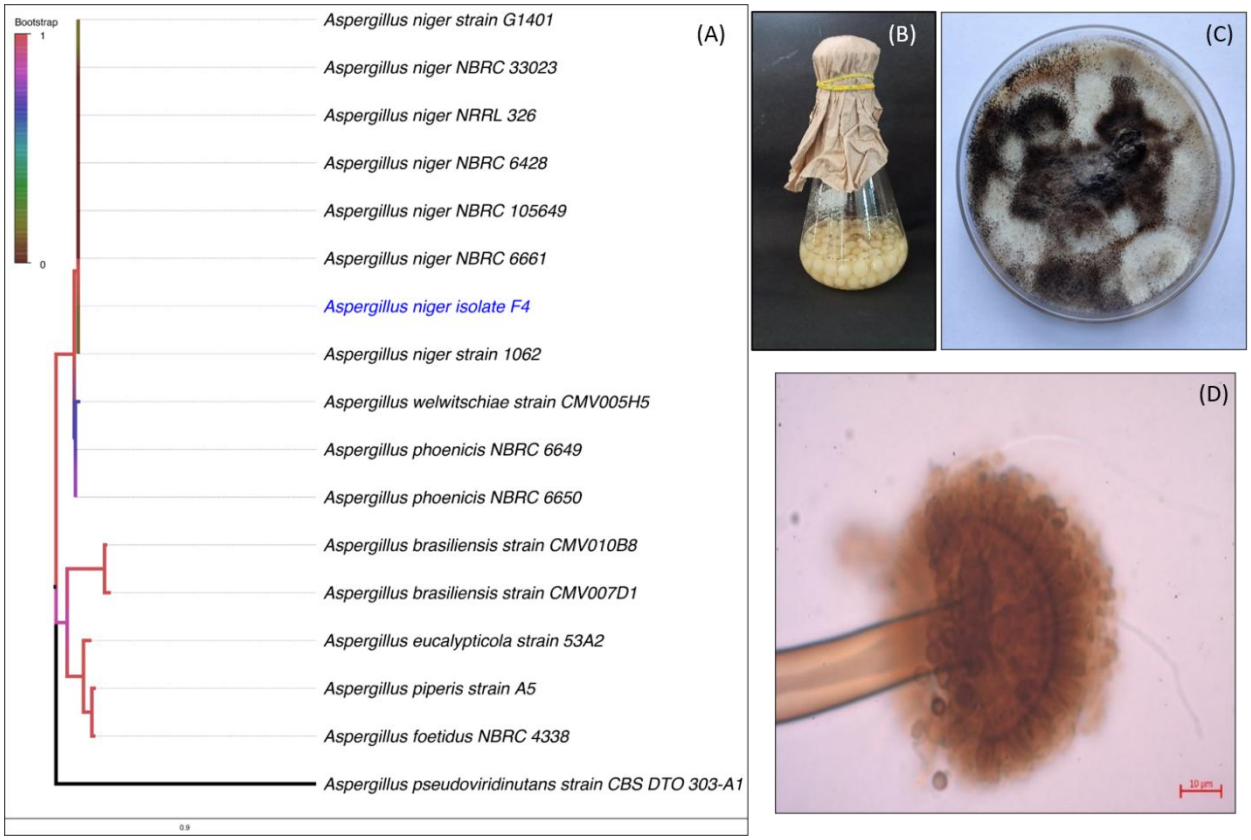

Fig.4.4. (A) The phylogenetic tree is the inference based on concatenated sequences from 3 genomic loci (ITS, benA and cmdA) constructed using the neighbour-joining method (bootstrapped 1000 times). (B) Seven-day old F4 culture filtrate in PDB. (C) F4 culture plate on PDA showing morphological characters. (D) Microscopic image of F4 culture under 40X magnification showing conidiophore and conidial characteristics.

4.3.7 Greenhouse experiment for determination biocontrol potential

4.3.7.1. Plant growth parameter

No variation was observed in shoot lengths of the rice plants across the treatment. However, root lengths varied significantly across treatments ($F_{4, 20} = 4.48$, $p < 0.05$) with no difference recorded between P and RD treatments (Fig. 4.5A). While, total

plant fresh weight varied significantly across treatments ($F_{4, 20} = 6.12$, $p < 0.05$), no difference was once again recorded between P and RD treatments (Fig. 4.5B).

4.3.7.2. Gall formation and reproduction

Results of the greenhouse experiment revealed that all the treatments had a significant effect on nematode mortality which was reflected in the reduction of number of galls per plant ($F_{3,16} = 4.125$, $p < 0.05$, Table. 4.1). Maximum reduction in the number of galls was found in case of root dipping (RD) treatment which was significantly lower than the other two treatments. No difference in gall count was recorded for the soil drenching (SD) and seed treatment (ST) applications. The control plants had maximum number of galls per plant (35.8±0.86)

Number of eggs + J2 per plant also was significantly reduced across all the treatments ($F_{3,16} = 69.23$, $p < 0.05$, Fig. 4.5C) with maximum reduction observed in case of RD treatment (2561.4±156.1) followed by ST (6086±610.6) and SD (6933.4±631.9), respectively. Although the difference in the number of eggs + J2 per plant was not significant between ST and SD.

A similar trend was also observed in case of reproduction factor (RF). All the treatments exhibited reduction of RF than the untreated control ($F_{3,16} = 69.23$, $p < 0.05$, Fig. 4.5C). Highest RF was recorded for the control plant (37.12±0.90, Fig. 4.5D). Maximum reduction was observed in case of RD treatment (5.12±0.31) which was significantly lower than both ST and SD treatments. No difference in RF was observed between ST and SD treatments.

Table. 4.1. Number of galls/plant in the in-vivo greenhouse experiment. The data represent means + SE from five replications. Different letters indicate statistically significant ($p < 0.05$) differences in gall count across the different treatment according to Duncuns new multiple range test

Treatments	Number of galls/plants
Plant + nematode (PN)	35.8±0.86[a]
Root dipping + nematode (RD)	2.8±1.11b[c]
Soil drench + nematode (SD)	16.8±0.37[b]
Seed treatment + nematode (ST)	17.2±1.17[b]

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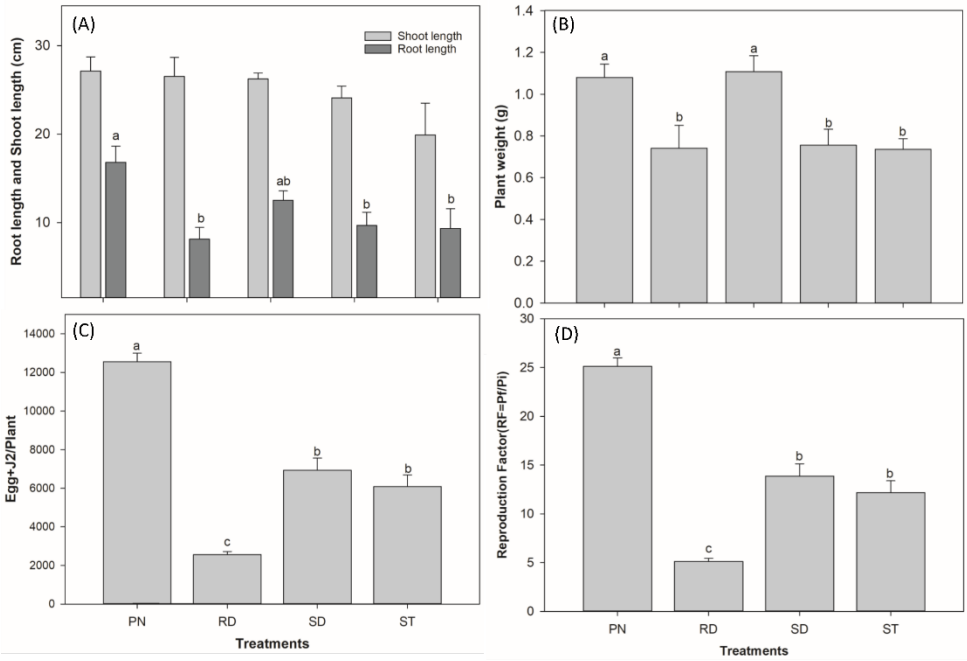

Fig. 4.5. Results of the *in vivo* pot experiment under greenhouse condition. Effect of different experimental treatments on (A) root length and shoot length (B) fresh weight, (C) Eggs + J2, and (D) Reproduction factor (RF), of RRKN at 28 DPI. The bars in the graph represent the mean + SE of the data from five replications. Different letters above the error bars indicated statistically significant ($p < 0.05$) differences in respective parameters across different treatments based on Duncan's new multiple range test. In case of shoot length, no significant ($\chi^2_{4,20}=3.9$, $p>0.05$) difference was observed across the treatments.

4.3.8. Effects on nematode development and plant defence

4.3.8.1 Effects on nematode development

Majority of the J2s that penetrated roots remained in that stage at 7DPI (Fig. 4.6A) in both treated and control plants. The number of J2s that penetrated that root varied between PN (control) and PNF treatments (t = 5.1914, df = 2.4556, p<0.05). At 14DPI, for both treatments, J3/J4 was the predominant life stage with significant difference (t = 10.661, df = 2.0748, p<0.05) in the number of nematodes recovered between treatments. Majority of the nematodes across treatments have progressed to the mature female stage by 21 DPI. An influx of newly hatched J2 was recorded from PN roots at 28 DPI. Mature female stage was also recorded at this time points. The number of nematodes in both time stages varied significantly between treatments (female: t = 2.9516, df = 2.0468, p<0.05 and juveniles: t = 12.784, df = 3.9802, p<0.05)

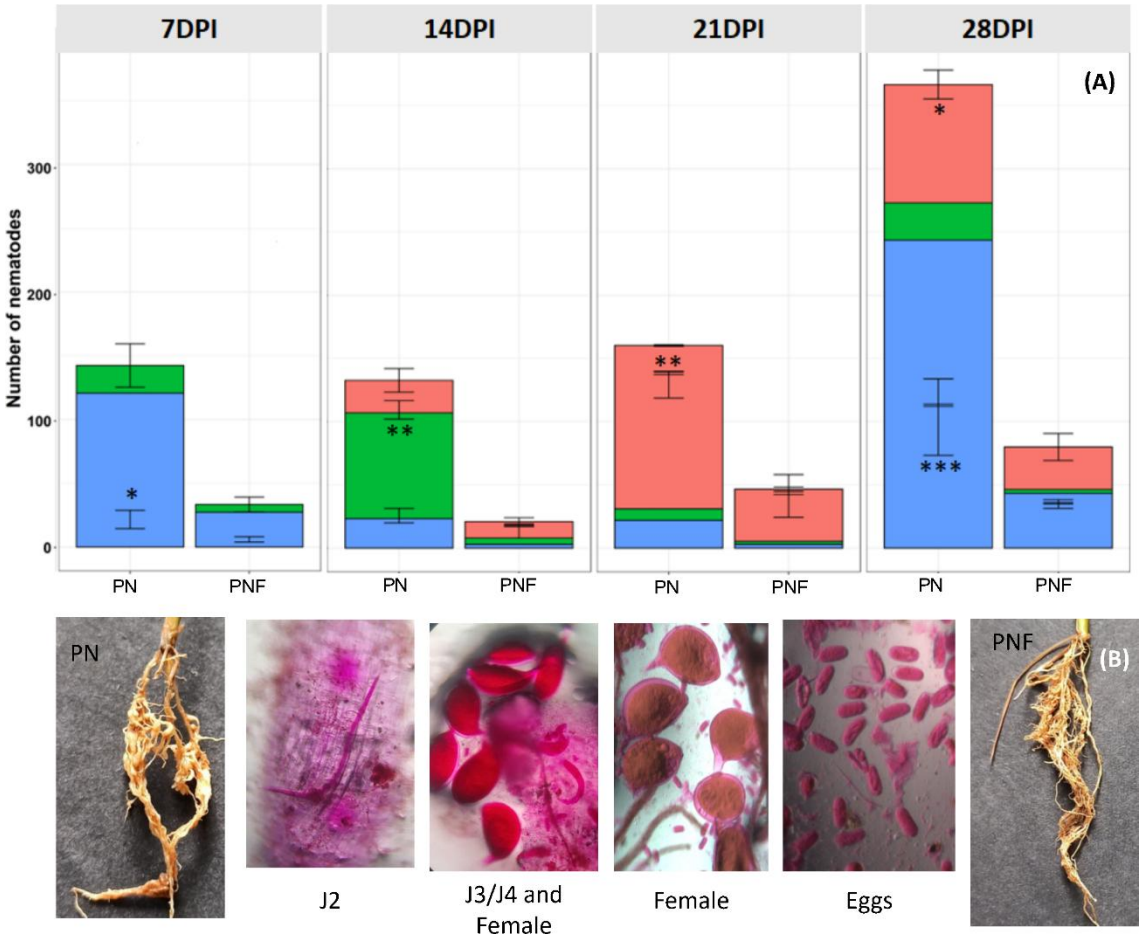

Fig.4.6. *M. graminicola* life stages at different time points (7, 14, 21, and 28 DPI) in (A) plant + nematode (PN, control) and plant + nematode + fungus (PNF) treated roots. Each stack represents the total number of nematodes at different life stages (colour coded). The bars represent the mean ± standard error of the data from five replicates. '*', '**' and '***' denotes statistically significant difference at $p< 0.05$, $p< 0.01$ and $p< 0.001$ levels as determined by welch two sample t-test due to unequal variation. (B) Photographs of root galls in two respective treatments PF and PNF, and photomicrograph of different life stages of RRKN stained with acid fuchsin. Female: matured female stage. J2: second-stage juveniles. J3/J4: intermediate stage juveniles.

4.3.8.2. Role of fungal culture filtrate in plant resistance related enzyme activities

Total root phenolic compounds ($F_{3,12}$ =220.6, $p<0.05$) and flavonoids ($F_{3,12}$ =182.1, $p<0.05$) varied significantly across treatments. Fungal treatments generally increased the total phenolic and flavonoid compounds in roots (Fig. 4.7B). Key plant defence enzymes varied significantly across treatments as well with F4 treatments aggravated the enzyme response in comparison to the control (PAL: $F_{3,12}$ =123.6, $p<0.05$; PPO: $F_{3,12}$ =1140, $p<0.05$; and POD: $F_{3,12}$ =57.35, $p<0.05$, Fig. 4.7 C, D). Plants exposed to both F4 fungus and the nematode showed the highest enzymatic response.

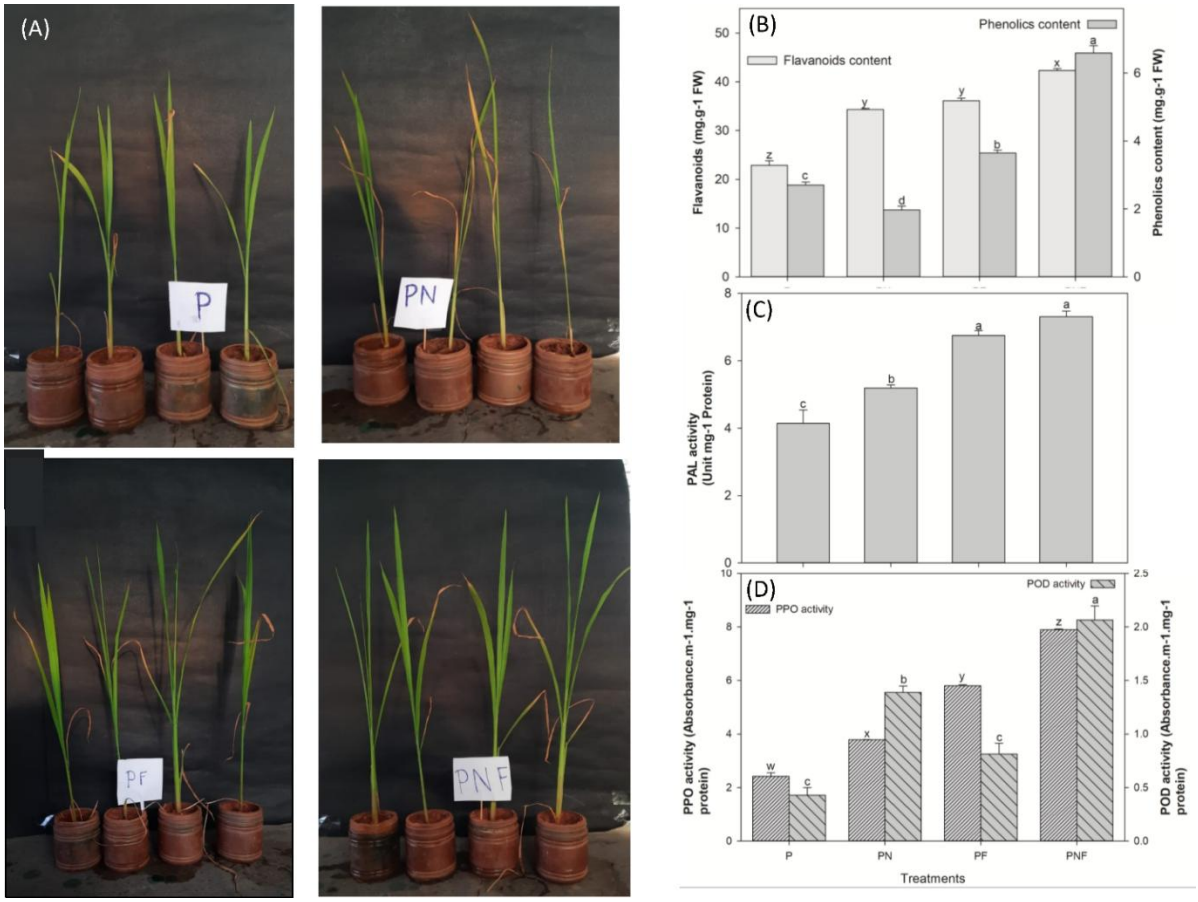

Fig. 4.7. Amount of secondary metabolite production, changes of defence enzyme activities in roots of rice plants subjected to different treatments. (A) Photograph of experimental setup. (B) Phenolics and flavonoid (mg/g FW) (C) PAL activity (U/mg protein) (D) PPO and POD activities (Abs./m/mg protein). Values were mean + SE of four replicates. Different letters above bars indicate statistically significant ($p < 0.05$) differences in respective parameters across different treatments based on Duncun's new multiple range test.

4.4. Discussion

Root galls developed during nematode infestation is a nutrient rich environment that can harbour numerous endophytic microorganisms. There have been notable findings regarding endophytes associated with nematode galls and their potential for biological control of nematodes (Tian et al., 2014). A metagenomic study conducted by Tian et al., (2015) revealed that nematode root galls had a higher diversity of endophytic bacteria compared to healthy roots, suggesting that the nematode gall-associated region may harbour unique endophytes with distinct characteristics. In our experiment, we identified four endophytic fungal isolates from RRKN root galls that exhibited >50% mortality of juveniles under *in-vitro* conditions. Among these, one isolate, identified as *Aspergillus niger* isolate F4 exhibited nearly 100% mortality of

M. graminicola juveniles under *in-vitro* and ~78% under *in-vivo* conditions, demonstrating its potential as a biocontrol agent against this major pest of rice. Our findings were in line with previous studies. For example, Goswami et al., (2008) reported the antagonistic effects of *Acremonium strictum* isolated from tomato roots, against juveniles and on the inhibition of egg hatching. In a similar study, Tian et al (2014) also demonstrated the biocontrol potential of *A. implicatum* against *M. incognita* in tomato. It is noteworthy that our findings constitute the first evidence of biocontrol potential of *A. niger* isolate F4, a gall specific endophytic fungus against *M. graminicola,* a major pest of rice.

In recent years, endophytes have emerged as an important tool for the biological control of plant-parasitic nematodes. Numerous examples exist where endophytes, isolated from diverse sources, have been employed to manage nematode infestation in different crops, either in isolation or in conjunction with other biocontrol agents. For instance, an endophytic fungus, *Alternaria* sp. Controls the pinewood nematode or pine wilt nematode (*Bursaphelenchus xylophilus*) population by producing alternariol 9-methyl ether (Lou et al., 2016). In another example *F. oxysporum*, isolated from banana, demonstrated nematistatic and nematicidal properties against the root-lesion nematode (*Pratylenchus goodeyi*). In addition, the application of endophytic bacteria *P. putida* and *P. aurantiacea* resulted in a 40.7% to 42.2% reduction in potato cyst nematode (*Globodera rostochiensis*) relative to the control roots (Trifonova et al., 2014).

Besides other nematodes, endophytes have also been employed for the control of root knot nematodes. For example, isolates of *T. asperellum* and *F. oxysporum* reduced the penetration of *M. incognita* juveniles and egg densities by 35–46% in tomatoes (Bogner et al., 2016). *Pseudomonas* sp. (EB3), *Bacillus* spp. (EB16, EB18), and *Methylobacterium* sp. (EB19) exhibited antagonism against the root knot nematode *M. incognita*, resulting in the reduction of number of adult females, egg masses, and gall index in okra plants (Vetrivelkalai et al., 2010). Examples exists, albeit few, on the use of endophytes for the control of *M. graminicola* infestation in rice. Padgham and Sikora (2007) published that the endophytic bacterium *B. megaterium* can reduce root penetration and gall formation by *M. graminicola* by more than 40%. However, the use of nematode gall specific endophytes in biological control of RKN is rare. Application of *Acremonium implicatum* in control of *M. incognita* in tomato was the only available example (Schouten 2016). Our study, to our

knowledge, for the first time shows the potential role of a gall associated fungal endophytes in the biological control of rice root knot nematode. It is also noteworthy that *A. niger* as nematode gall specific endophyte has never been reported before.

Endophytes suppress nematodes either by trapping (nematistatic) or killing them directly (nematicidal). The most prevalent nematode-trapping fungus, *Arthrobotrys* spp., has the distinctive capacity to generate sticky trapping nets when it comes into contact with nematodes (Niu and Zhang, 2011). Numerous endophytic demonstrated nematicidal capabilities by feeding and space competition, and synthesis of secondary metabolite or other toxic substances (Escudero et al., 2017, Schouten 2016). The result of our study suggests that nematode suppression by *Aspergillus niger* F4 involves multiple mechanisms. The *in vitro* bioassays have clearly demonstrated the nematicidal properties of the fungus, indicating the involvement of secondary metabolites, which aligns well with previous findings. For instance, *A. niger* culture filtrates have been found to produce oxalic acid (Jang et al., 2016), *A. candidus* produces citric acid (Shemshura et al., 2016), and *A. oryzea* produces kojic acid (Kim et al., 2016), all of which have known nematicidal properties. In a more recent study, Yeon et al., (2023) reported that *Aspergillus niger* F22 produces oxalic acid which exhibited strong bioactivity against *M. incognita* in tomato. Therefore, it seems prudent to assume that *A. niger* F4 also produces organic acid which is responsible for the bioactivity against *M. Graminicola*.

