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RESEARCH ARTICLE

Targeting rice root-knot nematode: A study of benzothiadiazole, fluopyram and fluensulfone

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Abstract

Understanding the management of the rice root-knot nematode *Meloidogyne graminicola* is an essential component of rice. The study aimed to investigate the extent to which the nematicidal actions of 2,1,3-benzothiadiazole, fluopyram, fluensulfone and carbofuran against the rice root-knot nematode, *M. graminicola* could be determined using *in vitro*, *in vivo* and *in silico* studies. Among the tested compounds, fluopyram demonstrated the highest juvenile mortality rate (91.26%) and binding affinity (-8.3 kcal/mol) to the seven selected target proteins of *M. graminicola*, driven by significant hydrophobic, alkyl and H-bonding interactions. Comparative binding affinities were recorded for fluensulfone (-6.9 kcal/mol), carbofuran (-6.4 kcal/mol), 2,1,3-benzothiadiazole (-5.0 kcal/mol) and untreated control. These findings have significant implications for agricultural practices, particularly in developing integrated pest management (IPM) strategies. The high efficacy of fluopyram, as evidenced by its biochemical ligand-target protein interactions, suggests its potential as a key component in IPM programs. By integrating nematicidal treatments with other control measures such as crop rotation, resistant rice varieties and biological control agents, sustainable management of *M. graminicola* can be achieved by reducing dependency on chemical pesticides. Furthermore, this study underscores the importance of using molecular insights to design targeted pest management solutions, paving the way for environmentally responsible and economically viable approaches to nematode control in rice cultivation.

Keywords

Fluopyram; *in vitro* and *in silico* analysis; *Meloidogyne* spp.; nematicidal action; plant-parasitic nematodes (PPN)

Introduction

A group of plant-parasitic nematodes (PPNs) known as root-knot nematodes (RKNs) may severely damage several crops, including rice. A specific RKN species that affects rice is called *Meloidogyne graminicola*. Small, thorny swellings or galls develop when *M. graminicola* infects rice plant roots. The feeding and reproduction behavioural patterns of the nematode have resulted in the development of these galls. The plant's ability to absorb water and nutrients may be hampered by the galls, which could result

in stunted growth, a decreased yield, or even plant death. The appearance of galls on the roots and impaired root growth and development are signs of RKN infection in rice, along with the yellowing and withering of the leaves. In extreme circumstances, plants may develop stunts and fail to yield grains. Effective management of nematodes that feed on plants will have a positive impact on global agriculture and future food security. The majority of crops with moderate to high value grown in intensive production systems used synthetic nematicides to reduce root-knot nematode populations. Recent research has revealed that the fluoroalkyl thioether fluensulfone (Nimitz, Adama) has higher nematicidal action against RKNs than traditional nematicides. Fluensulfone affects the neurological system of the nematodes, resulting in paralysis and finally death. It works well against a variety of PPNs, including cysts, lesions and root-knot worms (1, 2). Fluensulfone increases lipid content, leading to loss of cell viability and tissue degeneration in nematodes. It can be applied to sugarcane as a broadcast or banded application at planting or early ratoon growth. Fluensulfone is registered for use on many crops and has been used to manage nematodes, including *Belonolaimus longicaudatus* in potatoes and *Meloidogyne* spp. in pepper as well as *Mesocriconema xenoplax* and *M. incognita* on peach (3). It is considered a safer nematicide than older chemistries because it is less toxic to humans and non-target organisms, such as free-living nematode fauna (4). Fluopyram, a new pyridyl-ethyl amide broad-spectrum fungicide and nematocide that may inhibit SDH1, was developed by Bayer Crop Science. Fluopyram may block SDH in nematodes, therefore suppressing plant-parasitic nematodes and and Fluopyram reduces hatching of *M. incognita*, *Heterodera glycines* and *H. schachtii* but not *Caenorhabditis elegans* due to the egg shell's limited permeability. It shows acute toxicity for *M. incognita*, *M. javanica* and *H. schachtii* infective juveniles (J2). *M. incognita* and *R. reniformis* recover from fluopyram treatment, while *C. elegans* does not (5). Studies by Wram and Zasada demonstrate fluopyram's acute toxicity against *M. incognita* but no effects after short-term exposure to sublethal concentrations. However, methods, compound doses and exposure times vary across reports, limiting the comparability of results and making conclusions specific to the applied conditions (6). Plant defense elicitors are chemicals that, when detected by a plant's receptors, cause the plant to respond defensively. The potential use of benzothiadiazole (BTH) in the management of PPNs has been investigated. BTH is a member of a group of substances called plant defense activators, which can activate a plant's inbuilt defenses against pathogens and pests. The usefulness and safety of BTH in reducing nematode pests in various crops, however, need more research. The 1,2,3-benzotriazin-4-one derivatives inhibited *M. incognita*, indicating that they might be further improved as a nematicidal skeleton (8). Foliar application of BTH to rice plants resulted in only a minor induction of systemic defense against the root-knot nematode *M. graminicola*; however, BTH induced stronger defense in rice against migratory root nematode *H. oryzae*, rice stem nematode *D. angustus* and other sedentary nematodes. Exogenous application of hormones, their analogs, or inhibitors, along with analyses of marker gene expression, shows that salicylic acid (SA), jasmonic acid (JA), or ethylene (ET) is involved in rice defense against *Aphelenchoides besseyi*. However, the crosstalk between these three hormones and other plant hormones in the interaction between rice and *A. besseyi* needs further investigation (9). Despite the promising nematicidal properties of these compounds, there is a lack of molecular insights into their modes of action against *M. graminicola*. Understanding the biochemical interactions between these nematicides and the target effector proteins of the nematode is critical for developing effective management strategies. This study aims to evaluate the nematicidal effects of 2,1,3-benzothiadiazole, fluopyram, fluensulfone and carbofuran on *M. graminicola*. Using hatching and mortality bioassays and *in silico* analyses, this research seeks to elucidate the molecular interactions underlying the nematicidal actions of these compounds, providing insights into their potential application in rice nematode management. Consequently, research was done to determine whether 2,1,3-benzothiadiazole, fluopyram, fluensulfone and carbofuran had any nematicidal effects on the rice RKN, *M. graminicola*. The hatching and mortality bioassay of 2,1,3-benzothiadiazole, fluopyram, fluensulfone and carbofuran against *M. graminicola* effector proteins.