Preliminary microscopic observation revealed that numerous vacuoles are formed in the bodies of dead juveniles treated with F4 culture filtrate (Fig. 3D). This observation, albeit preliminary, is rare and was analogous to the only available report by Jang et al., (2016) who have observed the formation of vacuoles in *M. incognita* treated with *A. niger* F22 producing oxalic acid. Rajasekharan et al., (2017) reported the production of several medium and large sized vacuoles in the juveniles and eggs of the pine wood nematode *Bursaphelenchus xylophilus* and *M. incognita*, respectively when treated with 5-iodoindole. The rapid accumulation of vacuoles in the nematode body is referred to as methuosis, which is a type of programmed cell death leading to disruption of cellular membrane integrity. Hence, it is imperative to conduct a comprehensive chemical analysis of the F4 culture filtrate to identify the active constituents present.

In addition to the nematicidal properties of the fungus, attraction assays conducted in our study demonstrated that fewer nematodes (~18% of control) were attracted

towards the F4 treated roots, suggesting a possible repellent effect. Nematode development, however, was not suppressed in the fungus treated roots as revealed by the development assay. Both these results, when viewed in conjunction, suggest that *A. niger* F4 fungal broth contains chemicals as extracellular secondary metabolites that have repellent properties and the nematicidal activity.

Besides the direct effects, as evidenced in our study, inoculation of the fungus triggered plant defence response, potentially providing indirect protection against nematode infection. We observed an increase in the production of phenolics, flavonoids, and changes in the ethelene signalling pathway and jasmonic acid pathways, which subsequently led to enhanced activity of defence enzymes in the fungus-treated plants. These findings contribute to the numerous previous studies, demonstrating the induction of systemic resistance in plants as a result of inoculation of fungal endophytes (for a detailed discussion see Schouten, 2016).

Overall, the results of our study show the significant nematicidal activities of *A. niger* F4 both under *in vitro* and greenhouse conditions. Observation of vacuoles in the bodies of dead juveniles indicates the presence of chemical compounds in the culture filtrate with distinctive properties. Besides, inoculation by *A. niger* F4 provided indirect protection against nematode by triggering plant defence response. Future studies will be required to elucidate the active ingredients in the F4 culture filtrate. This study, to our knowledge for the first time, demonstrated the role of a gall specific endophytic fungus in biocontrol of plant parasitic nematodes.

4.5 Conclusion

Our study provided evidence that root galls developed during *M. graminicola* infection harbours distinct fungal endophytes communities with potent nematicidal properties. Of the total 32 fungal endophytes isolated from galled and healthy roots of rice, *Aspergillus niger* F4 exhibited nearly 100% mortality of *M. graminicola* juveniles under both *in-vitro* and *in-vivo* conditions. Preliminary microscopic observation revealed formation of numerous vacuoles within the bodies of dead juveniles. Available evidence, therefore, clearly established the potential of gall-specific fungal endophytes as biological control agent of *M. graminicola*, offering a novel management avenue for this important pest of rice.

Chapter 5: Objective 3

Chapter 5: Objective 3

Nematicidal Activity and Defence Priming by *Aspergillus niger* F4 Culture Filtrate Against *Meloidogyne incognita* in Tomato

5.1 Introduction

Root-knot nematodes (RKNs), *Meloidogyne* spp. Are among the most destructive and widely distributed plant-parasitic nematodes globally. They have a broad host range encompassing over 3,000 plant species, including cereals, vegetables, pulses, and horticultural crops. Annual global yield losses attributable to RKN infestations are estimated to exceed USD 100 billion (Kayani et al., 2017). RKNs have a obligate sedentary endoparasitic lifecycle. The infective second-stage juveniles (J2s) penetrate host roots in the apical zone and migrate intracellularly to the vascular cylinder inducing the development of multinucleated giant cell, which function as highly active metabolic sinks, supplying nutrients to the developing nematode (Xue et al., 2024). The resulting galls impairs water and nutrient uptake leading to considerable yield reductions and quality deterioration.

One of the most widespread and economically important RKN species, *M. incognita*, poses a major threat to tomato (*Solanum lycopersicum* L.), a high-value horticultural crop grown extensively across temperate and tropical regions. In heavily infested fields, yield losses in tomato can exceed 40–50%, depending on environmental conditions, cultivar susceptibility, and nematode population density (Nagendran et al., 2019). Despite the long-standing reliance on synthetic nematicides for nematode suppression, their use is increasingly constrained due to toxicity, environmental persistence, and regulatory restrictions. In recent years, a number of nematicides were banned (e.g., carbofuran, aldicarb) from agricultural uses, which has created a void in the repertoire of nematode management. The newer chemistries are not only highly expensive but often lack label information for application in vegetable crops. Thus, development of ecofriendly and cost-effective solutions for management of root knot nematodes in tomato is of paramount importance.

Biological control, on the other hand, offers a promising alternative. Use of antagonistic microorganisms or the metabolite they synthesis constitute an important

component of biological control and has received significant attention in recent years. Among the different microorganisms, endophytic fungi, that reside within plan asymptomatically, are of particular interest because of their dual role in direct nematode mortality and modulation of host defence (Porras-Alfaro and Bayman, 2011). They often produce secondary metabolites that play diverse roles including causing nematode mortality, disruption of the chemosensory behaviours of the nematodes and induce systemic plant resistance, mediated by salicylic acid, jasmonic acid, and ethylene pathways (Molinari and Leonetti, 2019). Indirect effects fungal endophyte includes activation of the plant systemic acquired resistance through Activation of pathogenesis-related (PR) proteins, antioxidant enzymes such as peroxidase (POD), polyphenol oxidase (PPO), and phenylalanine ammonia lyase (PAL), and the accumulation of phenolics and flavonoids which further enhances plant resistance to nematode infestation (Sahebani and Hadavi, 2008; Kang et al., 2014).

In Chapter 3, we isolated and characterized a gall-associated endophytic fungus, *Aspergillus niger* F4 (hereafter F4), from the rice root nematode *M. graminicola*-infected rice roots (Chapter 4). The culture filtrate of this isolate exhibited significant nematicidal activity under *in vitro* conditions with 100% J2 mortality observed after 48 h post treatment at 50% concentration. The culture filtrate also inhibited egg hatching by >95%. Under *in vivo* greenhouse pot study, significantly suppression of gall formation and nematode reproduction were also observed. Moreover, F4-treated plants showed enhanced defence enzyme activity and secondary metabolite accumulation, suggesting both direct and indirect modes of action (see Chapter 4).

M. incognita and *M. graminicola* are phylogenetically related members of the genus *Meloidogyne* (Holterman et al., 2009; Janssen et al., 2016). Given this phylogenetic relatedness, we hypothesized that *A. niger* F4 culture filtrate would exhibit cross-species nematicidal efficacy. As *M. incognita* is one of the most widespread and economically damaging root-knot nematode species infecting a wide range of agricultural and horticultural crops (Jones et al., 2013), evaluating the efficacy of F4 culture filtrate against this nematode has direct implications for its sustainable management across different cropping systems. Therefore, the present study was undertaken to investigate the biocontrol efficacy of *A. niger* F4 culture filtrate against *M. incognita* in tomato.

5.2 Methodology

5.2.1 Nematode Inoculum Preparation

Single egg-mass culture of *Meloidogyne incognita* was obtained from the experimental farm of the Indian Statistical Institute, Giridih, Jharkhand, India. The nematode culture was maintained on uninfected tomato plants grown in double steam-sterilized soil. Infective second-stage juveniles (J2s) were extracted from root galls using a modified Baermann funnel technique. Freshly hatched J2s were collected on a 400-mesh (37 µm) sieve for use in the bioassays.

5.2.2 Preparation of Fungal Culture Filtrate

The rice-root gall-associated fungal endophyte *Aspergillus niger* F4 (hereafter, F4) was used in this study. See Chapter – 3 for isolation and identification of the fungus. Briefly, F4 was cultured in 250 mL conical flasks containing 100 mL of Potato Dextrose Broth (PDB) and incubated at 28 °C in the dark in a shaker incubator at 120 rpm for 7 days. After incubation, the fungal broth was filtered through Whatman No. 1 filter paper and then passed through a 0.45 µm syringe filter to remove conidia and hyphal fragments. The resultant culture filtrate was used in the experiments.

5.2.3 In Vitro Dose-Dependent Nematicidal Assay

Bioassay method described in section 4.2.3. of Chapter- 4 was followed for the dose-dependent nematicidal bioassay. Briefly, the bioassay was conducted using four concentrations (5%, 25%, 50%, and 75%) of the F4 culture filtrate. Approximately 100 freshly hatched *M. incognita* J2s, concentrated in 20 µL of distilled water, were pipetted into each well of a six-well culture plate. The fungal culture filtrate was added to achieve the desired concentrations, and the total volume in each well was adjusted to 2 mL using sterile distilled water. The experiment was conducted with five replicates and repeated twice with distilled water served as the negative control. The number of dead J2s was recorded under a Carl Zeiss Axioscope Stemi-305 stereomicroscope at 24 and 48 h of post treatment. Nematodes were considered dead when no change in body posture was observed following addition of few drops of 1 N sodium hydroxide (NaOH) into each well (Chen and Dickson, 2000). Mortality rates were corrected using Abbott's formula. The median lethal concentration (LC_{50}) was calculated using probit analysis. The corrected mortality percentages were subjected to probit transformation. A regression line was then fitted to the probit values plotted against the $\log_{10}$ of the

culture filtrate concentrations. The LC_{50} value and its 95% confidence limits, was estimated from the regression equation using a probit model (Finney, 1971). All statistical analyses were performed using the 'ecotox' package in R (version 4.3.1).

5.2.4 Attraction and Penetration Assays:

Tomato seedlings with 1.5–1.8 cm long roots were selected for the assay. A 23% (w/v) Pluronic F-127 gel solution was prepared and cooled to 4 °C. Approximately 5000 freshly hatched J2s were suspended in water and mixed well with the gel to achieve a final concentration of 500 J2s/mL. Three millilitres of J2-infused Pluronic gel were dispensed into each well of a six-well culture plate. The roots of one set of seedlings were dipped in sterile distilled water (control). Roots dipped in F4 culture filtrate for 1 h served as the treatment. Both sets of seedlings were positioned centrally in each gel-filled wells. Root zones were examined under a stereomicroscope at 6, 12, 24, and 32 h post-inoculation. Attraction was assessed up to 24 h, while penetration was examined thereafter. To visualize penetrated nematodes, tomato roots were removed, stained with 0.35% acid fuchsin (following the modified protocol of Xue et al., 2024), and the number of penetrated nematodes was quantified under a microscope.

5.2.5 Plant Material, Treatments, and Pot Experimental Design

A greenhouse pot experiment was conducted to assess the impact of *A. niger* F4 on *M. incognita* infection in tomato. Susceptible tomato seeds (cv. Pusa Ruby) were surface-sterilized using 70% ethanol for 3 minutes, followed by 1% sodium hypochlorite for 1 minute, and rinsed thrice with sterile distilled water. Seeds were germinated on moist filter paper at 28 °C. After 7 days, germinated seedlings were transplanted into 13 cm × 13 cm plastic pots containing autoclaved soil and vermicompost (1:1), and kept at 28–30 °C in a seed germinator (REMI CHM-12 plus) for 30 days until reaching the 5–6 leaf stage.

Four treatments were included: (1) P: plant only (non-treated, non-inoculated control), (2) PN: plant + nematode, (3) PF: plant + F4 culture filtrate, (4) PNF: plant + F4 culture filtrate + nematode. For PF and PNF treatments, roots of seedlings were immersed in F4 culture filtrate for 6 h before transplanting; control treatments were mock-treated with sterile distilled water. The experiment followed a completely randomized design (CRD) with sterilized growth medium consisting of 300 cc of autoclaved soil (sand: silt: clay = 65:13.2:18.1, Ghosh et al., 2023) and vermicompost

(1:1). Plants were maintained at 30–35 °C and 75–80% RH. Two days after transplantation, 500 freshly hatched *M. incognita* J2s were inoculated into the rhizosphere of PN and PNF treatments. Plants were harvested on 2, 4, 6, and 8 days post-inoculation (DPI). The experiment was repeated twice. Nematode numbers and gall indices were recorded at 8 DPI.

5.2.5.1 Root Infection Assay

Gall numbers were counted visually at 4 and 8 DPI in PN and PNF treatments. Roots were stained with 0.35% acid fuchsin, destained, stored in glycerol, and examined under a Carl Zeiss Axioscope stereomicroscope for nematode developmental stages.

5.2.5.2 Plant Defence-Related Enzyme Activity

Roots harvested at 2, 4, 6, and 8 DPI from all treatments were used for estimating phenolics, flavonoids, and defense enzymes: catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), polyphenol oxidase (PPO), chitinase (CHI), and β-1,3-glucanase (GLU) with methods as described below.

6) *Determination of Phenolics and Flavonoids*

Total phenolic content was quantified using the Folin–Ciocalteu method as described by Silva-Beltrán et al. (2015). Briefly, 2 g of tomato root tissue was ground in liquid nitrogen and extracted with 20 mL of 80% methanol. The extract was incubated with Folin–Ciocalteu reagent at 25 °C for 30 minutes. Absorbance was measured at 765 nm using a UV-Vis spectrophotometer, and total phenolic content was calculated using a Gallic acid calibration curve.

Total Flavonoid content was determined following the method of Silva-Beltrán et al. (2015) with slight modifications. Root samples (0.3 g) were homogenized in 2 mL of methanol containing 1% HCl (v/v), incubated for 12 h at 25 °C, and centrifuged at 4200 rpm for 20 minutes. An aliquot of the supernatant was sequentially treated with 5% $NaNO_2$, 10% $AlCl_3$, and 1 M NaOH. The absorbance was measured at 510 nm, and flavonoid content was quantified using a rutin standard curve.

b) Enzyme Assays

Phenylalanine Ammonia-Lyase (PAL; EC 4.3.1.5) activity was assayed following the method of Zucker (1965) by monitoring the formation of trans-cinnamic acid from L-phenylalanine. The reaction mixture containing the enzyme extract and 50 mM L-

phenylalanine was incubated at 37 °C, and absorbance was measured at 290 nm.

Polyphenol Oxidase (PPO; EC 1.10.3.1) activity was estimated as per Lamikanra and Watson (2001). The reaction mixture included catechol as substrate, and the increase in absorbance was measured at 398 nm.

Peroxidase (POD; EC 1.11.1.7) activity was measured according to Cakmak and Marschner (1992) using guaiacol as the hydrogen donor. The oxidation of guaiacol in the presence of H_2O_2 was monitored at 470 nm.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured according to Nakano and Asada (1981). The assay was based on the decline in absorbance due to the oxidation of ascorbate at 290 nm in the presence of hydrogen peroxide. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, and 0.1 mM H_2O_2. The reaction was initiated by the addition of H_2O_2, and the decrease in absorbance at 290 nm was recorded for 1 minute.

c) Chitinase and β-1,3-glucanase Assay

Chitinase (EC 3.2.1.14) activity was determined by measuring the release of N-acetylglucosamine (NAG) from colloidal chitin, following the method of Imoto and Yagishita (1971) with modifications as described by Tikhonov et al. (2002). The reaction mixture containing colloidal chitin and enzyme extract was incubated at 37 °C, and the amount of NAG released was quantified colorimetrically. Absorbance was measured at 585 nm, and enzyme activity was expressed as µmol NAG released per minute per gram of fresh weight.

B-1,3-Glucanase (EC 3.2.1.39) activity was estimated using laminarin as the substrate, based on the method of Cota et al. (2007). The reducing sugars released were quantified using the dinitrosalicylic acid (DNS) reagent. After incubation, absorbance was measured at 540 nm, and enzyme activity was expressed as µmol glucose equivalents released per minute per gram of fresh weight (µmol min^{-1} g^{-1} FW).

D) Reactive Oxygen Species (ROS) Estimation

Hydrogen Peroxide (H_2O_2) Content were quantified spectrophotometrically following the method of Junglee et. Al. (2014) method. Fresh root tissue was homogenized in cold 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12,000 × g for 15 minutes at 4 °C. The supernatant was mixed with 10 mM potassium

phosphate buffer (pH 7.0) and 1 M potassium iodide (KI). The absorbance of the reaction mixture was measured at 412 nm. H_2O_2 content was calculated using a standard curve.

Superoxide Dismutase (SOD; EC 1.15.1.1) activity was assayed by its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in a riboflavin-mediated light reaction system following Cervilla et. Al. (2007) protocol. The reaction mixture contained phosphate buffer (pH 7.8), methionine, NBT, EDTA, riboflavin, and enzyme extract. After illumination under fluorescent light for 10 minutes, absorbance was recorded at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit NBT reduction by 50%.

5.2.6 Gene Expression Analysis of SAR and ISR-Associated Genes

At 8 DPI, root tissues from *PN* and *PNF* treatments were harvested and immediately frozen in liquid nitrogen for total RNA extraction. RNA was isolated using TRIzol reagent (Sangon Biotech, China), following the manufacturer's protocol, which is widely employed for high-yield RNA isolation from plant tissues rich in polysaccharides and polyphenols (Chomczynski and Sacchi, 1987). The extracted RNA was treated with Rnase-free Dnase I using the Rneasy Mini Kit (Qiagen, Germany) to eliminate any genomic DNA contamination, ensuring the integrity of downstream gene expression analysis.

RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA), and RNA integrity was verified by agarose gel electrophoresis. First-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the qScript cDNA Synthesis Kit (QuantaBio, USA), which utilizes a blend of oligo(dT) and random primers to ensure comprehensive reverse transcription.

Quantitative real-time PCR (qRT-PCR) was performed using gene-specific primers (listed in Supplementary Table S1) for a panel of defense-related and antioxidant genes, including *ACTIN*, *ERF1*, *JERF3*, *ACO*, *PR1-1b*, *PR3*, *PR5*, *PR1*, *PR2*, *PR9*, *CAT*, *PAL*, and *PPO*. Each 25 µL qPCR reaction mixture comprised SYBR® Premix Ex Taq™ (Takara, Japan), 2 µL of diluted cDNA (1:10), and 0.2 µM of each primer. Reactions were run on the ExpressPCR system (TATA-MD CHECK, India) using the following thermal cycling conditions: initial denaturation at 95 °C for 3 minutes; followed by 40 cycles of denaturation at 95 °C for 10 seconds, annealing at 60 °C for 34 seconds, and

extension at 72 °C for 45 seconds; with a final extension step at 72 °C for 10 minutes.

Gene expression levels were normalized to the internal reference gene *ACTIN*, which has been validated for stability under biotic stress in tomato (Expósito-Rodríguez et al., 2008). Relative expression levels were calculated using the 2^−ΔΔCt method (Livak and Schmittgen, 2001).

Table 5.1 gene and the primer details used for the gene expression analysis

No	Gene name	Accession Number	Function	Primers	Amplicon size
1	*PR1-1b*	NM_001247385.2	Synthesis of pathogenesis-related protein 1 (PR1)	F: GATCGGACAACGTCCTTAC R: GCAACATCAAAAGGGAAATAAT	171bp
2	*PR-3*	NM_001247474.2	Chitinase	F: AACTATGGGCCATGTGGAAGA R: GGCTTTGGGGATTGAGGAG	110 bp
3	*PR-5*	NM_001247422.3	Thaumatin-like	F: GCAACAACTGTCCATACACC R: AGACTCCACCACAATCACC	376 bp
4	*JERF3*	NM_001247533.2	Jasmonate Ethylene Response Factor 3	F: GCCATTTGCCTTCTCTGCTTC R: GCAGCAGCATCCTTGTCTGA	63 bp
5	*ACO*	XM_015225653.2	1-aminocyclopropane-1-carboxylic acidoxidase	F: CCATCATTTCTCCAGCATCA R: TTGGCAGACTCAAATCTAGG	98 bp
6	CAT	NM_001247257.2	Catalase 2	F: TGCTCCAAAGTGTGCTCATC R: TTGCATCCTCCTCTGAAACC	400 bp
7	Actin (Housekeeping)	NM_001321306.1	Actin-7-like	F: GATACCTGCAGCTTCCATACC R: GCTTTGCCGCATGCCATTCT	232 bp
8	*PR-1*	Y08804	Synthesis of pathogenesis-related protein 1 (PR1)	F: GGATCGGACAACGTCCTTAC R: GCAACATCAAAAGGGAAATAAT	174 bp
9	*PR-2* gene	NM_001247229	Synthesis of β-1,3-glucanase	F: AAGTATATAGCTGTTGGTAATGAA R: ATTCTCATCAAACATGGCGAA	381 bp
10	Ethylene-responsive factor 1 (*ERF1*)	NM_001247919.2	Encodes a key transcription factor belonging to the AP2/ERF superfamily	F: ACGAGCTTTCTTCTTTTCTCTC-TCTAAA R: GAAACTCGATATCCTTCTGTA-AAATCTTC	275 bp

5.2.7 Pot Study under Greenhouse Conditions

A pot experiment was conducted under controlled conditions to evaluate the nematode suppression potential of *Aspergillus niger* F4 in comparison with a chemical nematicide. Tomato seedlings at the 5–6 leaf stage were raised as described in Section 2.5 and transplanted individually into 1 kg capacity earthen pots containing a steam-sterilized 1:1 (v/v) mixture of soil and vermicompost.

The experimental treatments were as follows:

1. Negative control (water): No nematicide or biocontrol treatment was applied.

2. Positive control (Velum Prime): Fluopyram-based chemical treatment (Velum Prime®, Bayer Ltd.) was applied as a soil drench at the recommended concentration of 1.25 g/L.
3. Fungal treatment (F4): Seedling roots were immersed in the culture filtrate of *A. niger* F4 for 4 h prior to transplanting.

Each treatment was replicated five times, and the pots were arranged in a completely randomized design (CRD). At three days after transplantation, each plant was inoculated with 2000 freshly *M. incognita* J2s were, applied in suspension near the rhizosphere. At 45 DPI, tomato plants were gently uprooted, and root systems were washed under running tap water to remove adhering soil. Plant growth parameters, including shoot length and root length (in cm), were measured using a measuring scale. Shoot and root fresh weights (in g) were recorded separately using a precision digital balance immediately after harvest. For assessment of root galling, the number of root galls was counted per plant, and the gall index was calculated as the number of galls per gram of root fresh weight. Nematodes were extracted from 100 g of homogenized pot soil using the sucrose elutriation–centrifugation method (Barker et al., 1978), and the final population density was expressed as the number of second-stage juveniles (J2s) per 100 g of dry soil. To quantify the biocontrol efficacy, the percentage control effect was calculated based on the reduction in gall index using the formula:

$$Control\ Effect\ (\%) = \left(\frac{GI_{control} - GI_{tretment}}{GI_{control}}\right) \times 100$$

Where, $GI_{control}$ = the mean gall index of untreated, nematode-inoculated control plants, and $GI_{treatment}$ = the mean gall index in either the chemical or fungal treated plants.

5.2.8 Statistical analysis

R statistical software (R-3.6.2) was used to conduct all statistical analyses. All the data were first examined for homogeneity of variance and normality using the Shapiro Wilk normality test and Bartlett test before analysis of variance. Wherever required [log (x + 1)] transformation was used to improve normality and homogeneity of variance. If normality assumption is not achieved, the non-parametric Kruskal-Walis Chi square test was run. Significant variations between treatments were examined using the Duncan's multiple range test.

5.3 Results

5.3.1 In vitro nematicidal assay

To evaluate the nematicidal efficacy of *Aspergillus niger* F4 culture filtrate against *Meloidogyne incognita* second-stage juveniles (J2s), a dose- and time-dependent in vitro inhibition assay was conducted. The culture filtrate exhibited strong nematicidal activity, causing 97.74% mortality at 24 h post-treatment with a 50% concentration. Juvenile mortality increased progressively with both increasing concentrations and extended exposure durations.

At 24 h post-inoculation, mortality rates corresponding to different concentrations of the culture filtrate were as follows: 4.50% ± 1.20 at 5%, 75.99% ± 2.03 at 25%, and 98.83% ± 0.52 at 75%. Based on the dose–response relationship, the median lethal concentration (LC_{50}) of the culture filtrate against *M. incognita* J2s was calculated to be 17.43% (Fig. 5.1A).

5.3.2 Attraction and root penetration assays

A root attraction and penetration assay was conducted to evaluate whether *A. niger* F4 culture filtrate influences the ability of *M. incognita* J2s to locate and enter tomato roots. At both 6 and 12 h after the initiation of the assay, a significantly higher number of J2s were attracted to the water-treated (control) roots compared to the F4-treated roots (Treatment: $F = 56.096$, $p < 0.05$; Time: $F = 8.046$, $p < 0.05$). The attraction to control roots increased with time, suggesting an accumulation of nematodes near the root tip. By 24 h, the number of nematodes visible around the control root tip began to decline, indicating active root penetration (Fig. 5.1B).

At 24 h post-treatment, the number of J2s aggregated and penetrated into F4-treated roots was 60.86% lower than in the control. On average, 92 ± 5.29 nematodes were observed to be in contact with or inside the control root tips, whereas only 56 ± 2.90 were found in the F4-treated roots. At 32 h, the number further increased in the control roots to 98.66 ± 2.69, which was 53.38% higher than that in F4-treated roots (Fig. 5.1B).

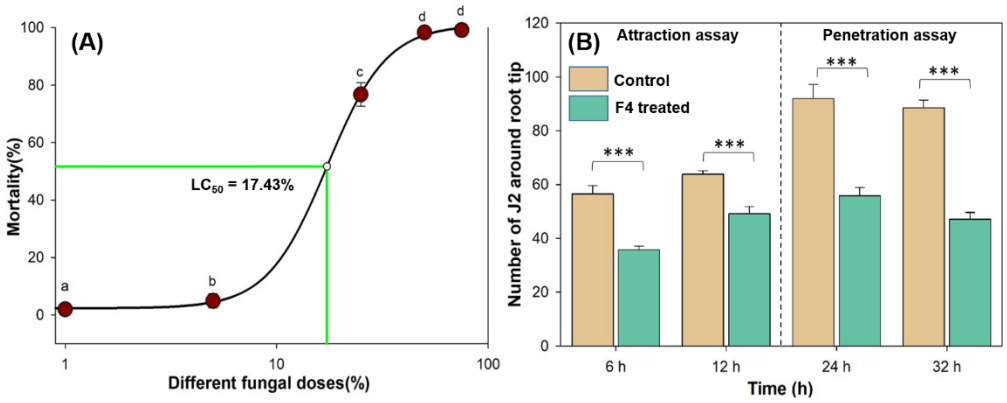

Fig. 5.1. Nematicidal activity and behavioural response of *M. incognita* J2s to *Aspergillus niger* F4 culture filtrate. (A) Dose-dependent in vitro mortality of *M. incognita* J2s following 24 h exposure to varying concentrations of *A. niger* F4 culture filtrate. The mortality percentage increased significantly with increasing concentrations of the culture filtrate. Each data point represents the mean ± SE of five replicates. Letters above the points indicate statistically significant differences according to Duncan's multiple range test ($P < 0.05$). The green line denotes the LC_{50} value, calculated to be 17.43%. (B) Behavioural response of *M. incognita* J2s in attraction and penetration assays at four time intervals (6, 12, 24, and 32 h post-inoculation). In the attraction assay (left panel), the number of J2s aggregating around the root tip was significantly higher in control roots than in F4-treated roots at 6 h and 12 h. In the penetration assay (right panel), the number of J2s observed inside or in contact with the root tissue remained significantly lower in F4-treated roots compared to control roots at 24 h and 32 h. Bars represent mean ± standard error of eight biological replicates. Asterisks denote statistically significant differences between treatments (***$P < 0.001$).

5.3.3 Root infection assay

The effect of *A. niger* F4 culture filtrate on the penetration and early development of *M. incognita* within tomato roots was assessed at 4 and 8 DPI. The number J2s inside tomato roots was significantly reduced in the F4-treated plants (PNF) compared to untreated (PN) controls at both time points. At 4 DPI, the number of J2s inside PN roots was 18 ± 1.6, whereas PNF roots had only 9 ± 1 J2s, indicating a 50% reduction (Table 5.2). This trend continued at 8 DPI, with 49.6 ± 1.2 J2s in PN roots compared to 26 ± 1.4 in PNF roots, reflecting a 47.58% reduction and suggesting a sustained inhibitory effect of the culture filtrate on nematode ingress and establishment.

While no significant difference in gall number was observed at 4 DPI between PN and PNF treatments, a clear difference emerged at 8 DPI. PN roots showed an average of 11 ± 2.9 galls, whereas PNF roots had significantly fewer galls (5 ± 0.9), indicating that F4 treatment delayed or suppressed gall formation, as less number of nematodes have penetrated the roots (Table 5.2).

The root biomass was unaffected by either nematode infection or F4 treatment at both time points. Root weights ranged between 0.4 ± 0.1 to 0.5 ± 0.2 g, with no

differences between PN and PNF treatments at both time points, indicating that the observed differences in nematode development were not confounded by changes in root growth.

Table 5.2. Effect of *A. niger* F4 culture filtrate on root penetration and early infection events of *M. incognita* in tomato at 4 and 8 DPI. PN = Plant + nematode (untreated control); PNF = Plant + nematode + F4 treatment. Values represent mean ± standard error (n = 5). * indicate statistically significant differences at $p < 0.05$.

Days	Root weight (gm)		Number of galls		Number of *M. incognita* J2s	
	PN	PNF	PN	PNF	PN	PNF
4 DPI	0.4 ± 0.2	0.4 ± 0.1	4 ± 0.8	0	18 ± 1.6*	9 ± 1
8 DPI	0.42 ± 0.2	0.5 ± 0.2	11 ± 2.9	5 ± 0.9	49.6 ± 1.2*	26 ± 1.4

5.3.4 Plant Defence-Related Enzyme Activity

6) Phenolic and flavonoid

Phenol and flavonoid contents in tomato roots were monitored at 2, 4, 6, and 8 DPI to evaluate the biochemical response induced by *Aspergillus niger* F4 and *Meloidogyne incognita*. Phenol levels remained relatively stable in control, PF, and PN treatments at early time points but increased significantly in PNF-treated plants from day 4 onwards, reaching 6.0 ± 0.3 $mg \cdot g^{-1}$ FW at day 8—more than twice that of the control (Fig. 5.5A). This suggests that F4 primes phenolic biosynthesis, which is further amplified upon nematode challenge. Phenolics are known to reinforce cell walls and interfere with nematode development, contributing to enhanced resistance.

Flavonoid content followed a similar trend (Fig. 5.5B), with significantly higher levels in PN and PNF plants across all time points. PNF-treated plants consistently showed the highest accumulation, reaching 32.5 ± 1.2 $mg \cdot g^{-1}$ FW at day 8. Flavonoids function as antioxidants and modulators of defense signaling, and their sustained induction in PNF plants suggests enhanced stress mitigation and defense priming by F4.

The stronger biochemical responses in PNF-treated plants correlate with reduced galling and nematode reproduction observed in greenhouse assays. These findings indicate that *A. niger* F4 enhances tomato defense by priming both phenylpropanoid and flavonoid pathways, supporting its potential as a biologically derived alternative to chemical nematicides. Further work identifying the active components of the culture filtrate may help optimize its field efficacy.

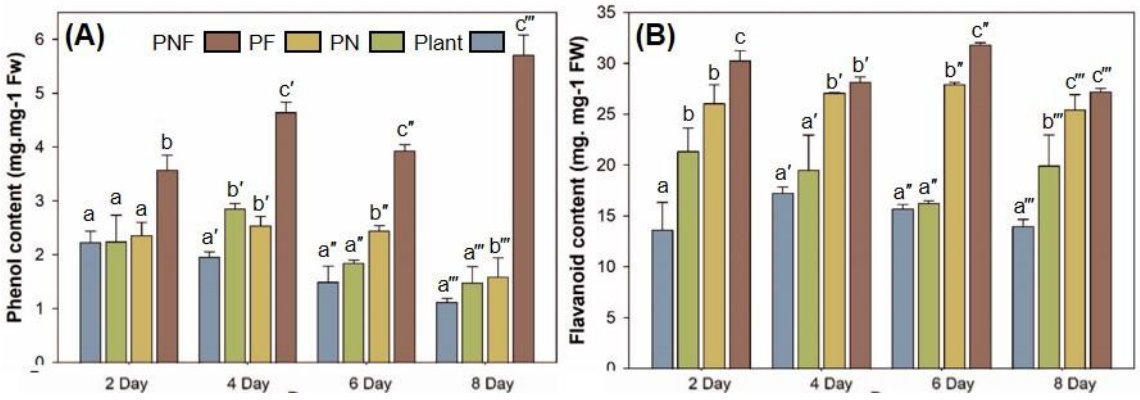

Fig. 5.2. Temporal changes in total phenol (A) and flavonoid (B) contents in tomato roots under different treatments. Values are expressed as mean ± SE (n = 5). Different letters above the bars indicate statistically significant differences between treatments at each time point according to Duncan's multiple range test ($P < 0.05$).

b) Defence Enzymes Activities

The activities of four key defence related enzymes, peroxidase (POD), polyphenol oxidase (PPO), ascorbate peroxidase (APX), and phenylalanine ammonia-lyase (PAL), were measured in tomato roots at 2, 4, 6, and 8 DPI under different treatments (Plant, PN, PF, PNF). Enzyme activities were consistently highest in the PNF treatment, indicating enhanced defence activation in response to combined F4 application and nematode challenge (Fig. 5.6).

POD activity (Fig. 5.3A) showed a progressive increase across all treatments, with the highest activity recorded in the PNF. At 2 DPI, POD activity in PNF plants was significantly higher than in PF, PN, and control plants. This trend continued across all time points, peaking at 8 days with approximately 20 units·min^{-1}·mg^{-1} protein in PNF roots. POD catalyzes the oxidation of phenolic substrates and is associated with cell wall strengthening and inhibition of pathogen invasion, suggesting that its induction contributes to nematode resistance.

PPO activity (Fig. 5.3B) followed a similar pattern. While modest increases were

observed in PF and PN treatments, PPO activity was significantly enhanced in PNF plants at all time points, reaching a maximum of ~16 $U \cdot mg^{-1}$ protein at 8 days. PPO plays a role in quinone formation and protein crosslinking, limiting nematode establishment and mobility in root tissues.

APX activity (Fig. 5.3C), an indicator of oxidative stress mitigation, was also significantly elevated in PNF-treated plants. At 2 DPI, APX activity in the PNF was nearly double that of the control. Activity remained significantly higher in PNF plants throughout the time course, peaking at 8 days. This suggests that F4 treatment enhances the antioxidant capacity of host roots, helping to manage ROS generated during nematode invasion and tissue damage.

PAL activity (Fig. 5.3D), a key enzyme in the phenylpropanoid pathway, showed consistent and significant induction in PNF plants from day 2 onwards. Although PN and PF treatments also showed moderate increases, the PNF combination resulted in the highest PAL activity at all-time points. As PAL catalyzes the conversion of phenylalanine to cinnamic acid—a precursor for lignin and various phenolics—its induction strongly supports enhanced secondary metabolite production and structural reinforcement of root tissues.

Together, the increased activities of POD, PPO, APX, and PAL in PNF plants indicate a coordinated activation of both oxidative and secondary metabolite-based defences. These biochemical responses align with earlier observations of elevated phenol and flavonoid levels and reduced galling and nematode reproduction. The data suggest that *A. niger* F4 primes tomato plants for faster and stronger activation of defence enzyme systems upon nematode attack, contributing to its effective biocontrol potential.

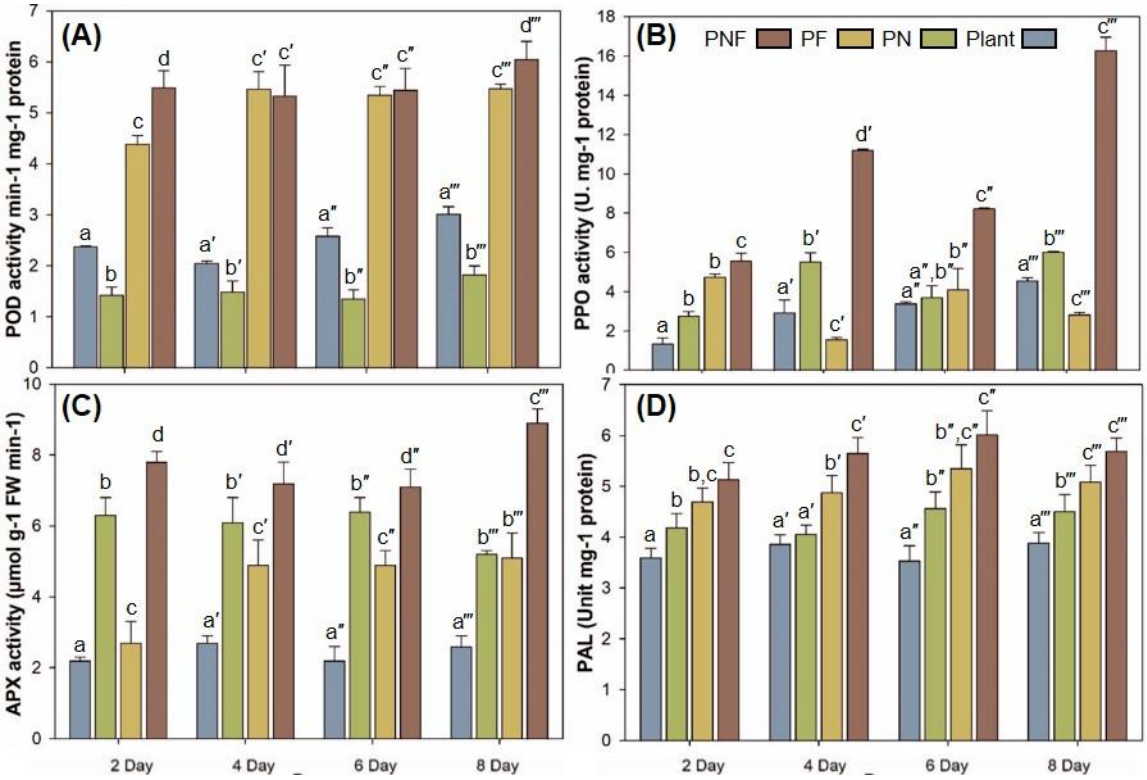

Fig. 5.3. Temporal activity of defence related enzymes in tomato roots under different treatments. Enzyme activities were measured at 2, 4, 6, and 8 DPI. (A) Peroxidase (POD), (B) Polyphenol oxidase (PPO), (C) Ascorbate peroxidase (APX), and (D) Phenylalanine ammonia-lyase (PAL) activities were quantified. Values represent mean ± SE (n = 5). Different letters above the bars indicate statistically significant differences between treatments at each time point according to Duncan's multiple range test ($P < 0.05$).

c) Activities of β-1,3-Glucanase and Chitinase

The activities of β-1,3-glucanase and chitinase were measured in tomato roots at 2, 4, 6, and 8 DPI under four treatments (Plant, PN, PF, PNF). B-1,3-glucanase activity (Fig. 5.4A) was significantly elevated in PNF plants at all time points, with maximum activity recorded at 8 days (~38 µmol·g^{-1}), followed by PF and PN. Control plants showed the lowest levels throughout. A similar trend was observed for chitinase activity (Fig. 5.4B), where PNF plants exhibited the highest activity, peaking at ~1.1 nkat·mg^{-1} protein at 8 days. PF and PN treatments showed moderate induction, while the control remained unchanged. In both enzymes, PNF-treated plants consistently showed significantly higher activity than other treatments across all time points.

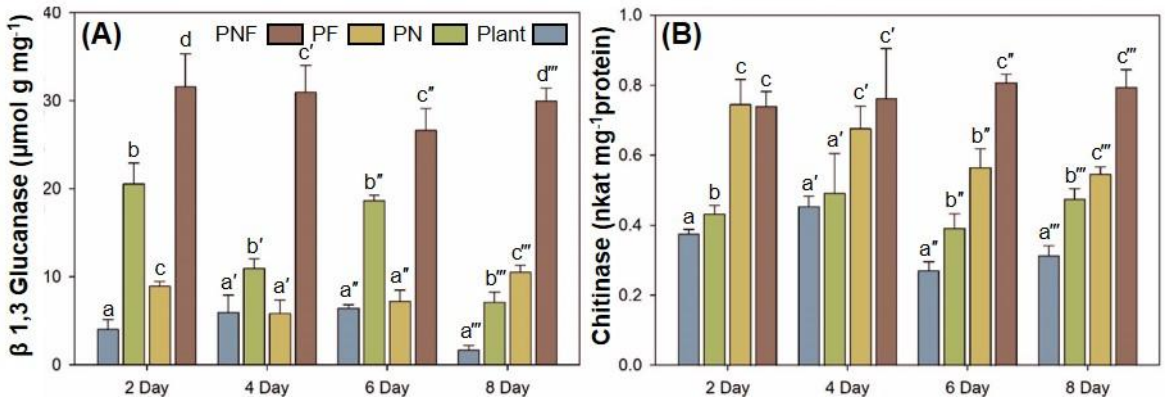

Fig. 5.4. Temporal activity of hydrolytic defence enzymes in tomato roots under different treatments. (A) β-1,3-Glucanase and (B) chitinase activities were measured at 2, 4, 6, and 8 DPI. Values represent mean ± SE (n = 5). Different letters above the bars indicate statistically significant differences among treatments at each time point according to Duncan's multiple range test ($p < 0.05$).

d) ROS generation abilities

Hydrogen peroxide (H_2O_2) and superoxide dismutase (SOD) levels were quantified in tomato roots at 2, 4, 6, and 8 DPI to assess oxidative stress and antioxidant responses under different treatments. As shown in Fig. 5.5A, H_2O_2 levels were highest in PN-treated roots across all time points, peaking at approximately 115 nmol·g^{-1} FW at 2 DPI. In contrast, PNF and PF treatments exhibited significantly lower H_2O_2 accumulation, with values ranging from 60–80 nmol·g^{-1} FW. Control plants consistently showed the lowest H_2O_2 levels.

SOD activity (Fig. 5.5B) followed a complementary pattern. PN-treated plants exhibited the highest SOD activity, reaching ~460 U·g^{-1} FW at 4 DPI. SOD activity in PF and PNF treatments was significantly lower than in PN at 4 and 6 DPI, but slightly increased by 8 DPI in the PNF group. Control plants showed the lowest SOD activity throughout the time course. Overall, the PN treatment induced the highest oxidative stress, whereas PF and PNF treatments maintained comparatively lower ROS levels, indicating moderate antioxidant enzyme activation.

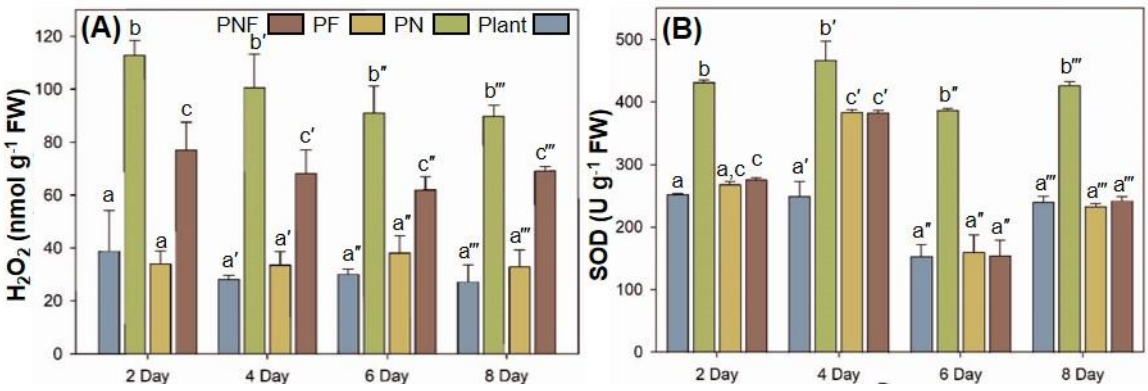

Fig 5.5. Temporal changes in hydrogen peroxide (H_2O_2) content and superoxide dismutase (SOD) activity in tomato roots under different treatments. (A) H_2O_2 content and (B) SOD activity were measured at 2, 4, 6, and 8 DPI. Values represent mean ± SE (n = 5). Different letters above the bars indicate statistically significant differences between treatments at each time point according to Duncan's multiple range test ($P < 0.05$).

5.3.5 Gene expression study

To elucidate the molecular basis of *A. niger* F4-mediated resistance in tomato, the expression profiles of key defence-related genes were analysed by quantitative RT-PCR. Expression levels were normalized against the housekeeping gene *Actin*. Among the genes evaluated, *ACO* (1-aminocyclopropane-1-carboxylic acid oxidase), *JERF3* (Jasmonate Ethylene Response Factor 3), and *PR1-1b* (Pathogenesis-Related protein 1 isoform) were significantly upregulated in F4-treated roots compared to the water-treated control. Statistical analysis using unpaired *t*-tests confirmed the upregulation of *ACO* ($t = 15.308$, $p < 0.01$), *JERF3* ($t = 13.813$, $p < 0.01$), and *PR1-1b* ($t = 18.093$, $p < 0.01$). These genes exhibited fold changes exceeding two-fold, with the strongest induction observed for *PR1-1b*. Other genes, including *PR1*, *PR2*, *PR3*, *PR5*, and *ERF1*, did not show statistically significant changes in expression (all $P > 0.05$), although a mild upward trend was noted for *PR3* and *PR5* (Fig. 5.6).

The upregulation of *ACO* and *JERF3* points to a prominent role of the jasmonic acid (JA) and ethylene (ET) signalling pathways in F4-mediated defence activation. *ACO* catalyses the final step in ethylene biosynthesis, which is critical for inducing structural and biochemical defences at nematode infection sites. Elevated *ACO* expression in F4-treated roots likely enhances local ethylene production, contributing to cellular defences such as callose deposition, lignification, and inhibition of nematode feeding site formation. *JERF3*, a transcription factor integrating JA/ET signals, is known to activate broad-spectrum defence responses, including the

transcription of downstream *PR* genes and oxidative stress-related enzymes. Its strong induction suggests that F4 treatment primes the host for an enhanced response upon nematode attack.