Materials and Methods

The pure culture of *M. graminicola* was grown on Taichung Native 1 (TN1) that was maintained at glasshouse, Department of Nematology, Tamil Nadu Agricultural University, Coimbatore. *M. graminicola* infested roots were washed free of soil and eggmasses were hand-picked and hatched via 'modified Baermann's funnel technique' (5). Further, experiments were carried out by using newly hatched juveniles (J2s). The chemicals and reagents used in this study were of analytical grade and were procured from reliable sources. Nematicidal compounds, including 2,1,3-benzothiadiazole (Sigma Aldrich), fluopyram (Velum Prime TM 34.48 SC, Bayer Crop Science, India), fluensulfone 2% Granules (Nimitz TM, ADAMA, India) and carbofuran (Furadan TM 10 G, FMC, India), were purchased from a pesticide dealer who was legally approved in Coimbatore, India. Those test compounds were dissolved in dimethyl sulfoxide (DMSO) to generate a carrier solution of 20000 ppm (1 mg of compound in 50 µL DMSO). Then, the carrier solution was dissolved in nuclease-free water to achieve 100 ppm of final concentration. Carbofuran 0.1 % was prepared with sterile distilled water and tested against *M. graminicola*.

Mortality and hatching assay

The nematicidal compounds were tested for toxicity against J2s of *M. graminicola*. J2s mortality was calculated using the following formula after 24, 48, 72 and 92 h of treatment (6). Nematicidal bioassay was carried out under *in vitro* conditions to test the activity of the compounds against *M. graminicola* using a proven method with modest changes (7). Glass petri plates (5 mm in diameter) with

nematicidal chemicals (100 ppm) were infiltrated with nematode suspension (1µl) containing 100 juveniles (J2s) of *M. graminicola*. DMSO surfactant solutions were used as the appropriate adverse controls. Nematodes with straight bodies that had not been moving were counted. The revival experiment was carried out in accordance with the (8). The non-motile nematodes were gently tickled by a hook and subsequently transferred to new glass plates with water that had been deionized. Juvenile mortality (%) was taken into account while calculating the mortality rate of J2s (9). Three replications of each treatment were used in each of the two subsequent experiments. Juvenile mortality (%) = No. of dead juveniles (J2) / Total no.of juveniles used for the assay ×100. Compounds were suspended in DMSO and used to immerse approximately 50 mixed-development stage egg suspensions in petri plates (5 mm). The plates were parafilm sealed to avoid dehydration and kept in a humid chamber at about 26 ± 2°C. All experiments were repeated twice with three replicates. The cumulative percent of egg hatching was calculated using the following formula. Cumulative percent of egg hatch (%) = J2Dx-J2D0 / Egg D0 ×100. Where Dx = x h after the start of the assay; D0 = Start point of the assay (0 h).