PR1-1b is a classical marker of pathogenesis-related responses and is often associated with systemic acquired resistance (SAR). Its significant upregulation indicates that in addition to JA/ET signalling, elements of the SAR pathway may also be triggered by the F4 treatment. However, the lack of significant induction in *PR2*, *PR5*, and canonical *PR1* suggests that the defence response may be spatially restricted or temporally staggered, reflecting a partial activation of SAR rather than a full systemic response.

These transcriptional responses correlate well with phenotypic outcomes observed in the greenhouse experiment. F4 treatment led to a significant reduction in gall index and nematode population density in soil, although its efficacy was slightly lower than that of the commercial nematicide. Specifically, F4-treated plants showed a gall index of 7.28 ± 0.40 and a control effect of 85.62 ± 6.20%, compared to 5.25 ± 0.55 and 89.25 ± 6.61% in nematicide-treated plants. The difference, while statistically evident, is biologically meaningful given that the commercial product contains a high concentration of purified active ingredients, whereas the F4 treatment used unrefined culture filtrate. The expression data support the hypothesis that F4 acts not only through direct antagonism of *M. incognita* but also by priming host defences via JA/ET signalling pathways.

In conclusion, the gene expression analysis provides mechanistic insights into how *A. niger* F4 modulates plant immunity. The pronounced activation of *ACO*, *JERF3*, and *PR1-1b* underscores its potential to function as a biological elicitor capable of enhancing host resistance to root-knot nematodes. These findings reinforce the biological control potential of F4 and highlight its promise as a source of novel nematicidal compounds. Further studies involving time-course expression profiling, hormone quantification, and purification of active metabolites from the culture filtrate could pave the way for developing effective, eco-friendly nematode management strategies.

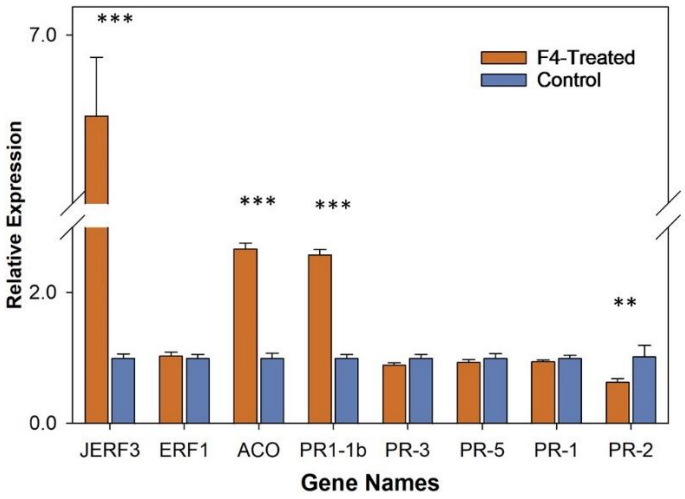

Fig. 5.6. Relative expression of defence-related genes in tomato roots treated with *A. niger* F4 culture filtrate**.** Gene expression was assessed by quantitative RT-PCR and normalized against the expression of the housekeeping gene *Actin*. The bar graph shows the mean relative expression levels (± SE). See Table 5.1 for the selected genes and their functions. Significant upregulation was observed in *ACO*, *JERF3*, and *PR1-1b* ($p < 0.001$), as determined by unpaired *t*-tests. *** indicate highly significant differences relative to control ($P < 0.001$).

5.3.6 Pot Study under Greenhouse Conditions:

Treatment with *A. niger* F4 culture filtrate significantly improved tomato plant growth and reduced gall formation by *M. incognita* compared to the untreated control. Shoot length in the untreated control was 34.06 ± 0.94 cm, whereas plants treated with the chemical nematicide and F4 culture filtrate exhibited increased shoot lengths of 41.10 ± 2.05 cm and 39.33 ± 1.48 cm, respectively with no significant difference between them. A similar trend was observed for root length, with the shortest roots recorded in control plants (12.82 ± 0.40 cm), and significantly longer roots in F4-treated (17.86 ± 0.24 cm) and nematicide-treated (16.20 ± 0.27 cm) plants.

Shoot and root biomass also improved significantly following treatment. Shoot fresh weight increased from 6.39 ± 0.80 g in the control to 8.82 ± 0.40 g and 8.65 ± 0.66 g in F4 and nematicide treatments, respectively. Root fresh weight was lowest in control plants (2.15 ± 0.17 g), while F4-treated plants recorded the highest root biomass (4.07 ± 0.19 g), followed by nematicide-treated plants (3.69 ± 0.09 g).

Nematode infection resulted in severe galling in control plants, with a mean gall index of 50.75 ± 0.96. In contrast, galling was significantly reduced in both treatment, with the nematicide treated plants showing a gall index of 5.25 ± 0.55 and the F4 treatment showing 7.28 ± 0.40. Based on these reductions, the control effect was calculated to be 89.25 ± 6.61% for the nematicide treatment and 85.62 ± 6.20% for the

F4 treatment, indicating that the culture filtrate of *A. niger* F4 was as effective as the chemical control in reducing *M. incognita* infestation in tomato.

Overall, no statistically significant differences were observed between the F4-treated and nematicide-treated plants across most parameters, including shoot and root lengths, shoot and root fresh weights (Table 5.3 and Fig. 5.6). While both treatments significantly reduced galling and improved plant growth compared to the untreated control, a slight difference was observed between the *A. niger* F4 culture filtrate and the commercial nematicide. The gall index in F4-treated plants was 7.28 ± 0.40, compared to 5.25 ± 0.55 in the nematicide-treated. Correspondingly, the control effect achieved by the F4 treatment was 85.62 ± 6.20%, slightly lower than the 89.25 ± 6.61% observed for the nematicide. While the difference was statistically significant, it was biologically expected, considering that the commercial product contains high concentrations of purified active ingredients, while the F4 treatment contains only the culture filtrate without further enrichment. Nevertheless, the efficacy demonstrated by the F4 culture filtrate highlights its strong potential as a biologically derived alternative to synthetic nematicides. Further identification of the active compound(s) within the culture filtrate and bioassay-guided fractionation may enhance its nematicidal potency and pave the way for the development of novel, eco-friendly nematode management solutions.

Table 5.3. Effect of *A. niger* F4 culture filtrate and chemical nematicide on plant growth, gall formation, and control efficacy in tomato at 45 DPI.

Treatments	Shoot Length (cm)	Root Length (cm)	Shoot Fresh Weight (g)	Root Fresh Weight (g)	Gall Index	Control Effect (%)
Control	34.06 ± 0.94^{a}	12.82 ± 0.40^{c}	6.39 ± 0.80^{b}	2.15 ± 0.17^{c}	50.75 ± 0.96^{a}	–
Nematicide	41.10 ± 2.05^{b}	16.20 ± 0.27^{b}	8.65 ± 0.66^{a}	3.69 ± 0.09^{b}	5.25 ± 0.55^{b}	89.25 ± 6.61^{a}
F4	39.33 ± 1.48^{b}	17.86 ± 0.24^{b}	8.82 ± 0.40^{a}	4.07 ± 0.19^{a}	7.28± 0.40^{c}	85.62 ± 6.20^{a}

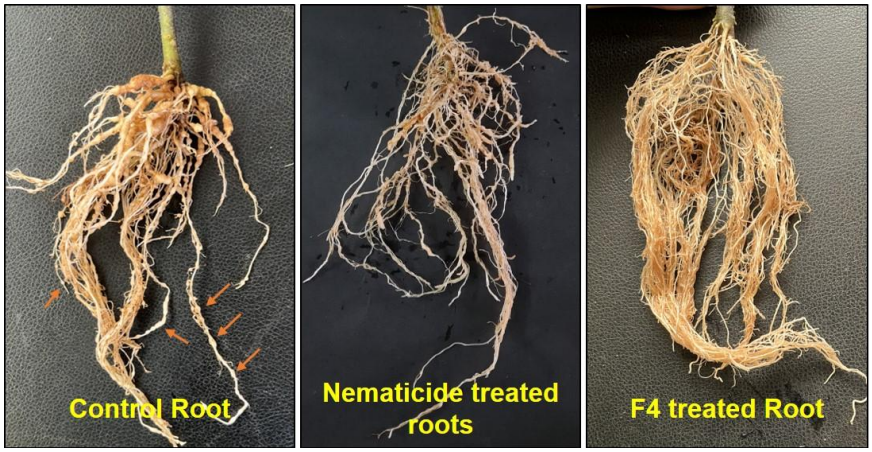

Fig. 5.6. Comparative root morphology of tomato plants under different treatments at 45 DPI with *Meloidogyne incognita***.** Representative images show root systems from untreated control (left), nematicide-treated (middle), and *Aspergillus niger* F4-treated (right) tomato plants. Control roots exhibit extensive galling, indicated by orange arrows, due to nematode infestation. In contrast, roots from nematicide- and F4-treated plants display minimal galling and improved root architecture.

At 45 DPI, the number of *M. incognita* second-stage juveniles (J2s) recovered from soil varied significantly among treatments (Fig. 5.7). Control pots exhibited a high nematode population density, with J2 counts ranging widely and a median exceeding 200 J2s per 100 g of soil. In contrast, both the chemical nematicide and *l* F4 treatments resulted in a significant reduction in J2 recovery, with the lowest numbers recorded in nematicide-treated soil (Fig. 5.5). The slightly higher J2 count observed in the F4-treated plants is likely due to variation in nematode recovery across replicates. Overall, both treatments caused a significant reduction in J2 numbers relative to the control, highlighting the strong nematicidal potential of the F4 culture filtrate. These findings are consistent with gall index and control effect data, further supporting the role of *A. niger* F4 in managing *M. incognita* infestation in tomato.

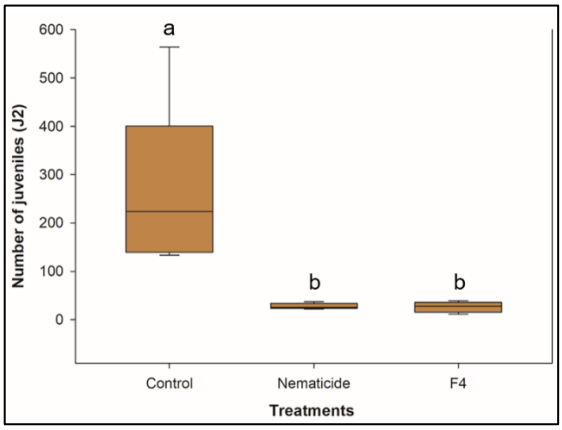

Fig. 5.7. Number of *M. incognita* J2s recovered from soil at 45 DPI. Boxplot shows the distribution of J2s recovered per 100 g of soil under three treatments: untreated control, chemical nematicide, and *Aspergillus niger* F4 culture filtrate. The control plants exhibited a significantly higher number of recovered J2s, while both nematicide and F4 treatments led to substantial reductions in soil nematode population. Median values are indicated by horizontal lines within boxes; whiskers represent data range excluding outliers.

5.4 Discussion:

The results of the present study clearly demonstrate that the culture filtrate of *Aspergillus niger* F4 exhibits potent nematicidal activity against *Meloidogyne incognita*, both in vitro and under greenhouse conditions. The culture filtrate displayed strong nematicidal activity in vitro, with a calculated LC_{50} value of 17.43% and near-complete juvenile mortality at higher concentrations. Greenhouse study demonstrated significant reduction of nematode penetration, gall formation, and population build-up in soil. Biochemical assays confirmed that F4 treatment induced the plant defence responses. Overall the results provided clear evidence of the dual mode of action of F4 treatment: direct nematicidal effects and activation of host-mediated defence responses.

The culture filtrate significantly inhibited root penetration and delayed gall formation, reducing the number of invasive J2s and galls per plant. These findings are in line with earlier reports on fungal endophytes, such as *Fusarium moniliforme* and *Trichoderma harzianum*, which have been shown to restrict nematode ingress and feeding site establishment (Le et al., 2009; Sahebani & Hadavi, 2008). Notably, the reduction in gall index and soil J2 population in F4-treated plants was comparable to that of fluopyram-based chemical nematicide. This is a significant finding as it suggests the presence of one or more secondary metabolites in the F4 culture filtrate with potent nematicidal activity. While the direct application of crude culture filtrates is not a feasible or scalable option for field deployment, the observed efficacy provides

strong justification for further chemical characterisation. Identification and purification of the active compound(s) from the F4 filtrate could enable the development of biologically derived nematicidal molecules. Such lead compounds offer a promising alternative to conventional nematicides, particularly in light of growing regulatory restrictions and environmental concerns associated with synthetic chemistries (Kumar et al., 2020).

In addition to its nematicidal effects, F4 treatment significantly enhanced the tomato plant's innate defence responses. Enzyme assays revealed strong induction of peroxidase, polyphenol oxidase, ascorbate peroxidase, phenylalanine ammonia-lyase, chitinase, and β-1,3-glucanase, all of which play key roles in restricting nematode infestation and development. These responses were further substantiated by elevated levels of total phenolics and flavonoids in F4-treated roots, compounds known to reinforce cell walls and mediate chemical defence against nematode infestation (Radwan et al., 2012; Sahebani & Hadavi, 2008). These findings align with those reported for other endophyte-derived formulations, such as *Fusarium moniliforme* and *Chaetomium globosum*, which enhance biochemical defences in host plants (Le et al., 2016; Zhou et al., 2016).

The role of *A. Niger* F4 in activating inducible defence pathways was further validated at the transcriptional level. Gene expression analysis revealed significant upregulation of *ACO* and *JERF3*, implicating the jasmonic acid (JA) and ethylene (ET) signalling pathways in F4-mediated resistance. These pathways are well established as central components of induced systemic resistance (ISR), which is typically activated by beneficial microbes (Pieterse et al., 2014). The concurrent induction of *PR1-1b*, a marker for pathogenesis-related protein accumulation, suggests cross-talk between JA/ET and salicylic acid (SA) pathways, although canonical SAR markers such as *PR1* and *PR2* were not significantly induced. This supports the hypothesis that F4 primes a broad-spectrum but primarily ISR-dominant defence phenotype in tomato, conferring durable resistance to *M. incognita*.

Future work should focus on bioassay-guided fractionation of the culture filtrate to identify and purify the active nematicidal compound(s). High-resolution mass spectrometry and NMR-based structural elucidation may enable characterisation of the underlying metabolites, which could then be synthesised or fermented at scale. Furthermore, field trials under different agro-climatic conditions will be essential to assess formulation stability, persistence, and efficacy under natural infestation

pressures. Investigating the interaction of F4 metabolites with soil microbiota and non-target organisms will also be critical to ensure ecological safety and regulatory acceptance.

In conclusion, *A. niger* F4 culture filtrate demonstrated significant nematicidal properties against *M. incognita* in tomato with comparable efficacy to that of commercial nematicide. It further demonstrated the ability to induce predominantly ISR mediated plant defences in tomato against this very important plant parasitic nematode. These attributes position it as a promising source of lead molecules for the development of next-generation, biologically derived nematicides. Future work should focus on the purification of bioactive components, formulation development, and field location filed trials for translating these findings into a deployable biocontrol product.

Chapter 6: Objective 4

Chapter 6: Objective 4

Metabolomic Characterisation and Functional Elucidation of Bioactive Compounds in *Aspergillus niger* F4 Culture Filtrate

6.1. Introduction:

Plant-parasitic nematodes are known as one of the most devastating pests to global agriculture, causing estimated annual losses exceeding $157 billion worldwide (Jones et al., 2013; Singh et al., 2015). Among these pathogens, the root-knot nematode (RKN) *M. graminicola* substantially impacts rice cultivation practices, including yield reduction by 20-80% diverse rice-growing regions (Kyndt et al., 2014; De Waele and Elsen, 2007). The management of these pathogens has traditionally depended on synthetic nematicides, which raises substantial concerns of environmental persistence, resistance development and non-target effects demanding the exploration of sustainable alternatives (Nguyen et al., 2019; Holajjer et al., 2016).

Conventional management solutions predominantly depend on synthetic nematicides, which, while effective, provide significant hazards to human health and the environment, and have increased limitations due to regulatory and resistance challenges. Therefore, there is an urgent necessity to create sustainable alternatives that are environmentally friendly and agriculturally efficient.

Fungal endophytes are microorganisms that asymptomatically inhabit within plant tissues have emerged as a possible source of natural biocontrol agents. These fungi enhance plant development and resistance under stress while producing a variety of secondary metabolites that exhibit bioactivity against plant diseases and pests, including nematodes (Schouten, 2016; Aly et al., 2011). As reviewed in Chapter-2 (Sec 2.4.2), fungal endophytes, especially those from the genus Aspergillus, synthesise different compounds, including alkaloids, polyketides, terpenoids, and furan derivatives, which can disrupt nematode lifecycle, development, and infectivity. These metabolites may operate via mechanisms including the loss of cuticle integrity, the creation of oxidative stress, or the modification of host plant defence pathways, such as systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Wang et al., 2022; Wang et al., 2025).

Our earlier research as described in Chapters 3 and Chapters 4, has shown that the culture filtrate of root gall associated endophytic fungi *Aspergillus niger* F4 exhibits significant nematicidal activity against both *Meloidogyne graminicola* and *M. incognita*. In-vitro assays demonstrated significant juvenile mortality in both *M. graminicola* and *M. incognita*, whereas *in-vivo* experiments with rice and tomato, respectively, validated a substantial decrease in galling and nematode reproduction post-*A. niger F4* treatment. Furthermore, transcriptome study of treated plants revealed increased expression of SAR and ISR marker genes, indicating that the F4 culture filtrate not only directly affects nematodes but also stimulates plant immune responses. These data furnish substantial evidence for the existence of active secondary metabolites in the F4 culture filtrate. Despite this, the identification of the responsible metabolites and the precise mode of action remain till un-resolved. A thorough understanding of these biochemical components and their mechanisms is essential for validating the utility of *A. niger* F4 as a biocontrol agent and advancing its development into a field-ready, metabolite-based nematicide.

This chapter aims to address the existing knowledge gap through the pursuit of two primary objectives: 1) identify and characterise the secondary metabolites found in the culture filtrate of *Aspergillus niger* F4, employing advanced analytical techniques such as LC-MS, FTIR, NMR, and 2) functional elucidation of bioactive compounds in the culture filtrate. Various methods were employed including laboratory bioassays, quantification of reactive oxygen species (ROS), fluorescence and electron microscopy, as well as nematode gene expression profiling to clarify the cellular damage and stress pathways triggered by F4-derived metabolites. The identification and mechanistic understanding of active metabolites in the F4 culture filtrate will facilitate the development of novel, biologically derived formulations for nematode control, offering an economical and ecologically sustainable alternative to synthetic nematicides.

6.2 Materials and methods

6.2.1 Preparation of large-scale fungal culture filtrate

A. niger F4 was inoculated in potato dextrose broth (PDB) medium (200g peeled potato decoction 20g dextrose, pH 7.2, 1000 ml H2O) at 30 °C and 160 rpm for 7 days. Then, the *A. niger F4* broth was filtered with Whatman 1 filter paper followed by

passing through 0.45-micron syringe filter. This filtrate was concentrated to one-third of its original volume using a rotary evaporator under reduced pressure. The resulting concentrate was stored at room temperature and used for subsequent downstream assays.

6.2.2 Solvent fractionation assay of F4 culture filtrate

Six litres of culture filtrate from *Aspergillus niger* F4 was collected post- 7-incubation and subsequently filtered using muslin cloth and Whatman No. 1 filter paper to eliminate fungal biomass. The filtered broth was concentrated at 55 °C under decreased pressure with a rotary vacuum evaporator (Eyela, Model No. N1-NW; Buchi Rota-Vapor R-3, Switzerland), followed by the extraction of secondary metabolites of fungal culture filtrates, following standard protocols (Bogner et al., 2017; Bills & Gloer, 2016). To extract bioactive compounds, the concentrated culture filtrate underwent successive liquid-liquid partitioning using organic solvents of ascending polarity. The solvents employed included petroleum ether, n-hexane, chloroform, ethyl acetate, n-butanol, acetone, and methanol. This sequential extraction method optimises the recovery of chemically different metabolites, that range from non-polar lipophilic molecules to polar phenolics, alkaloids, glycosides and steroids (Sasidharan et al., 2011; Bills & Gloer, 2016; Gebreyohannes et al., 2019).

6.2.3 Identification of bioactive fraction:

To evaluate the nematicidal potential of each solvent fraction from the *Aspergillus niger* F4 culture filtrate, in-vitro bioassays were performed against freshly hatched *M. graminicola* juveniles., following nematode mortality assay protocols (Moo-Koh et al., 2022; Bogner et al., 2017; Degenkolb & Vilcinskas, 2016). Dried fraction from each solvent was dissolved in 0.5% DMSO to prepare three serial dilutions: 100%, 50%, and 25% (w/v or v/v depending on extract type). For the solvent fraction with low water solubility (e.g., ethyl acetate, petroleum ether), 5 µL of the fraction was initially dissolved in 0.5% DMSO with 0.6% Tween-20 as an emulsifying agent and concentration was made up to desired concentration. Tween-20 facilitates dissolvability of the hydrophobic compounds without showing toxic effects on nematode juveniles at low concentrations (Mishra et al., 2020; El-Sayed et al., 2021). The assay was conducted in U-bottom, 96-well microtiter plates. 100 µL of distilled water containing ~100 freshly hatched J2s were added in each well. Another 100 µL of the solvent-fraction-solution *(crude solvent fraction)* was added, bringing the total

volume of 200 µL. A control was there with 0.5% DMSO (with or without 0.6% Tween-20). For each treatment four biological replicates were performed. The 96-well plates were incubated in the dark at 30°C for 24 hours. The number of dead nematodes were counted under the Zeiss-Stemi 305 stereomicroscope (10× Carl Zeiss, Germany), and mortality percentage was adjusted with Abott's formula (Abbott, 1925). Data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Bartlett's test). One-way ANOVA was performed to assess treatment effects, followed by Duncan's Multiple Range Test (DMRT) for mean separation at $p < 0.05$. Statistical analyses were conducted using R (version 4.5.0), and results are expressed as mean ± standard error (SE). Treatments sharing the same letter were not significantly different.

6.2.4 Purification of the bioactive fraction

The extracted phase of methanol and water showed the highest nematicidal activity and was used for the further purification. The extracted fractions by methanol were loaded to a silica gel column, then eluted with ethyl acetate: acetone (9.9:0.1; v/v) and ethyl acetate: methanol: acetone (9.5:0.5:0.5; v/v), as mobile phase. Five subtractions were collected. Three subfractions were evaporated at 55 °C to dry, then dissolved in distilled water for the nematicidal activity assay as described above. The presence of compounds was checked with TLC (Silica gel 60 F_{254}) were run in Hexane: Ethyl Acetate solvent, Methanol: Water solvent combinations and spots were visualized under UV (Dutta et., al 2024).

6.2.5 Identification of the compounds

Purified methanol fraction of *A. niger F4* culture filtrate was subjected to High-Resolution LCMS ESI HRMS for further identification. Analysis was done with Waters Mass Spectrometer (Waters Corporation, Milford, MA, USA), Model: Xevo G2-XS Qtof for detecting metabolomics profiling at the Central Instrumentation Facility, CSIR–Indian Institute of Chemical Biology (IICB), Jadavpur, India. The identification of compounds was executed by comparing the mass spectra with the spectral data of the Metlin library and NIST from the RAW data of chromatogram provided by UNIFI software specific to the instrument. Acquity UPLC Class-1 used as UHPLC system, BEH C18, 1.7 µm, 2.1 × 100 mm analytical column was used. Two solvents were used in mobile phase; solvent A: Water + 0.1% Formic Acid and Solvent B: Acetonitrile + 0.1% Formic Acid. The column temperature was set at 40 °C with a flow rate of 0.3

mL/min. Volume of sample injection was 5 µL. The gradient program ran 0–2 min: 5% B, 2–12 min: linear increase to 95% B, 12–15 min: hold at 95% B finally 15.1–20 min: re-equilibration at 5% B. MS was performed both in positive ESI+ and nagetive ESI- modes. Lock Mass was set for mass correction with Leucine enkephalin at ($[M+H]^+$ = 556.2771). The acquisition Range was m/z 50–1500 in the centroid resolution mode (MSE with low and high energy function)

FTIR (Fourier Transform Infrared Spectroscopy analysis) analysis (ATR mode) of the purified crystals of methanol fraction of *A. niger F4* culture filtrate was done by FTIR Spectrophotometer (Model: Tensor 27 Bruker, Germany) available at the IICB, Kolkata, India to detect the functional groups existing in the present compounds.

^{1}H NMR analysis of the purified methanol fraction was performed using a JEOL spectrometer (Model: JNM-ECZ400S/L1, 400 MHz) at the Nuclear Magnetic Resonance Facility, CSIR–Indian Institute of Chemical Biology (IICB), Jadavpur, Kolkata, India. The sample was first dissolved in MeOD (deuterated methanol), and then the spectrum was attained at ambient temperature (~25 °C) using a standard one-pulse (1D) acquisition program. The chemical shifts (δ) were recorded on a scale from 0-10 ppm for proton (^{1}H) NMR. The field strength was set at 9.3957 T. Tetramethylsilane (TMS) was used as the internal standard in the spectrophotometer to check the chemical shift referencing, and spectra were processed with JEOL Delta software.

6.2.6 Confirmation of bioactive compound

A dose–mortality assay was performed using the primary identified compound to assess the nematicidal efficacy of sphinganine ((2S,3R)-2-amino-1,3-octadecanediol; ≥98% purity; Cat. No. 860498P-10MG, Sigma-Aldrich, St. Louis, MO, USA) against second-stage juveniles (J2s) of *Meloidogyne graminicola*. Sphinganine was dissolved in cell culture-grade DMSO to make a 1 mg/mL stock solution, followed by serial dilution to attain working concentrations of 0.0625,0.125,0.25, 0.5 and 0.75 µg/mL. The highest concentration of DMSO (<0.05%) found in the minimal sphinganine dosage acted as the control. The *M. graminicola* juvenile mortality was performed in 96 well plate following Dutta et al. (2023). Freshly hatched *M. graminicola* J2s were kept in minimum amount of distilled water and then adjusted the concentration of sphinganine. Three biological replicates of each concentration were taken. The 96-well plates were kept at dark for 24 hours. Number of dead and alive nematodes were

counted under the Zeiss-Stemi 305. Mortality was adjusted with Abott's corrected formula. Statistical analysis was conducted following methods described in section 6.2.3.

6.2.7 MTT assay of the selected compound

Cytotoxicity of the test compound was assessed using the Cell Proliferation Kit I (MTT) (Roche, Cat. No. 11 465 007 001) following the manufacturer's instructions. HT29 (also named A549) cells were cultured using 0.25% trypsin-EDTA, they were harvested, counted (with Bio-Rad TC20™ Automated Cell Counter), and seeded into 96-well flat-bottom cell-culture plates with the adequate density of 100µL of growth medium in each well. The cells adhered with overnight incubation at 37°C in a CO_2 incubator. After that, the culture medium was aspirated and it was replaced by 100 µL of fresh medium containing sphinganine dissolved in 0.05% cellular grade DMSO at various concentrations. 0.05% DMSO in media was treated as control. Another control with only culture medium was also taken. The control and treated cells were incubated for 24 hours at 37°C. Twenty-four hours post treatment, 10 µL of MTT 171abelling reagent (adjusted to final concentration of 0.5 mg/mL) was added to each well, and the plate was incubated for an additional 4 hours at 37°C in a CO_2 incubator. Finally, media was removed carefully, formazan crystals were dissolved with solubilization buffer and plate was read by using Bio-Rad iMark™ microplate reader at 595 nm. Statistical analysis was conducted following methods described in section 6.2.3.

6.2.8 Effect of F4 Filtrate on the Morphology of M. graminicola J2

6.2.8.1 Light Microscopy of *M. graminicola* juveniles

The morphology of *M. graminicola* J2 exposed to 50% of *A. niger F4* culture filtrate 24 h post inoculation was examined by Zeiss-Stemi 305 stereomicroscope (10× Carl Zeiss, Germany). For detail analysis temporary slides were prepared and observed in higher magnification (40×) using Carl Zeiss Axio ScopeA1 (Carl Zeiss, Germany)

6.2.8.2 Scanning Electron Microscopy (SEM) of *M. graminicola* juveniles

The changes in morphological characteristics of *M. graminicola* juveniles were observed with and without F4 culture treatment through a SEM (scanning electron microscopy). In the treatment set, J2s were treated with 50% of *F4* culture filtrate for 4 hours. Nematode treated with distilled water served as the control set. Two set of nematodes were washed separately, thrice with sterile distilled water and then fixed with 2% glutaraldehyde, diluted with 0.1 M phosphate buffer solution (pH 7.2) at 25°C

for 4 h. The next day the fixed nematodes to dehydrated with the gradient ethanol series (from 30, 50, 70, 90, and 100%). Then, the dried sample slides were assembled on stubs, coated with gold and observed using a ZEISS EVO LS 10 SEM.

6.2.8.3 Temporal Reactive Oxygen Species (ROS) generation Assay

ROS production in *M. graminicola* J2 treated by 50% *F4* culture filtrate detected at different time points by staining with DCFH-DA (2′,7′-Dichlorofluorescin diacetate, Sigma-Aldrich, USA)), a cell permeable florescent probe (Liu et. Al., 2001). A set of *M. graminicola* J2s were treated with 50% of *F4* culture filtrate for eight hours, samples were taken for the assay at consequent hour intervals of 1, 2, 4, 6, 8 hrs. A control set of juveniles were incubated in M9 buffer was the control and its data was taken finally at 8th hour's post inoculation (HPI). At 1, 2, 4, 6, 8 HPI, 250 µl DCFH-DA was added to each well and incubated at 37 °C for 30 min in dark. After staining, nematodes were washed with M9 buffer for three times. ROS production was measured using a multi-mode-microplate reader (FLUOstar Omega, BMG Labtech) and observed under a fluorescence microscope (Olympus CKX53).

6.2.9 Gene Expression analysis of M. graminicola with A. niger F4 treatment

Approximately 20,000 freshly hatched J2s of *M. graminicola* were washed with minimum amount of nuclease-free water, pelleted by centrifugation at 5000 rpm for 5 minutes in and distributed into 1.5 mL microcentrifuge tubes. The study was conducted with two treatments: control (treated with sterile nuclease-free water) and F4 treated (nematodes treated with 50% (v/v) culture filtrate of *A. niger* F4). Depending on the temporal ROS generation assays (see previous section for method and Fig. 6.8A for results) and dose–mortality response, 2-hours treatment of nematodes were selected as the time point for the gene expression analysis.

A set of genes involved in core physiological functions, such as stress response (lig, eftu), lipid metabolism (fat), neuromuscular signalling (unc-26, unc-68, ace1, odr), and parasitism-related processes (*sra*, *pap*, *rck*), were selected for the gene expression analysis for the study based on their putative roles in nematode survival and host interaction. List of genes selected, their role and primer sequences are detailed in Table-6.1.

Total RNA was extracted from the control and F4 treated J2s using HiPurA Pre-filled Catridges for plant RNA purification (Himedia) and Insta NX Mag 16 automated

nucleic acid extractor (Himedia) according to the manufacturers' protocol. The yield and quality of the RNA were examined in a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) followed by 1% (w/v) agarose gel electrophoresis. The extracted RNA (~ 400 ng/µL) was converted to cDNA using InvitrogenTM SuperScriptTM VILOTM cDNA synthesis kit. The cDNAs were stored at – 20 °C for further downstream applications.

For the gene expression analysis, a qRT PCR based assay was performed in thermal cycler CFX96 (BioRad). The reaction mixture (20 µL) contained 1 µL of cDNA (~20 ng), forward and reverse primers each of 0.8 µL, 10 µL of Takara TB green Premix Ex TaqTM dye and the rest was adjusted with nuclease-free water. The PCR program was set at hot start at 95 °C for 2 min followed by 40 cycles of 94 °C for 30 s and 60 °C for 45 s, then 72 °C for 45 s. Amplification specificity was determined by adding a melt curve programme (95 °C for 15 s, 60 °C for 15 s), followed by a slow ramp from 72 to 95 °C). With the help of CFX Maestro software (BioRad), quantification cycle (Cq) values were retrieved, then fold change in target gene expression was calculated by the $2-\Delta\Delta Cq$ method. Gene expression was normalized using *actin* as a housekeeping gene for *M. graminicola*.

Table 6.1 Selected genes for expression profiling in *M. graminicola* juveniles treated with *A. niger* F4 culture filtrate.

Gene	**Function**	**Primer Name(s)**	**Primer Sequence(s) (5′→3′)**
ODR	Olfactory signalling and chemosensation; involved in host recognition	Mg_odrF / Mg_odrR	TATGCACGAAACATGTGCAA / TTACATCATTCCCGCCTTTT
Rck	Receptor for Activated C Kinase; regulates multiple signalling pathways	Mg_rackF / Mg_rackR	TCTCTTCATGGTCACGGACA / GGTCCCAAAGACGTAGAGTTTT
Ace1	Acetylcholinesterase; neuromuscular function and pesticide target	Mg_ace1F / Mg_ace1R	TTCTTATAATTCTGATTGGCCTTT / TCTTGGACCAGTTCCTAATCG
Fat	Polyunsaturated fatty acid synthesis; affects dauer formation, immunity	Mg_fatF / Mg_fat6R	GCCAACCAATATGTGCAAAG / AAAGCTAAATTTCCTTTACGTTCA
apl	Synaptic vesicle cycling, neurotransmission	Mg_aplF / Mg_aplR	CCATGTAAACAACCAGCAACA / TGCCTCTAGTAGCCTTTCAATG
tns	Tension-like proteins; structural role in maintaining nematode body shape	Mg_tnsF / Mg_tnsR	CTTTTATATGAACAGGAACAGAATG AA / GAAAGTTTAACTGGCCCAGAA
unc68	Ryanodine receptor; involved in calcium signalling and muscle function	Mg_unc68F / Mg_unc68R	GGAATGGGGCAAGCAACTAT / CAACTTTTCCAATTCCTTTTCG
unc26	Phosphatidylinositol phosphatase; crucial for synaptic vesicle recycling	Mg_unc26F / Mg_unc26R	TGAAGAAGGAATAGAAGAAGATTGG / CCTTCAGCTAAATTTACAAGTTCAT

SRA	Serpentine receptor class A; G-protein-coupled receptors	Mg_sraF / Mg_sraR	GATGCATTAATTGAATACGTAATTTCC / CCAATTTATTTAATTAGAAGAGGTCGT
Pap	Prolyl aminopeptidase; protein processing and stress response	Mg_papF / Mg_papR	CTGAAGGAGAGGAAGACCATTT / TGAGGATTAACATGACACAAAGC
eftu	Elongation factor Tu; protein synthesis	Mg_eftuF / Mg_eftuR	GGTTGATGTTCATGAAGGAATG / GCATCTGCAGGTGGAAGTTT
wdr	WD-repeat domain proteins; signal transduction, stress response	Mg_wdrF / Mg_wdrR	TTTGTGATGGAATTGCTGCT / ATGTCCAACAGTGCCTACCC
lig	Ligase family; DNA repair or ubiquitin-related pathways	Mg_ligF / Mg_ligR	CAGAAATTCCAACACCTCCA / TCTCCAACACGTGAACTTGC

6.3 Results

6.3.1 Identification of bioactive fraction:

The nematicidal activity of sequential solvent fractions derived from the *A. niger F4* culture filtrate against *M. graminicola* J2s was evaluated, and the mortality responses are presented in Figure 6.1. The results revealed significant differences in J2 mortality across the solvent fractions ($F_{5,18} = 768$, $p < 0.05$), highlighting the influence of solvent polarity on the extraction efficiency of bioactive metabolites from the F4 culture filtrate. Among the tested fractions, water and methanol extracts exhibited the highest nematicidal activity (98.29 ± 2.3% and 82.51 ± 4.8% mortality, respectively), with the maximum mortality recorded in the water fraction. This underscores the ability of highly polar solvents to extract bioactive nematicidal molecules from *A. niger* F4.

Although the highest mortality was observed in the water fraction, the methanolic extract represents a valuable intermediate, offering considerable nematicidal activity along with enhanced chemical stability and compatibility for downstream characterisation, compared to aqueous extracts which are more prone to degradation and microbial contamination (Zhang et al., 2019; Wang et al., 2022). The acetone fraction showed moderate efficacy (74.21 ± 1.9% mortality), while the ethyl acetate extract exhibited limited nematicidal activity (21 ± 1.7%). The hexane fraction recorded the lowest mortality, suggesting that non-polar extracts contain few or no nematicidal molecules.

The strong activity observed in the methanolic fraction suggests the presence of moderately polar secondary metabolites such as alkaloids, phenolic compounds, aromatic polyketides, esters, and long-chain fatty acids, which have been previously reported to possess activity against various plant pathogens (Narsing Rao et al., 2017; Gupta et al., 2014; Mannaa & Kim, 2018; Samarakoon et al., 2022). Earlier studies

have shown that methanolic extracts of fungal metabolites, including gliotoxins, oxylipins, and fumigaclavines, exert nematotoxic effects by disrupting neuromuscular coordination, causing cuticle disintegration, and inducing oxidative stress (Singh et al., 2021; Yadav et al., 2020). The significant mortality observed in the methanolic fraction supports the hypothesis that *A. niger* F4 produces similar or novel classes of nematicidal compounds that can be efficiently extracted with methanol. Given its potent activity and operational advantages, the methanolic fraction is considered a promising candidate for subsequent chemical characterisation, purification, structural elucidation, and eventual development into a biopesticide. Methanol-based extraction also offers practical benefits for natural product research due to its reproducibility, solvent compatibility, and ease of removal under vacuum (Zhang et al., 2019).

6.3.2 Identification of the compounds

The purified methanolic fraction from *A. niger* F4 culture filtrate was subjected to comprehensive spectroscopic analyses including LC-MS/MS, FTIR, and ^{1}H NMR to elucidate the chemical structure of the bioactive compound.

6.3.2.1 LC-MS/MS Analysis

High-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS) revealed a major molecular ion peak $[M+H]^+$ at m/z 302.3048, suggests the presence of a compound with ~301 Da molecular weight (Fig. 6.2.A). With the help of NIST, METLIN database and confirmation with previous literature the calculated exact mass for the molecular formula $C_{18}H_{37}NO_2$ is 302.3059, with the experimental value showing a minimal error of -3.6 ppm, confirming high mass accuracy. The fragmentation pattern also showed characteristic peaks at m/z 284.29, 266.28, and 303.30, indicating sequential cleavages near hydroxylated positions and the amine group typical feature of a sphingoid backbone structure. Notably, the molecular formula and i-FIT value of 834.8 matched closely with the commercial standard of (2S,3R)-2-amino-1,3-octadecanediol (sphinganine) (≥98% purity, Cat. No. 860498P-10MG, Sigma-Aldrich, St. Louis, MO, USA), providing strong evidence for structural identification (Fig. 6.2.B).

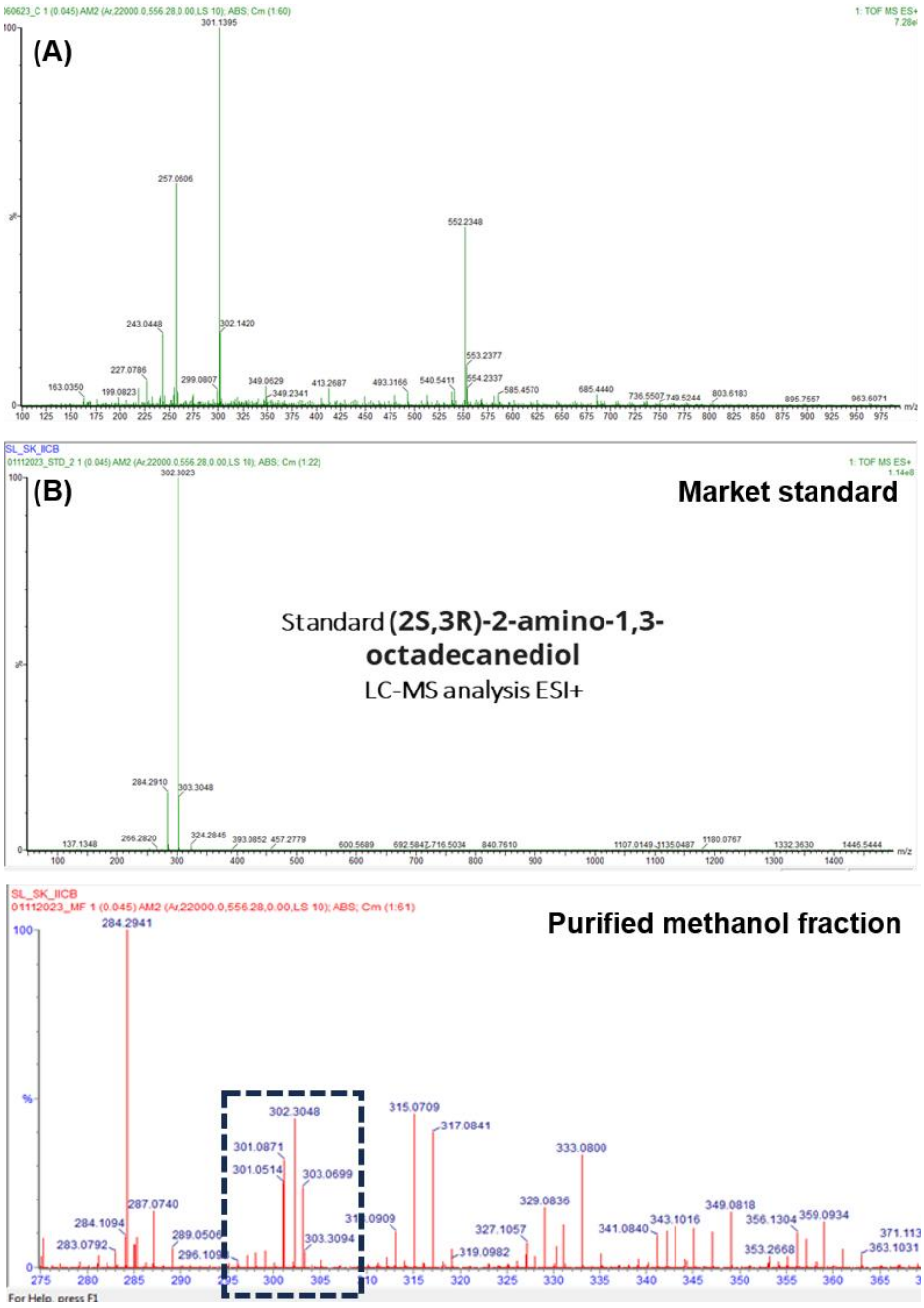

Fig. 6. 2 (A) High-resolution mass spectra of the purified methanol fraction. Peaks indicating metabolites detected in positive electrospray ionization mode (ESI^+). The spectrum shows the relative intensities of ions plotted against their corresponding mass-to-charge (m/z) ratios. Data were acquired using time-of-flight mass spectrometry (TOF MS) with a scan range of m/z 100–1000. (B) LC-MS analysis of standard (2S,3R)-2-amino-1,3-octadecanediol compared with purified methanol fraction; The positive (ESI+) of the reference standard (2S,3R)-2-amino-1,3-octadecanediol reveals a prominent $[M+H]^+$ peak, consistent with the calculated molecular weight of the protonated species ($C_{18}H_{40}NO_2$, theoretical m/z = 303.3059, dashed rectangle). The accompanying isotope pattern and fragmentation ions (e.g., m/z 284.2910 and 284.2941) support the molecular structure.

6.3.2.2 FTIR Spectroscopic Analysis

The FTIR spectrum (Fig. 6.3) provided important information regarding the functional groups present in the purified methanol fraction. A broad absorption spectrum at 3360 cm^{-1} indicated the O-H stretching vibrations of present in hydroxyl (-OH) and amine (-NH) group. Strong absorption bands at 2920 cm^{-1} and 2852 cm^{-1} corresponded to asymmetric and symmetric C-H stretching vibrations, respectively, a characteristics of long chain aliphatic backbone. A prominent peak at 1640 cm^{-1} was assigned to the N-H bending vibration. Additional absorption bands between 1234 cm^{-1} and 1090 cm^{-1} were attributed to C-N and C-O stretching vibrations, aligning with the presence of amino alcohol functional groups (Silverstein et al 2005). Collectively, these spectral features associate perfectly with the sphinganine structure, corroborating the findings from the LC-MS/MS analysis.

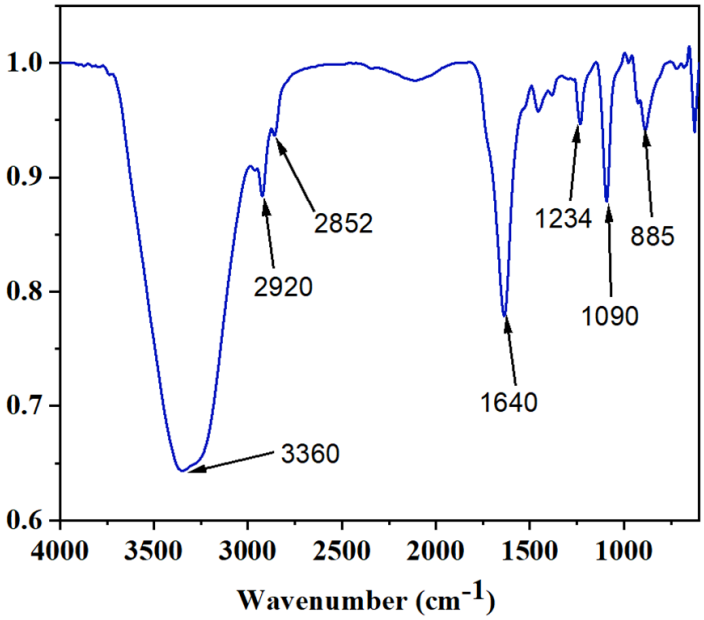

Fig. 6. 3 FTIR spectrum of the purified methanol fraction of *A. niger* F4.