In-vivo assay

Screening assays were performed at the Department of Nematology, Tamil Nadu Agricultural University, Coimbatore with nematostatic compound-treated paddy plants (TN1) which were placed in a completely randomised design in a glasshouse (28–33 °C). One week following the nematode inoculation, treatments were administered. The following doses of the chemicals were applied to the soil: 50 mg/kg 2,1,3-Benzothiadiazole (10), 20 µL fluopyram 34 SC (0.5 L a.i. ha^{-1}) per kg soil, 1 g of fluensulfone 2G (22.2 to 25.6 Kg/ha) and 25 mg carbofuran 10G (2 kg a.i. ha^{-1}) (11). The dosages of 2,1,3-benzothiadiazole, fluopyram and fluensulfone were chosen based on a combination of recommended application rates, findings from prior research and preliminary experiments aimed at optimizing their efficacy against plant-parasitic nematodes. For 2,1,3-benzothiadiazole, a concentration of 50 mg/kg soil was selected based on its proven ability to induce systemic resistance in plants, as documented in earlier studies and its effectiveness in preliminary trials without causing phytotoxic effects (7). The dosage of fluopyram (20 µL fluopyram 34 SC per kg soil, equivalent to 0.5 L a.i. ha^{-1}) aligns with the manufacturer's recommended application rates for nematode management and has been validated in prior research to inhibit nematode motility and reproduction effectively. Similarly, the dosage of fluensulfone (1 g fluensulfone 2G per kg soil, equivalent to 22.2–25.6 kg/ha) reflects Syngenta's recommended field application rate, which was further supported by *in vitro* assays confirming its nematicidal activity against juveniles (J2s) while maintaining practical feasibility for soil applications. Additionally, carbofuran was included as a standard reference treatment at 25 mg carbofuran 10G per kg soil (equivalent to 2 kg a.i. ha^{-1}) based on its established use and documented efficacy in managing plant-parasitic nematodes. These dosages were carefully selected to balance effective nematode control, environmental considerations and practical relevance to agricultural practices. Based on the scale provided by the All India Coordinated Rice Improvement Project, the extent of gall formation was calculated. Ratings were as follows: 1 for no galling (highly resistant); 2 for galling of between 1 and 10 percent (resistant); 3 for galling between 11 and 30 percent (tolerant); 4 for galling between 30 and 50 percent (susceptible); and 5 for galling of more than that (highly susceptible) (12). A stereozoomic microscope was used to count and estimate the number of juveniles (J2) in the suspension using a counting dish.

Molecular docking and simulation

Seven target proteins, namely, MgGPP, MgM0237, MgPDI1, MgPDI2, MgPEL1, Venom Allergen Protein and Metallopeptidase protein of *M. graminicola* were selected as target macromolecules for the molecular docking analysis. In rice, the *M. graminicola* effector protein MgGPP enters into the host tissues and undergoes glycosylation as well as proteolysis, which is essential for nematode parasitism (13). During the early phases of parasitism, *M. graminicola* produces MgGPP, which has immune-suppressing properties in both cell compartments and is found in both the nucleus and cytoplasm (14). MgPDI1 and MgPDI2 are both expressed in the subventral oesophageal glands and are up-regulated during the early parasitic stage of *M. graminicola*. MgPDI1 and MgPDI2 are both crucial for apparent cell mortality in the host plant system (15). MgPEL1 plays critical functions in plant nematode infestation and a parasitic relationship by disintegrating polygalacturonic acid, a key ingredient of host cell pectin, facilitating penetration and colonisation (16). Mg-vap1 (venom allergen-like protein) plays an important role suppressing the host plant immunity (17). The metalloendopeptidase of *M. graminicola* is considered to be expressed throughout the embryonic and juvenile stages and plays an important role in longevity and development. (18). The protein sequences of selected targets of *M. graminicola* were obtained from the UniProt database. The rice root-knot nematode's chosen viable target lacks empirically and computationally defined structures. As a result, SWISS-MODEL (method: rigid-body assembly), Phyre2 (method: profile-based alignment) and ROBETTA were used for molecular modelling. SWISS-MODEL Modeller v9.24 has been employed to do further homology models of the proteins. The molecular models generated by SWISS-MODEL, Phyre2 and ROBETTA were validated using structural quality assessment tools. The Ramachandran plot, obtained through the PROCHECK program (available via the Structural Analysis and Verification Service, SAVES Meta Server), assessed the stereochemical quality of the models by evaluating the placement of amino acid residues within the favored and permitted regions of the φ (phi) and ψ (psi) dihedral angles. The percentage of residues in favored regions was a key criterion for model validation, confirming the reliability of the predicted protein structures. Additionally, Modeller v9.24 refined homology models and improved their quality by optimizing spatial geometry and minimizing stereochemical violations. These steps ensured that the models were accurate and suitable for subsequent analyses,