6.3.2.3 ^{1}H NMR Spectroscopic Analysis

The spectrum of ^{1}H NMR further determined the proposed structure through the identification of major proton signals (Fig. 6.4). Chemical signals observed at 0.911, 0.970, 1.283 and 1.379 ppm match typical signal from the methylene ($–CH_2$) group in the long hydrocarbon chain or aliphatic group, while the terminal methyl protons resonated at ~ 0.8 ppm. The downfield chemical shift was observed at 3.349, 3.656 and 3.788 ppm are observed, assigned with protons on carbons attached to

electronegative ions –OH and –NH_2 groups, which is characteristics of the hydroxyl and amine groups. A distinctive triplet at ~3.3 ppm precisely supports the presence of a terminal –CH_2–OH group. Chemical shift observed at 1.992, 2.197, 2.33 and 2.546 ppm may originate from –CH_2 groups adjacent to the –OH groups in long aliphatic chain. These protons are more deshielded than regular aliphatic methylene protons. The combination of these signals suggested a long chain aliphatic group consisted with a C18 backbone. The ^{1}H NMR data correlated with the structure of sphinganine (2S,3R)-2-amino-1,3-octadecanediol), thereby supporting the identification of the compound.

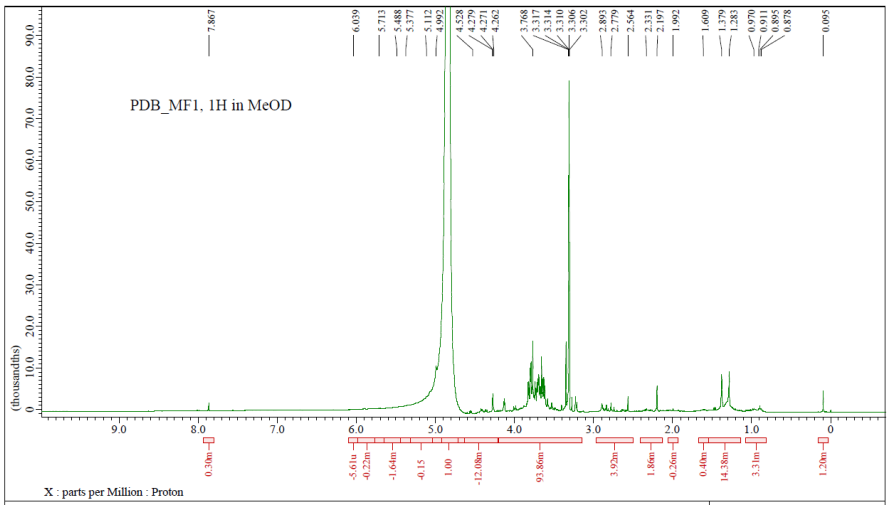

Fig. 6.4 ^{1}H NMR spectra of the purified methanol fraction of *A. niger F4*

The detailed spectroscopic investigation of the purified methanol fraction from *A. niger* F4 culture filtrate resulted in strong evidence for its identification as sphinganine or (2S,3R)-2-amino-1,3-octadecanediol ($C_{18}H_{37}NO_2$, MW: 301.29 Da). This compound is classified within the sphingolipid category, particularly as a sphingoid base. It has a long-chain aliphatic backbone that includes amino and hydroxyl functional groups. Sphingolipids, especially sphinganine, have gained attention for their wide-ranging biological activities, such as antimicrobial, antifungal, and nematicidal effects across different biological systems (Jadhav et al., 2016; Noda et al., 2020). The discovery of sphinganine from *A. niger* F4 is noteworthy and documented for the first time, as it represents as one of the limited instances of this compound being synthesized by filamentous fungi, particularly within the *Aspergillus*

genus.

Recent studies have shown the strong nematicidal effects of sphingoid bases on plant-parasitic nematodes. Guo et al. (2016) observed that *Bacillus cereus* strain S2 demonstrated significant nematicidal activity against *Meloidogyne incognita* through the production of sphingosine, which is a structural analogue of sphinganine. The authors demonstrated that sphingosine had a direct impact on nematode viability by compromising cellular membranes due to its amphipathic characteristics. Additionally, Lee et al. (2022) discovered 3-furoic acid from the sea-derived *Aspergillus luchuensis* Hy-6, demonstrating its efficacy as a nematicidal agent against plant-parasitic nematodes in cucumber. This finding underscores the potential of *Aspergillus* species to generate metabolites with nematicidal capabilities.

The amphiphilic characteristics of sphinganine, which includes both hydrophobic (long aliphatic chain) and hydrophilic (amino and hydroxyl groups) components, indicate possible membrane-disrupting abilities that could play a role in its nematicidal potential. Sphingolipids interact with membrane components, resulting in changes to membrane fluidity and permeability, potentially causing cellular dysfunction in target organisms (Jadhav et al., 2016). Furthermore, Mendelson et al. (2021) illustrated that sphingolipid metabolites can activate programmed cell death pathways in nematodes, suggesting an additional potential mechanism for their nematicidal effects.

The process of sphinganine biosynthesis in *A. niger* probably involves the synthesis of serine and palmitoyl-CoA via serine palmitoyltransferase, following the reduction of 3-ketosphinganine through the action of 3-ketosphinganine reductase (Merrill, 2002). Multiple studies have reported that fungi have the enzymatic mechanisms required for sphingolipid synthesis, especially those related to stress responses and adaptation to challenging environments (Singh & Del Poeta, 2011). The synthesis of sphinganine by *A. niger* F4 could be influenced by various environmental parameters, including nutrient availability, pH levels, and cultivation conditions, underscoring the metabolic adaptability of this fungal species.

Wang et al. (2019) demonstrated that sphingolipids derived from fungi displayed notable nematicidal effects on root-knot nematodes in tomato plants, leading to a decrease in gall formation and enhancement of plant growth parameters. In a similar vein, Xu et al. (2023) illustrated that sphingoid bases derived from microbial sources can efficiently manage plant-parasitic nematodes in agricultural environments,

presenting a significantly lower environmental impact than traditional nematicides.

6.3.3 Confirmation of bioactive compound

Juvenile mortality was observed 24 hours post inoculation (HPI) of sphinganine. The percentage of juvenile mortality demonstrated a significant dose-dependent (Fig. 6.5.A) relationship ($F_{5,12}$= 1072, $p<0.05$). At minimum applied dosage of 0.0625 µg/mL a J2 mortality of 58.0±3.1% was observed. However, ~100% morality was achieved at the highest tested concentration of 0.75 µg/mL. The LC_{50} concentration of sphinganine was found to be 0.0603 µg/mL, highlighting the potent nematicidal property of this compound.

The nematicidal effect of sphinganine is consistent with observations regarding structurally analogous sphingoid bases like sphingosine. Liu et al. (2021) found that *Bacillus cereus* strain S2 generated sphingosine, exhibiting strong nematicidal effects against *M. incognita*, probably by compromising membrane integrity and disrupting signalling pathways. Sphinganine, a biosynthetic precursor in sphingolipid metabolism, may operate through similar mechanisms, potentially incorporating into the nematode lipid bilayer and modifying membrane fluidity, which could result in cellular dysfunction and death (Hannun & Obeid, 2008; Cowart & Hannun, 2007).

6.3.4 MTT assay with bioactive compound

The cytotoxic potential of sphinganine on HT-29 human intestinal epithelial cells was tested at four concentrations: 0.3 ug/ml, 0.5 ug/ml, 0.7 ug/ml, 0.9 ug/ml (LC_{50} = 0.06 ug/ml). The results showed (6.5.B) that all the selected concentration of sphinganine did not have any significant effects on the viability of HT-29 cells ($F_{4,15}$ = 0.712, $p > 0.5$). The results align with previous studies indicating that sphinganine exhibited low cytotoxicity in non-transformed epithelial and fibroblast cell lines (Kimberly et al., 1995). This finding suggests that sphinganine can be considered a safe candidate for further development as a biocompatible nematicidal agent. However, following the available guidelines for pesticide safety (e.g. OECD Test Guideline 129, EPA OCSPP 870.2400, and REACH Annexes VII–X) further in vitro assays with hepatic (e.g., HepG2), dermal (e.g., HaCaT), and renal (e.g., HEK293) cell lines will be required to generate a comprehensive cytotoxic evaluation of sphinganine.

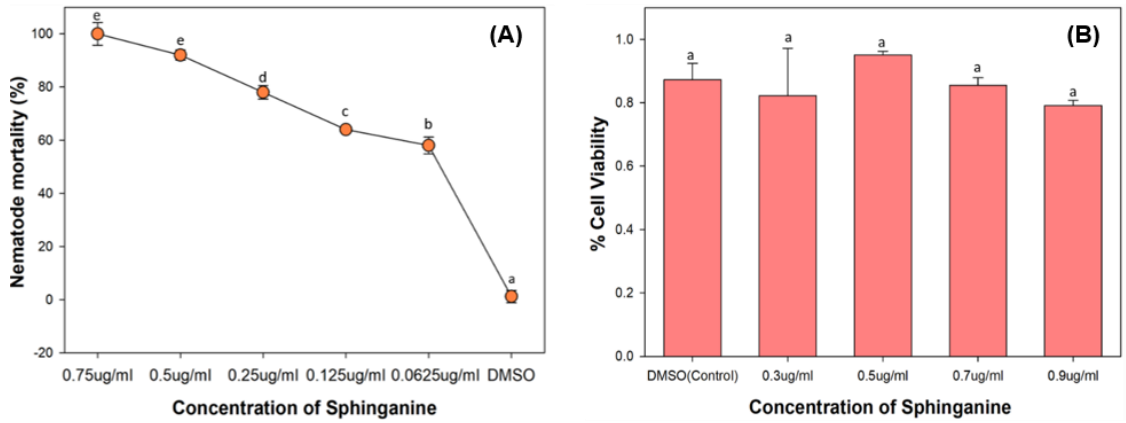

Fig. 6.5 (A) Nematode mortality (%) following exposure to increasing concentrations of sphinganine (0.0625–0.75 µg/ml) for 24 h. DMSO served as the solvent control. A significant dose-dependent increase in mortality was observed (ANOVA: $F_{5,18} = 768$, $p < 0.05$).LC_{50} was estimated at 0.06 µg/µl. Same letter(s) indicate no statistical significance ($p > 0.05$) using Duncan's multiple range test (DMRT). (B) Cell viability test of HT 29 mammalian cell line after treatment with sphinganine at varying concentrations (0.3–0.9 ug/ml), with DMSO control. Bars represent mean ± SE of four replicates. No significant differences were observed among treatments (ANOVA: $F_{4,15} = 0.712$, $p = 0.596$).) Same letter(s) indicate no statistical significance ($p > 0.05$) using Duncan's multiple range test (DMRT).

6.3.5 Morphological Changes of M. graminicola J2s with A. niger F4 culture filtrate treatment

The mode of action of F4 culture filtrate was investigated using microscopic imagery such as light and scanning electron microscopy (SEM) as well as detection of intracellular ROS production. Collectively these studies revealed that the treatment of F4 culture filtrate leads to marked morphological changes and generates considerable oxidative stress in the *M. graminicola* juveniles.

6.3.5.1 Light Microscopy of *M. graminicola* juveniles

Bright-field imaging of treated nematodes showed significant morphological changes, such as internal vacuolization, cytoplasmic granulation, and a total loss of motility (Fig.6.6). The control nematodes exhibited clearly defined cellular boundaries and demonstrated active movement. The vacuoles observed in nematode juveniles following F4 treatment are characteristic of "methuosis," a form of non-apoptotic regulated cell death involving vacuole accumulation and disruption of membrane integrity (Rajasekharan et al., 2017). The cytological collapse seen in treated samples was consistent with the morphological symptoms recorded in *M. incognita* when exposed to fungal metabolites like 2-furoic acid from *Aspergillus fumigatus* 1T-2 (Gao et al., 2022).

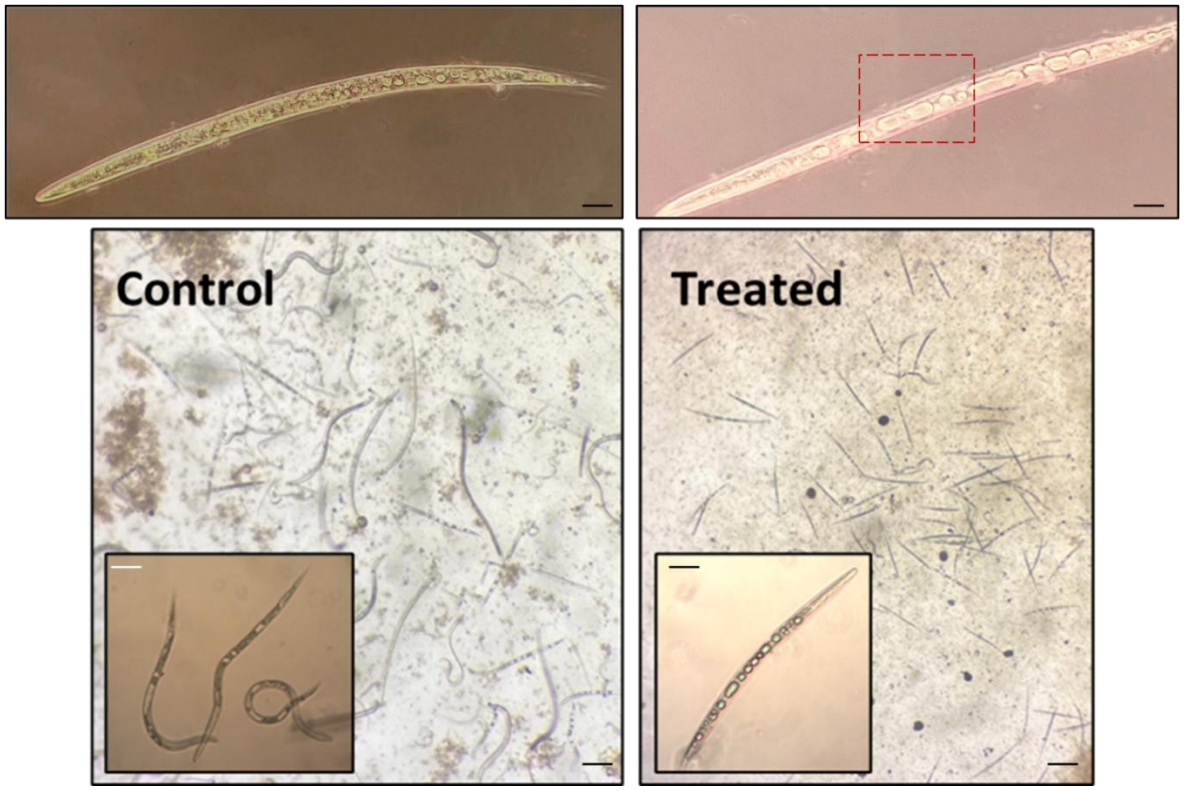

Fig. 6.6 Light microscopy images of *M. graminicola* juveniles;
Top left and right represents high-magnification (40×) images of *M. graminicola* J2. The untreated nematode (left) exhibits a typical spindle-shaped morphology with visible gut granules. The treated nematode (right) shows significant vacuolation and disrupted internal structure (inset image). In bottom panels, overview of nematode populations in control and treated groups. Control J2s display active coiling and normal movement (left), while treated J2s appear paralyzed and morphologically dissimilar, predominantly adopting a straightened posture (right). Inset images represent magnified views of representative individual nematodes. "–" represents Scale bars; Scale bars=30 µm (overview), 10 µm (inset image).

6.3.5.2 Scanning Electron Microscopy (SEM) of *M. graminicola* juveniles

SEM analysis showed that the control juveniles had a smooth, intact cuticle, while the nematodes subjected to F4 filtrate exhibited significant surface collapse, ruptured annulations, and areas of peeling cuticle (Fig.6.7). Disruption of cuticular integrity in nematodes is a characteristics response to exposure to nematicidal compounds, as previously reported in nematodes exposed to sphingosine-producing *Bacillus cereus* (Gao et al., 2016) and fungal-derived diketopiperazines (Wang et al., 2022). Notably, such structural deterioration of the nematode's outer layers compromises barrier function, leading to osmotic imbalance and facilitating the penetration of toxic metabolites, a process fundamental to both microbial and synthetic nematicide activity.

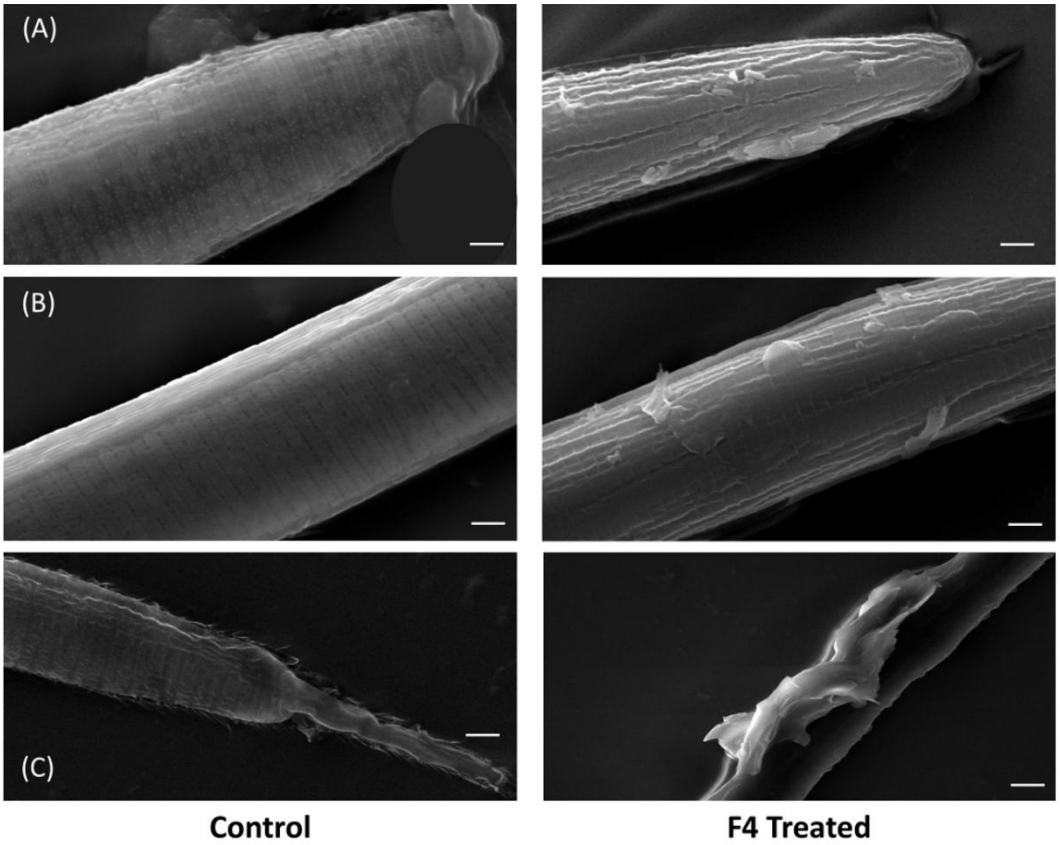

Fig.6.7 Scanning electron microscopy images illustrating the ultrastructural; (A–C) SEM micrographs of the nematode cuticle under control (left) and F4-treated (right) conditions (A) Intact cuticle structure in control nematodes with smooth longitudinal striations, whereas treated samples exhibit severe surface damage and fissures at the head region of the juveniles. (B) Higher-magnification view showing uniform cuticle ridges in control nematode's abdomen region and extensive delamination and tearing in treated nematode. (C) Posterior body regions showing distinct ruptures and collapse in the treated group whereas in control the intact tails are visible. "–" represents Scale bars= 2 µm.

6.3.5.3 ROS generation assay

The production of intracellular ROS was evaluated using the H2DCFDA fluorescence probe. Treated nematodes showed a significant and time-dependent rise in ROS accumulation, reaching its maximum at 4 hours after treatment (Fig. 6.8.A). Confocal microscopy revealed a strong green fluorescence predominantly concentrated in the pharyngeal and intestinal areas of juveniles treated with F4, whereas the control nematodes exhibited minimal fluorescence (Fig. 6.8.B). The sharp increase in ROS suggests damage caused by oxidative stress, aligning with mechanisms documented in previous research. Liu et al. (2021) illustrated the role of ROS in causing mitochondrial collapse and resulting in nematode lethality after exposure to *Purpureocillium lilacinum*. In a related study, Kim et al. (2023)

demonstrated that iodoindoles and abamectin triggered a type of non-apoptotic cell death known as methuosis in *Bursaphelenchus xylophilus*, characterised by ROS overproduction and vacuole accumulation, which closely resembles the phenotypes seen in the treated nematodes in our study.

The interplay between ROS-induced cytotoxicity and structural compromise indicates that the F4 filtrate operates through a dual mechanism—compromising membrane integrity while also inducing oxidative damage. This dual modality is gaining recognition in the effects of both microbial and plant-derived nematotoxins (Santos et al., 2023; Kim et al., 2023), and may reflect a conserved susceptibility pathway in nematodes linked to redox imbalance and membrane destabilisation.

Overall, the microscopy and biochemical data together indicate that the culture filtrate from *Aspergillus niger* F4 causes lethal damage in *M. graminicola* juveniles by inducing structural disintegration of the cuticle and oxidative stress, as shown by increased ROS levels.

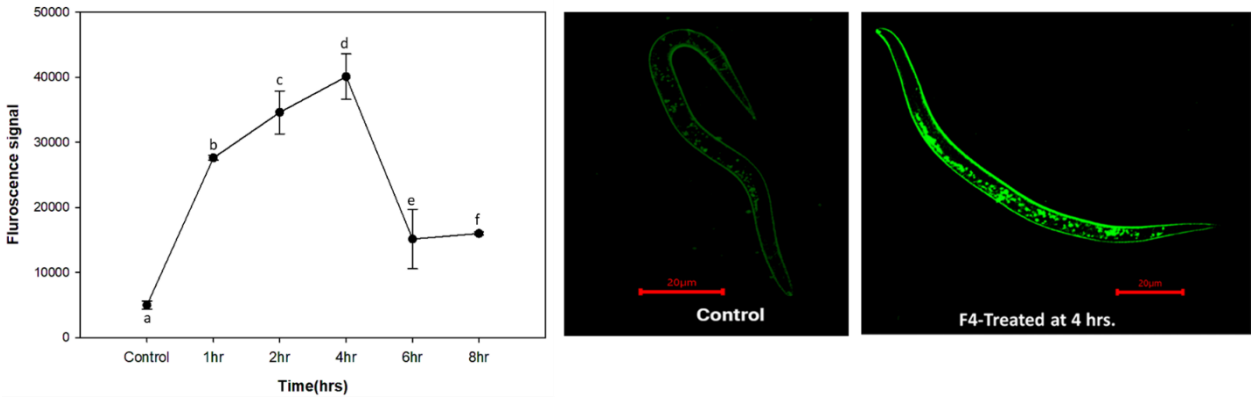

Fig.6.8 (A) Quantitative ROS generation assay over time, following exposure to F4. Data represent mean ± SD (n = 3). Different lowercase letters indicate statistically significant differences among time points ($F_{5,24}$ = 1227; $p < 0.05$) Different letters indicates significant difference done by Duncan's DMRT test. (B) Representative fluorescence microscopy images of J2s stained with H2DCFDA. Control nematodes show weak fluorescence, indicating basal ROS levels. In contrast, F4-treated nematodes (4 h) display intense, widespread green fluorescence throughout the body, confirming ROS overproduction. Scale bars = 20 µm.

6.3.6 Gene Expression analysis of *M. graminicola* with A. niger F4 treatment

The gene expression study on *M. graminicola* juveniles treated with *A. niger* culture filtrate demonstrated significant differential expression patterns in nine target genes (among 13 targeted genes) relative to untreated controls (Fig.6.9). The relative

gene expression levels, normalised to control conditions (established at 1.0), exhibited significant upregulation in most of the analysed genes under F4 treatment conditions. The most significant overexpression was noted in the *fat* gene, which demonstrated a 3.8-fold increase in expression (±0.5) compared to controls. *Ace1* gene exhibited a 3.2-fold overexpression (±0.4), while *sra* gene had a 2.9-fold increase in expression (±0.3). The *eftu* and *wdr* genes exhibited moderate overexpression at 2.0-fold (±0.2) and 2.9-fold (±0.1), respectively.

Several genes exhibited modest yet consistent upregulation such as: *apl* at 1.8-fold (±0.1), *tns* at 1.9-fold (±0.1), *unc26* at 2.8-fold (±0.3), *pap* (prolyl aminopeptidase) at 1.8-fold (±0.1), and *lig* (ligase family) at 1.9-fold (±0.1). The *odr* gene and *rck* gene demonstrated slight elevation at 1.4-fold and 1.1-fold, respectively, but *unc68* (ryanodine receptor) displayed the lowest response with approximately 0.8-fold expression compared to controls. Control samples exhibited stable baseline expression levels for all studied genes (about 1.0 ±0.1), validating the robustness of the experimental design and normalisation methods.

Exposure to *A. niger* F4 culture filtrate induced significant transcriptional changes in *M. graminicola* juveniles, indicating a multi-faceted physiological stress response. The significant overexpression of the *fat* gene indicates that *M. graminicola* juveniles initiate polyunsaturated fatty acid production pathways in reaction of exposure to *A. niger* F4 culture filtrate. This metabolic reaction is likely to signify a cellular defence mechanism, as polyunsaturated fatty acids are essential for membrane stability and stress resilience in nematodes (Watts & Browse, 2002). Augmented fatty acid metabolism may preserve membrane integrity under chemical stress induced by fungal metabolites.

The overexpression of *ace1* (acetylcholinesterase) suggests possible neurotoxic effects of *A. niger* F4 culture filtrate on the nervous system function of *M. graminicola*. Acetylcholinesterase is essential for neuromuscular function and acts as a key target for numerous nematicidal agents (Opperman & Chang, 1991; Corbett et al., 1984). The over expression presumably signifies a compensatory mechanism to sustain cholinergic signalling amid chemical stress. This discovery aligns with recent transcriptomic analyses by Hada et al. (2021), which revealed analogous modulation of the acetylcholinesterase gene in *Meloidogyne incognita* upon exposure to nematicidal agents.

The overexpression of *sra* genes signifies improved environmental sensing skills in

reaction to fungal compounds. G-protein-coupled receptors are crucial for nematode chemosensation and environmental adaptability (Bargmann, 2006). The comparatively overexpression indicates that juveniles are actively reacting to chemical signals in the *A. niger* culture filtrate, possibly as a component of an avoidance or stress response mechanism.

In contrast, *odr* gene expression showed only modest elevation, indicating selective modulation of chemosensory pathways. *Odr* genes are essential for host recognition and environmental perception in plant-parasitic nematodes (Wyss, 2002; Perry & Moens, 2013). Dutta & Akhil (2021) conducted comparative studies on the infection potential of *M. incognita* and *M. graminicola* in basmati rice, revealing species-specific variations in chemosensory gene expression patterns, indicating that *M. graminicola* may utilise unique chemoreceptor mechanisms relative to other root-knot nematode species. The differential response observed between *odr* and *sra* pathways in this investigation indicates pathway-specific sensitivity to *A. niger* metabolites, suggesting that the fungus filtrate primarily influences non-olfactory chemosensory processes while preserving fundamental *odr* function. This selective modulation pattern corresponds with the species-specific chemosensory adaptations shown in comparative *Meloidogyne* investigations; wherein distinct nematode species demonstrate differing sensitivities to ambient chemical signals.

The moderate overexpression of *wdr* gene facilitates the activation of stress response pathways, as these proteins participate in signal transduction cascades and cellular stress responses (Li & Roberts, 2001). This increase presumably enhances the coordination of many stress response pathways triggered by exposure to fungal metabolites. The elevation of *eftu* (elongation factor Tu) indicates heightened protein synthesis activities, potentially reflecting a stress response necessitating higher protein production or a compensatory mechanism for protein degradation induced by fungal toxins (Browning et al., 2001). This response aligns with biological efforts to sustain homeostasis amid chemical stressors.

The increased expression of *pap* (prolyl aminopeptidase) signifies increase in protein processing activity, potentially essential for handling damaged proteins or activating stress-response proteins during chemical stress conditions (Cunningham & O'Connor, 1997).

The overexpression of the *apl* and *unc26* genes, which are implicated in synaptic vesicle cycling and neurotransmission, indicates that *A. niger* culture filtrate

influences nematode nervous system function. The *unc26* gene encodes a phosphatidylinositol phosphatase essential for synaptic vesicle recycling (Harris et al., 2000), whereas *apl* is linked to synaptic vesicle cycling mechanisms. Their synchronised upregulation signifies adaptive responses to preserve neuromuscular function under chemical stress. In contrast, *unc-68* expression was slightly downregulated, implying that calcium signalling via the ryanodine receptor is either unaffected or regulated through alternative pathways (Maryon et al., 1996).

The increase of *tns* gene expression implies that *M. graminicola* juveniles undergo mechanical or structural stress due to culture filtrate treatment. These proteins serve structural functions in preserving the body form of nematodes and may be increased to mitigate morphological alterations caused by fungal chemicals (Benian et al., 1996).

The gene expression profile of *M. graminicola* juveniles exposed to *A. niger* F4 culture filtrate reveals widespread physiological disruption involving metabolism, neuromuscular signalling, environmental sensing, and protein synthesis. Key transcriptional changes, such as upregulation of *fat*, *ace-1*, *unc-26*, *sra*, and *pap*, indicate a coordinated stress response to multiple toxic cues, while selective modulation of *odr* and *unc-68* suggests pathway-specific targeting.

These findings provide molecular evidence that the F4 filtrate exerts a multi-target mode of action, consistent with earlier morphological and behavioural observations. Such broad-spectrum interference reduces the likelihood of resistance development and underscores the potential of *A. niger* F4 as a sustainable biocontrol agent against plant-parasitic nematodes.

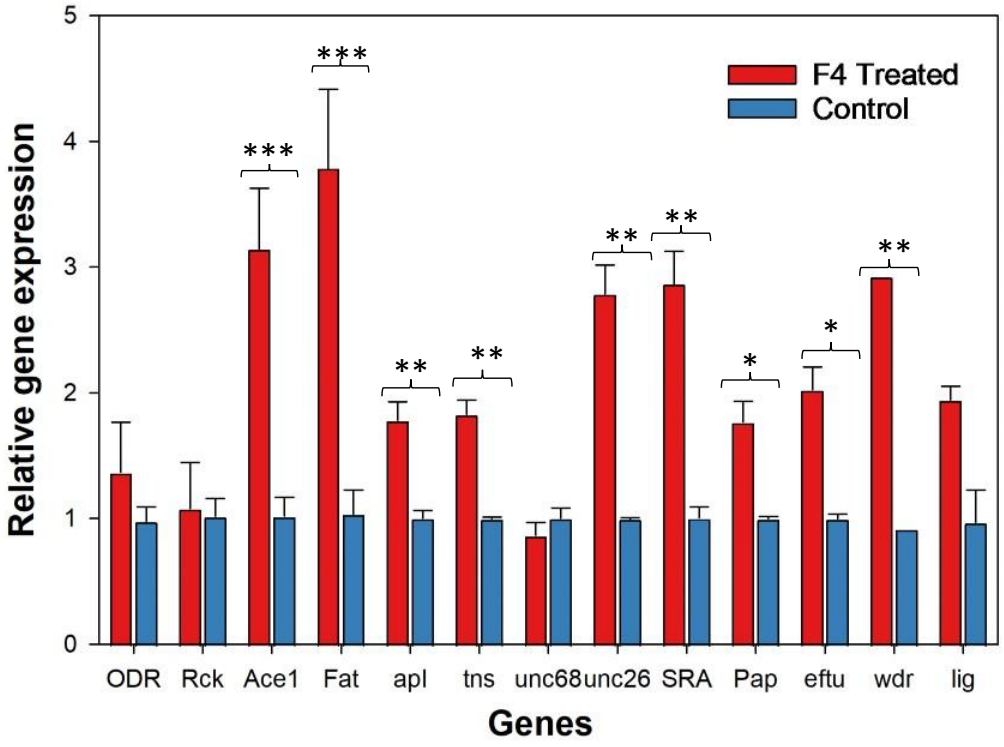

Fig 6.9 Relative gene expression of selected genes in *M graminicola*; qRT PCR analysis of thirteen genes associated with nematode development, metabolism, and stress response in control (blue) and F4-treated (red) groups. Data represent mean ± SD of three independent biological replicates. Asterisks indicate statistically significant differences compared to the control group (*p < 0.05, **p < 0.01, ***p < 0.001; Student's t-test).

6.4 Conclusion

This chapter establishes the nematicidal potential of *A. niger* F4 culture filtrate through a comprehensive metabolomic and functional characterisation approach. Methanolic extraction followed by LC-MS, FTIR, and ^{1}H NMR analyses identified sphinganine as the major bioactive compound. Sphinganine exhibited potent dose-dependent juvenile mortality in *M. graminicola* (LC_{50} = 0.0603 µg/mL), while showing negligible cytotoxicity in human epithelial cells.

Microscopic and ROS assays revealed that F4 treatment disrupts cuticular integrity and induces oxidative stress, while gene expression profiling indicated a systemic physiological response affecting metabolism, neuromuscular function, and stress signalling in nematodes. These multi-level effects suggest that *A. niger* F4 acts via a multi-targeted mode of action, reducing the risk of resistance development.

Overall, this study demonstrates that *A. niger* F4 culture filtrate is a promising candidate for developing a sustainable, metabolite-based nematicide, with strong potential for integration into eco-friendly nematode management strategies.

Chapter 7: Objective 5

Chapter 7: Objective 5

Develop and optimise a stable and efficient formulation of the F4 culture filtrate for field-level application in nematode management

7.1 Introduction

Plant-parasitic nematodes, especially root-knot nematodes (*Meloidogyne* spp.), represent a major threat to worldwide agricultural productivity. They infect plant roots by penetration, and results into gall formation, with additional interference in plant's water and nutrient absorption. Finally leads to decreased crop yield and quality. Meloidogyne graminicola and M. incognita are among the most detrimental nematode species in rice and tomato cultivation, capable of imposing significant economic losses under favourable conditions.

Chemical nematicides such as oxamyl, fluensulfone and fosthiazate have been widely used in root-knot nematode control. While these compounds offer rapid and broad-spectrum activity, concerns regarding eco-toxicity, environmental persistence, and regulatory restrictions have prompted the exploration of safer alternatives (Mishra et al., 2023). Moreover, none of the currently available chemical nematicides are labelled for application in rice cultivation, thereby limiting their formal use in this crop. Additionally, their high cost renders them unaffordable for India's small and marginal farmers, who constitute the majority of rice growers and are often unable to invest in such expensive inputs (Kumar et al., 2020).

In contrast, biological nematicides, including products formulated on *Purpureocillium lilacinum*, *Bacillus firmus*, and *Myrothecium verrucaria*, offer eco-friendly options with different modes of action involving parasitism, enzymatic degradation, or secondary metabolite production (Knowles, 2008).

The identification and validation of root gall-associated *A. niger* F4 as an effective biocontrol agent against *Meloidogyne graminicola* has created new avenues for the sustainable management of root-knot nematodes in rice agroecosystems (Sec-3). *In-vitro* and greenhouse experiments have shown that the culture filtrate of F4 causes

significant mortality of *M. graminicola* juveniles and disrupts egg hatching, along with significant reduction of nematode infection and galling when administered through root dipping and soil drenching (See Chapter 4, section 4.3.7.2). Its efficiency in *M. incognita* management in tomato plants, both *in vitro* and in planta, further emphasises its extensive nematicidal potential (see Chapter 5 for details).

Although promising experimental outcomes, the practical efficacy of any microbial biocontrol agent in agricultural systems depends on the establishment of an appropriate and stable formulation. An optimal microbial formulation must ensure ease of storage, transport, and field-level application, while maintaining the viability and bioefficacy of the biocontrol agent under diverse environmental conditions. It should exhibit a long shelf-life at ambient temperatures, protect against desiccation and UV degradation, enable uniform dispersion with minimal phytotoxicity, and remain compatible with conventional agricultural practices. Additionally, it must be safe for non-target organisms and economically viable for adoption by small and marginal farmers (Gouda et al., 2016; Kumar and Dara, 2021). A diverse array of formulations for microbial and metabolite-based products has been created and used in the commercial sector. These encompass liquid, solid, wettable powder (WP), and water-dispersible granule (WDG) forms, in addition to more sophisticated types such as encapsulated goods, emulsifiable concentrates, flowables, and slow-release tablets or discs.

Biopesticide formulations varies in effectiveness and field application. Liquid formulations provide rapid bioavailability; however, they are constrained by a brief shelf life and instability. Solid forms, such as granules and pellets, offer improved stability and soil dispersion but necessitate optimisation for dispersion. Wettable powders are compatible with spraying, however they are dusty and necessitate agitation. Water-dispersible granules (WDG) integrate powder dispersion with granular stability, ensuring user safety and consistent application. Advanced formulations such as encapsulated beads, emulsifiable concentrates, and slow-release tablets offer precise, controlled distribution; nevertheless, they tend to be expensive and are typically designed for specialised purposes (Gouda et al., 2016; Kumar and Dara, 2021).

A comparative analysis of formulation types indicates that the selection of a delivery system is influenced by various factors, including the biological properties of the active agent (spore-forming versus metabolite-based), intended application

method (foliar, soil, or seed treatment), shelf-life specifications, environmental conditions, and user safety considerations. Although liquid and WP formulations provide quick bioavailability, they are frequently surpassed by granular or WDG formulations regarding storage stability, handling convenience, and field effectiveness. Advanced encapsulation and slow-release technologies enhance the environmental longevity of biocontrol chemicals, although may be constrained by expense and production intricacy.

Thus, objective of this study concentrates on the development and optimization of a WDG formulation derived from the culture filtrate of *Aspergillus niger* F4 (hereafter F4). This method aims to integrate the biological effectiveness demonstrated in laboratory and greenhouse research with a delivery technology that guarantees optimal field applicability, prolonged stability, and safety for users. The resulting formulation is expected to act as a prototype for the application of root gall-associated fungal biocontrol agents in integrated nematode management for rice and other crop systems.

7.2 Methodology

7.2.1 Preparation of fungal culture filtrate

The F4 was grown in potato dextrose broth (PDB) and incubated on a rotary shaker at 120 rpm for 7 days at 30°C in darkness. Subsequent to incubation, the fungal broth was first filtered using Whatman No. 1 filter paper, followed by a 0.22-micron filter membrane to exclude fungal hyphae, spores, and other particle contaminants. The resultant filtrate was collected and preserved at room temperature in screw cap bottle until used for formulation.

7.2.2 Adsorption onto a carrier

Fumed silica (Sigma Aldrich) was utilised as the adsorbent in the WDG formulation. The criteria for fumed silica stipulated a particle size of under 5 microns, a pH between 6 and 7, and an absorption capacity greater than 200%. Initial saturation studies demonstrated that 45 g of fumed silica could absorb roughly 100 g of fungal culture filtrate. The resulting fumed silica infused with fungal broth was termed "Blank Powder" (BP). The percentage of the active component in BP was determined as follows: Percentage of Active Ingredient in BP = (Weight of absorbed culture filtrate / Total weight of BP) × 100 = (100 g / (45 g silica + 100 g filtrate)) × 100 = 68.97%

7.2.3 Preparation of Water-Dispersible Granule Formulations:

Seven distinct batches (B-1 to B-7) of WDG formulations were produced, with each batch weighing 20 g. The specific makeup of each batch is as follows:

1. Blank Powder (BP): 14.4 g (applicable to all batches)
2. Inert Component: Lactose Monohydrate (1.6–2.6 g various upon batch), (Knowles, 2008)
3. Wetting Agents: Sodium Dioctyl Sulfosuccinate (0.8 g) or Sodium N-Methylolyl Taurate (0.8 g), (Mishra et al., 2023)
4. Desiccant: Sodium Polycarboxylate (1.6 g), (Knowles, 2008)5.
5. Binding Agent: Cellulose, Starch, or Silica (0–1 g relative to batch), (Mishra et al., 2023)
6. Antimicrobial Agent: Butylated Hydroxytoluene (0.2 g), (Mishra et al., 2023)
7. Lubricant: Magnesium Stearate (0–1 g contingent upon batch), (Woods, 2003)
8. Water: 3.7–4.0 g (variable by batch)

The components for each batch were combined meticulously to guarantee uniformity. Thereafter, each combination was granulated employing conventional granulation methods and dried inside an oven (40-45°C) to achieve a final moisture content <5% (Parveen, 2023; Mishra et al., 2023; Sawant et al., 2023). The dried granules were sieved to provide a homogeneous size distribution of 0.1–3 mm, ensuring dust-free and free-flowing properties essential for field application (Mishra et al., 2023).

7.2.4 Evaluation of formulation potential

Formulations were assessed according to their physical characteristics, including dispersion rate in water, particle size distribution, stability under storage conditions, and moisture content, stability under different temperature. Furthermore, bio efficacy assessments were performed against *M. graminicola* in greenhouse pot studies to confirm nematicidal efficacy. Then effective formulations were tested in field. Phytotoxicity and compatibility with agricultural techniques were also recorded (Parveen, 2023; Sawant et al., 2023).

7.2.4.1 *In-vitro* juvenile mortality of M. *graminicola*

While 1gm of formulation from each batch contains ~49.66% BP% (w/w) of active ingredient. 1gm of each formulation batch is dissolved in 100ml of distilled water. Which will make 10000 ppm of formulation concentrate. Similarly, another concentrate of 5000ppm was also prepared. The slurry of the formulation was tested on ~100 J2 of *M. graminicola* at two high dosage concentrations. In six well culture plate the mortality was tested stated as sec: Number of juveniles were counted 24 hours post inoculation under Carl Zeiss Stemi Stereoscope (10x).

7.2.4.2 Greenhouse experiment

An *in-vivo* pot experiment was conducted under greenhouse conditions to assess the nematicidal efficacy of the F4 WDG formulation (B6) using the susceptible rice cultivar PB1121. Trays (18 inch × 18 inch × 3 inch) trays were filled with sterile soil. ~5000 J2s of *M. graminicola* was added into the soil. To mimic direct seeded rice ecologies, seeds were sown directly in the trays. Fater which, the soil was drenched with aqueous suspension (5 g/L) of B6 formulation (100 mL per tray). The experiment was conducted with three replications. The soil was kept moist throughout the experimental period. Following 21 days of growth, the plants were harvested, and the roots were carefully washed and prepared for nematological analysis. The total count of *M. graminicola* individuals at various developmental stages (J2, J3/J4, and mature females) were conducted under a stereomicroscope following acid fuchsin staining. Root galling was documented, and plant growth was evaluated visually. The untreated trays (plant + nematodes) functioned as the control.

7.2.4.3 Field application

A field trial was performed to assess its efficacy of the F4 WDG formulation (B6) against *M. graminicola* in rice. A sick plot was selected and the initial nematode population in the plot soil was evaluated using Cobb's sieving and the Baermann funnel technique (initial population = 60.04±0.72 J2 per gramme of soil). The trial was structured using a randomised block design with three treatments: B6 formulation at 5000 ppm, Commercial-grade nematicide (Velum® Prime, Bayer India, fluopyram 34.48% w/w SC according to the manufacturer's specifications), Untreated control.

In this study, Velum® Prime (fluopyram 34.48% SC) was used in rice fields owing to the limited availability of nematicides specifically targeted against *M. graminicola*.

Although not officially labelled for use in rice, Mahalik et al. (2024) recently reported its effective suppression of *M. graminicola* when applied at the recommended dose of 625 g/1000 L (2–2.5 mL/L), applied twice at a 5 day interval.

As *M. graminicola* poses a significant threat in direct-seeded rice (DSR) systems, the DSR method for rice cultivation was used for the field experiment. Seeds were sown in a regular pattern with a plant to plant spacing of 15 cm and row to row distance of 20 cm, resulting in approximately 30 plants per 1 × 1 m² plot. Each treatment was conducted with three replicate plots. Bunds were established around each plot to prevent cross-contamination. Standard agronomic practices were followed throughout, and no additional plant protection measures were applied. The most effective formulation batch, B6, was applied at a concentration of 5 g/L (equivalent to 2.48 g/L of active ingredient). Each plot received 5 L of spray solution, applied twice, matching the application schedule of Velum® Prime.

Eighty days after sowing, plants were carefully uprooted and evaluated for nematode infestation. Nematode populations (J2 and eggs) per plant were quantified using acid fuchsin staining and microscopic observation under a Carl Zeiss Stemi stereoscope. The reproduction factor (Rf = final population/inoculum density) was calculated for each treatment. Root galling was assessed by counting the number of galls/ g of root. Plant growth and root morphology were visually examined. All data were analysed using one-way ANOVA, followed by Tukey's HSD for post hoc comparisons among treatment means.

7.3 Results

7.3.1 Physio-chemical properties of formulation batches

Physicochemical Assessment of WDG Formulation Pre- and Post-Accelerated Thermal Storage (ATS). The physico-chemical properties of F4 WDG formulations were assessed prior to and after to accelerated thermal storage (54 ± 2 °C for 14 days) in accordance with CIPAC and BIS standard methodologies. Each test was performed in triplicate.

1. **Suspensibility** (%): Evaluated according to CIPAC MT 184. A 1% (w/v) suspension of the WDG formulation was made in regular hard water (CIPAC D water). Following a 30-minute settling period, a sample was extracted from the central region and dried to a consistent weight. Suspensibility was defined as the percentage of active component retained in suspension compared to the overall content.

2. **The pH** of a 1% aqueous suspension of the WDG was measured using a calibrated digital pH meter (CIPAC MT 75).
3. **Persistent Foam** (mL): In accordance with CIPAC MT 47, 200 mL of a 1% formulation suspension was agitated vigorously for 1 minute in a graduated cylinder. The foam height was measured immediately and after one minute. Persistent foam was quantified in millilitres.
4. **Wettability** (seconds): A mass of 5 g of the WDG formulation was carefully positioned on the surface of 100 mL of distilled water in a beaker without stirring. The duration for total submersion was recorded with a stopwatch (CIPAC MT 53).
5. **Wet Sieve Test** (% residue retained): In accordance with CIPAC MT 59, 10 g of WDG was suspended in water and filtered through a 75 µm sieve. The collected material was desiccated and measured. The outcome was articulated as the percentage of residue retained on the sieve, signifying dispersion uniformity.
6. **The degree of dispersion** was assessed by comparing the quantity of formulation that passed through the sieve, as per the wet sieve test, to the total formulation weight, expressed as a percentage (CIPAC MT 174).
7. **Dustiness**: Dustiness was assessed via a dustiness tester in accordance with CIPAC MT 171. A specific quantity of formulation was released from a predetermined height into a dust chamber. The produced dust was gathered on a pre-weighed filter and measured gravimetrically. Results were presented in mg or relative units based on the mass gain.

The physico-chemical parameters of the F4 WDG formulation were evaluated before and after accelerated thermal storage (ATS) at 54 ± 2 °C for 14 days.

- **Suspensibility** improved from 67.94% to 70.72%, reaching the FAO minimum specification of 70%.
- **pH** decreased from 10.15 to 7.3, falling within the acceptable range of 6.0–8.5.
- **Persistent foam** volume was reduced from 24 mL to 4 mL, meeting the CIPAC MT 47 guideline (≤25 mL).
- **Wettability** improved from 36 s to 25 s, which complies with the CIPAC MT 53 threshold of ≤60 s.
- **The wet sieve residue** decreased marginally from 1% to 0.5%, remaining well within the acceptable limit (≤2%) as defined by the CIPAC MT 59 standard, indicating satisfactory dispersibility.
- **Degree of dispersion** increased from 85.91% to 94.22%, well above the optimum standard of ≥90% (CIPAC MT 174).
- **Dustiness** decreased from 10.2 to 8.8, which is within acceptable safety limits per CIPAC MT 171.

7.3.2 Evaluation of Formulation potential

The potential of three selected batches B-2, B-3 and B-6 were evaluated under different *in-vitro* and *in-vivo* conditions to check their effect on *M. graminicola* J2

mortality.

7.3.2.1 *In-vitro* juvenile mortality of *M. graminicola*

The nematicidal efficacy of three chosen WDG batches (B2, B3, and B6) formulated from F4 culture filtrate was assessed against *M. graminicola* juveniles *in vitro*. Each formulation was evaluated at two concentrations, 5g/L and 10g/L of formulation batches. According to the formulation composition, each gramme of the batch formulation included approximately ~49.66% (w/w) of the active component in the test solution. The 5g/L and 10g/L test solutions accurately 3.4g/L and 6.8g/L of active ingredient, facilitating standardised comparisons among treatments. All formulations caused significant ($F_{3,12}$=40.16; $p<0.05$) mortality of J2 within 24 hours in a dose-dependent manner. At 10 g/L, both B2 and B6 attained nearly total nematode mortality (~99–100%), although B3 exhibited marginally reduced efficacy (~85%). At 5g/L, B6 exhibited highest efficacy (>95%), succeeded by B2 (~85%) and B3 (~70%). From this *in-vitro* observation only B-6 was taken further for greenhouse and field experiments.

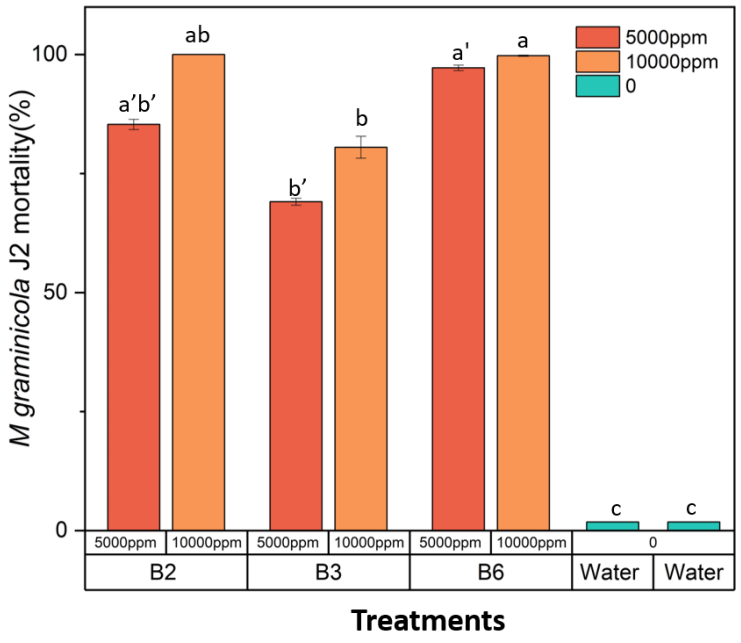

Fig. 7.1 *In-vitro* mortality of *M. graminicola* J2s. The bars in the plot corresponds mean ± SE of estimated J2 mortality (%). Different letters suggest significant difference across the treatment, by Tukey's HSD post HOC test.

7.3.2.2 Greenhouse assay

At 21 days post-inoculation with *Meloidogyne graminicola*, plants treated with the B6 formulation had a significantly decreased nematode population across

developmental stages. Microscopic analysis of root tissues demonstrated a significant inhibition of nematode development: treated plants exhibited decreased populations of second-stage juveniles (J2); (B6: 11.45±1.18, Control: 26.77±2.01), third- and fourth-stage juveniles (J3/J4), and mature females (B6: 26.1±2.4, Control: 58.77±3.18) in comparison to untreated controls. The total number of nematodes in treated plants were roughly one-third of those in untreated plants.

Notably, the average number of galls in treated PB1121 plants decreased significantly from 10 ± 0.3 (control) to 4.2 ± 0.5 (Table 7.1). Besides nematode suppression, treated plants demonstrated improved root and shoot growth relative to control plants.

Table 7.1 Number of galls/ roots found in PB1121 with and without treatment

Rice Variety	Control	B6 treated
PB1121	10±0.3	4.2±0.5

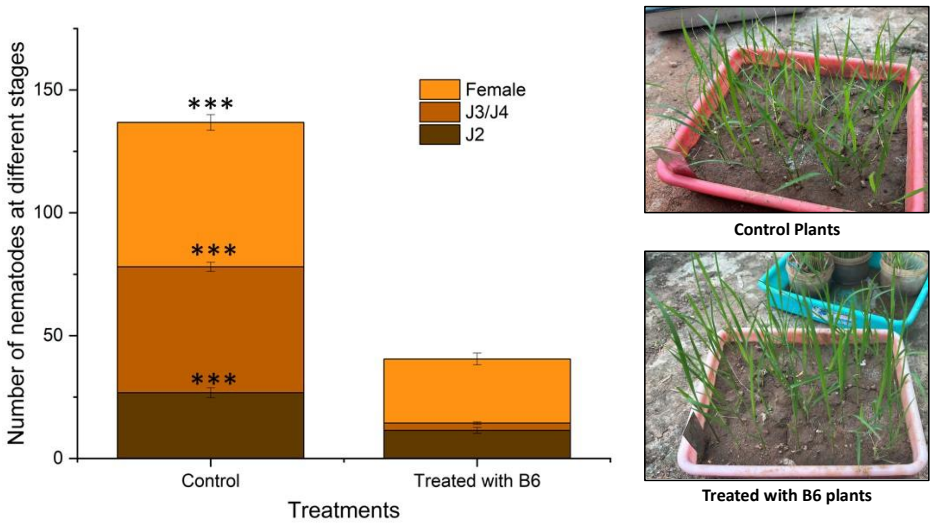

Fig. 7.2 *In-vivo* evaluation of *A. niger* F4 WDG formulation (B6) on nematode infestation; Left: Quantification of *M. graminicola* nematodes at different developmental stages (J2, J3/J4, and females) in control and B6-treated plants, 21 days post-inoculation. A significant reduction in total nematode count, especially in juvenile and female stages, was observed in B6-treated roots (***p < 0.001). Right: Images of rice plants of treated and control groups.

7.2.3.3 Field Application

Similar to the greenhouse experiments, field experiments demonstrated a significant decrease in nematode populations in plots under B6 soil drenching treatment. The total number of nematodes per plant (J2 and egg masses) was significantly reduced ($F_{2,28} = 61.27$; $p < 0.05$) in the B6 formulation treatment. Most importantly, no difference was observed between the B6 and Velum® Prime treated plots. The reproduction factor (Rf = Pf/Pi) diminished to approximately 45 in the B6 treatment, in contrast to around 90 in the control and roughly 30 in the Velum® Prime plots.

The number of galls per gram of root decreased significantly in B6-treated plants (1.8 ± 0.37/g root) compared to the control (2.5 ± 0.67/g). The roots of treated plants (B6 and Velum® Prime) displayed a healthier structure with enhanced secondary root branching, whereas control plants revealed stunted and highly galled root systems. Additionally, a substantial reduction in egg numbers per plant was recorded in both B6 and Velum® Prime treatments relative to control, indicating strong inhibition of nematode reproduction in treated plots.

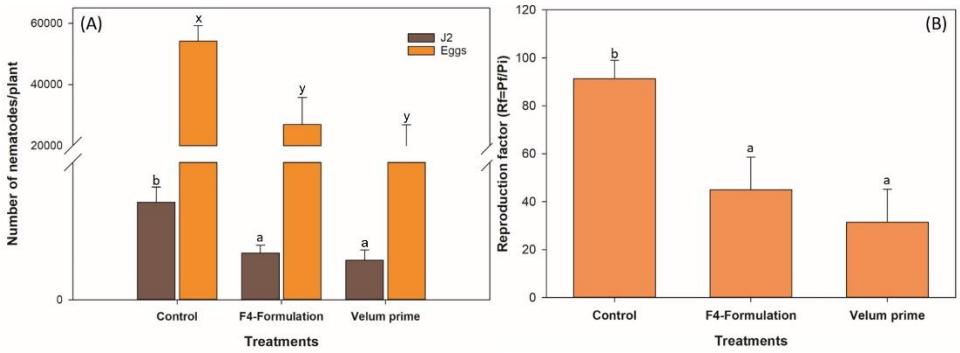

Fig. 7.3 Field efficacy of *A. niger* F4 WDG formulation compared to a commercial nematicide (Velum® Prime) against *M. graminicola* in rice cultivar PB1121. (A) Total number of *M. graminicola* nematodes (J2 and eggs) per plant, quantified 80 days post-transplantation. Bars represent mean values ± SE. (B) Reproduction factor (Rf = Pf/Pi) of *M. graminicola* calculated for each treatment. Bars with different letters indicate significant differences among treatments ($p < 0.05$). For both (A) and (B) different letters within the same nematode stage (J2 or eggs) indicate statistically significant differences ($p < 0.05$) according to Tukey's HSD test.

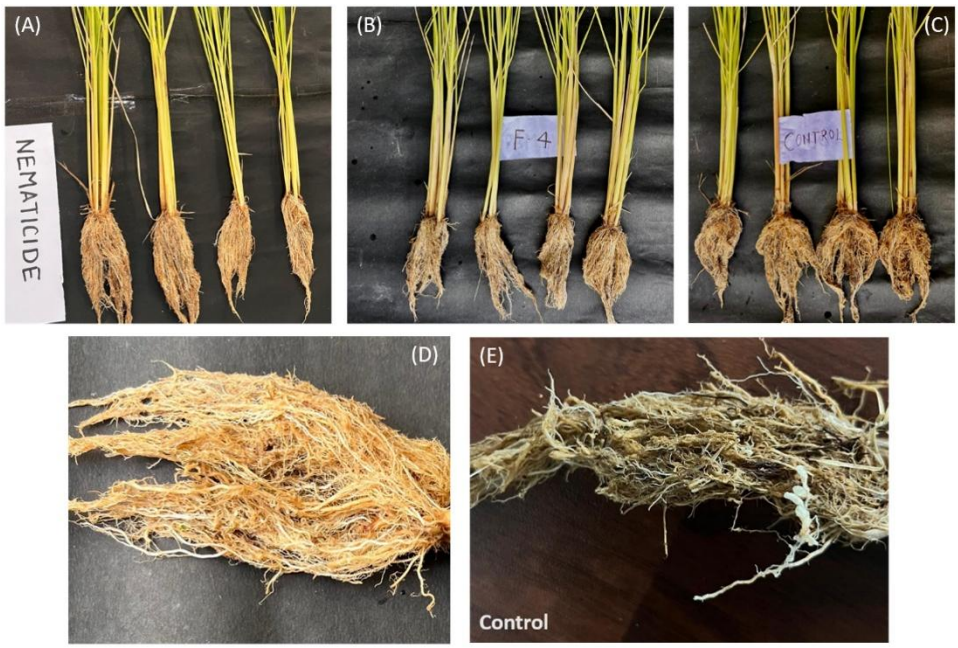

Fig. 7.4 Comparative evaluation of field data (A) Roots from plants treated with the chemical nematicide (Velum® Prime); (B) roots from plants treated with *A. niger* F4 WDG formulation (B6); (C) untreated control plants; (D) enlarged view of root system from B6-treated plants showing improved secondary root development and reduced galling; (E) Magnified view of control roots showing galling and poor root integrity due to *Meloidogyne graminicola* infection.

Fig. 7.5 (Left) Experimental layout of 1 m² plots showing replicated treatment blocks for nematicide, F4 formulation, and untreated control; initial nematode population was estimated at 60.04 ± 0.72 J2/g soil. (Right) Visual comparison of rice plant vigour at 80 days after transplanting; improved growth observed in F4-treated plots relative to the nematicide and control treatments, indicating effective suppression of nematode infection and improved plant health.

7.4 Discussion

The development and application of *A. niger* F4 as a water-dispersible granule (WDG) represent a significant advancement in the biocontrol of *M. graminicola*, a major constraint in rice production systems. The in vitro evaluation of the B6 formulation shown substantial nematicidal activity, achieving mortality rates over 95% at 5000 ppm and ~99% at 10000 ppm. The observed effects were uniform across replicate batches and were associated with the existence of bioactive secondary metabolites previously reported in *A. niger* other isolate (Zhang et al., 2008). Also aligns with the presence of bioactive compounds in chapter-6. The increased death rates found in the in-vitro assays confirm that the active components retained their effectiveness when included into a dry WDG matrix, hence validating the formulation design.

In greenhouse evaluations, both root dipping and soil drenching methods were tested independently to assess the delivery efficacy of F4 culture filtrate. In both the application methods B6-treated plants exhibited a significant reduction in overall nematode count relative to the untreated control, particularly in advanced developmental stages (J3/J4 and females), indicating an interruption in nematode development post-penetration (Fig. 7.2). In this context, statistical analysis revealed no significant difference between the two application methods in terms of nematode reproduction factor and total J2+egg counts. Given the logistical ease and broader field-scale applicability, the soil drench method was selected for further field trials. This is consistent with previous studies indicating that water-dispersible granule (WDG) formulations perform optimally when applied as soil drenches, ensuring uniform distribution of bioactive metabolites and better interaction with soil-borne pathogens (Ravindra et al., 2018; Mishra et al., 2023).

Importantly, the application of WDG via soil drenching also presents a practical advantage in direct-seeded rice (DSR) systems, which are increasingly adopted in upland and rainfed rice-growing regions due to their reduced labour demand, water-saving potential, and adaptability to mechanised farming. DSR eliminates the nursery phase, where early-stage *M. graminicola* infection often occurs, making direct soil application of biocontrol agents at seeding or transplanting stages critical for intercepting nematode invasion. Recent agronomic trends indicate a steady shift toward DSR in India and Southeast Asia, particularly in upland areas where traditional

puddling is less feasible (De Waele and Elsen, 2007; Haque et al., 2018). Therefore, soil drenching with a F4 WDG formulation at early crop stages offers a strategic intervention to suppress nematode populations before extensive root colonisation can occur, addressing both nursery-originated infections and early field infestations.

Field validation under natural infestation conditions further corroborated the biocontrol efficacy of F4. Plants treated with the B6 formulation exhibited a significant reduction in nematode populations (J2 and egg mass counts) and reproductive factor (Rf) relative to the control (Fig. 7.4), with outcomes comparable to the commercial nematicide Velum® Prime (fluopyram 34.48% SC).

In the absence of a label-registered nematicide for *M. graminicola* control in rice under Indian pesticide regulation, we employed Velum® Prime (fluopyram 34.48% SC) as a benchmarking standard. Although not officially approved for rice use against *M. graminicola*, Velum® Prime has demonstrated strong efficacy against root-knot nematodes in other crops, including cucurbits and solanaceous vegetables (Oka et al., 2020), experimentally it was also administered in rice for control of *M. graminicola* (Mahalik et., al 2024).

The B6 formulation was administered at a dosage of 5 g/L, equating to 2.48 g/L of active ingredient (derived from 49.66% w/w broth content), a concentration that is equivalent to or lower than those applied in analogous trials. Dawabah et al. (2019) documented the application of a talc-based formulation of *A. niger* F22 at concentrations of 2.5–5 g/L to inhibit *M. javanica* in tomato plants. Similarly, bioformulations of *Trichoderma harzianum* and *Purpureocillium lilacinum* have demonstrated excellent nematode suppression in rice and other crops at concentrations of 2–6 g/L when administered using root dipping and soil drench techniques (Ravindra et al., 2018; Debnath et al., 2012).

The efficacy of the B6 formulation can be partially attributed to its physicochemical properties. Mishra et al. (2023) and Parveen (2023) illustrate that water-dispersible granules (WDGs) offer advantages in terms of handling, dispersion, and the stability of active ingredients when compared to conventional wettable powders or liquid concentrates. The incorporation of fumed silica as an adsorbent provided an extensive surface area for binding culture filtrate, while the application of wetting agents and stabilisers promoted swift dispersion and preserved activity within soil matrix. The utilisation of root dipping alongside soil drench exemplifies efficient methodologies established in other studies concerning *Trichoderma harzianum* and *Purpureocillium*

lilacinum (Sawant et al., 2023), promoting early penetration of the root zone and direct antagonism of infective juveniles.

6. 5 Conclusion

The present study successfully developed and optimised a water-dispersible granule (WDG) formulation of *Aspergillus niger* F4 culture filtrate (batch B6), demonstrating its stability, field applicability, and potent nematicidal activity against *Meloidogyne graminicola*. The formulation retained bioefficacy under accelerated storage conditions and exhibited superior performance in both greenhouse and field assays, significantly reducing nematode populations and galling, with effects comparable to the commercial nematicide Velum® Prime. The B6 formulation thus offers a promising, eco-compatible alternative for nematode management in direct-seeded rice systems.

Chapter 8: Summary

Chapter 8: Summary & Conclusion

This thesis explored the potential of root gall-associated fungal endophytes as biological control agents against the rice root-knot nematode, *Meloidogyne graminicola*, through a multidisciplinary framework that integrated metagenomic, microbiological, biochemical, and formulation-based approaches.

In **Objective 1**, fungal community dynamics were assessed using both high-throughput sequencing and culture-dependent methods. Root galls formed during *M. graminicola* infection were found to harbour distinct fungal endophyte assemblages compared to healthy roots. While healthy roots were dominated by mutualistic genera, nematode-infected galls displayed a significant shift toward taxa with known stress tolerance and biocontrol potential, including *Fusarium*, *Aspergillus*, and *Pestalotiopsis*. These findings confirmed that nematode infection restructures endophytic communities, potentially enriching taxa with antagonistic traits.

Objective 2 focused on the isolation and functional screening of these gall-associated fungal endophytes. Among 32 isolates, *Aspergillus niger* F4 demonstrated potent nematicidal properties, causing near-complete mortality of *M. graminicola* juveniles under both in-vitro and in-vivo conditions. Microscopy revealed the formation of intracellular vacuoles in treated juveniles, indicating a strong physiological disruption and possible induction of non-programmed cell death.

In **Objective 3**, the nematicidal activity of *A. niger* F4 was evaluated against *M. incognita* in tomato. The unrefined culture filtrate significantly suppressed nematode infection and improved plant growth. This suppression was mediated via a dual mode of action, direct nematicidal activity and the activation of host systemic defences. Elevation of phenolics, flavonoids, antioxidant enzymes, and defence-related genes such as *PR1-1b*, *ACO*, and *JERF3* confirmed induced systemic resistance (ISR) in the treated plants.

Objective 4 undertook a metabolomics-guided characterisation of the bioactive compounds in the F4 culture filtrate. Sphinganine, identified as the major constituent, displayed a dose-dependent nematicidal effect at micomolar concentrations while exhibiting negligible toxicity to human cells. Functional assays corroborated a multi-targeted mechanism of action involving cuticle disruption, reactive oxygen species

generation, and transcriptional modulation of key physiological pathways in nematodes.

In **Objective 5**, the culture filtrate was formulated into a stable water-dispersible granule (WDG) for field application. The optimised batch (B6) retained its nematicidal activity under storage conditions and exhibited robust efficacy under greenhouse and field conditions. Application of the formulation significantly reduced nematode infestation levels and improved crop performance, with outcomes comparable to commercial chemical nematicides.

Overall, this study demonstrated that gall-associated fungal endophytes, particularly *Aspergillus niger* F4, are promising agents for the biological control of *M. graminicola*. The work provides a detailed mechanistic insight into the interaction between fungal metabolites and nematode physiology, highlighting the potential of integrating metabolite-based biocontrol into nematode management frameworks.

Future Research Directions

Further work is necessary to elucidate the full spectrum of bioactive metabolites present in the F4 filtrate and to establish their synergistic interactions. Genome mining and transcriptomic profiling of the fungal isolate could reveal regulatory pathways underpinning metabolite biosynthesis. Long-term field trials across diverse agroclimatic regions will be essential to validate the performance and ecological safety of the developed formulation. Finally, integrating fungal metabolites into existing integrated pest management (IPM) programmes will require regulatory assessments and stakeholder engagement to ensure scalable adoption.

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Dutta, M., Das, S., Sarkar, S., Karmakar, P., Mahato, B., & Ghosh, S. (2023). Culture filtrate of *Aspergillus niger* F4 elicits plant defence and shows nematicidal effect against *Meloidogyne incognita* in tomato. *Biological Control*, 186, 105341. https://doi.org/10.1016/j.biocontrol.2023.105341.

Other Publications

Das, S., Dutta, S., Ghosh, S., & Mukherjee, A. (2024) Chitinolytic microorganisms for biological control of plant pathogens: A comprehensive review and meta-analysis. *Crop Protection*, 106888.

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