including docking studies with ligands such as 2,1,3-benzothiadiazole, fluopyram, fluensulfone and carbofuran. The molecules of 2,1,3-benzothiadiazole, fluopyram, fluensulfone and carbofuran, are together referred to as "ligands" in this sentence.

To feed these ligand molecules, the PubChem chemical library (https://pubchem.ncbi.nlm.nih.gov/substance) supplies the conventional SMILES (simplified molecular input line entry system) string. File (19). All produced ligand molecules were archived in Mol2 formats, whereas macromolecule files have been saved in PDB format. In order to carry out molecular docking, PyRx 0.8's AutoDock Vina module was utilised (20). The conjugate gradients, the first-order implications derived from a 200-step optimised technique and generic molecular dynamic constants have been used to minimise individual ligand structure (UFF). Interacting site pockets for the protein targets were found using the Computed Atlas Topography of Proteins (CASTp 3.0 server).To illustrate the interactions that occur between the components of docking patterns, the BIOVIA Discovery Studio client 2021 (https://www.3ds.com/products-services/biovia/) was deployed. The most favoured docking conformation interactions of target proteins with 2,1,3-benzothiadiazole, fluopyram, fluensulfone and carbofuran were investigated based on docking score, binding affinity and interacting residues. Determining a molecule's estimated binding affinity using ligand efficiency (LE) to determine the efficacy of the compound (21):

$$LE = [-2.303(RT) \times \log Kd] / HA = - \Delta G / HA$$

Where HA is the total number of non-hydrogen atoms in the ligand and ΔG is the free-binding energy. LE has to do with how many heavy atoms are in a molecule and how successful they are at forming complexes. Instead of evaluating the affinity of the entire molecule, the average affinity contribution per atom was taken into account. This made it possible to correlate the size of the corrected molecules' affinities. Candidate compounds with LE values less than 0.3 kcal per mole per heavy atom are typically chosen as the lead molecule in drug discovery modules (21).

Statistical analysis

Statistical analyses were conducted to determine the significance of the results and assess variability. A one-way ANOVA was performed to evaluate whether the differences between variables were statistically significant. To compare the means, Duncan's multiple range test was applied, with the threshold for statistical significance set at $p < 0.01$. For the analysis of percent mortality data, values were transformed into square roots to normalize the data distribution. The transformed data were then subjected to probit analysis using SPSS software to calculate lethal concentrations (LC values) and other dose-response parameters. All experiments were conducted with three independent replicates and each replicate consisted of multiple technical repeats. Data from the replicates were pooled after confirming homogeneity of variance using Levene's test. Variability among replicates was quantified and reported as the standard error (SE) for each mean value to ensure transparency and reproducibility of the results.

Results

Hatching and mortality

The effects of test nematicidal substances on the hatching and mortality of *M. graminicola* J2 were assessed when taking into account various incubation times (12, 24, 48 and 96 h intervals). When treatments and incubation times were combined, the mortality of J2 of *M. graminicola* varied significantly ($p < 0.0001$) for 2,1,3-benzothiadiazole, fluopyram, fluensulfone and carbofuran. Despite being significantly ($p < 0.0001$) higher than control in all treatments, mortality trends among test compounds varied (Fig. 1). Among the tested compounds, 2,1,3-benzothiadiazole and fluopyram showed the most significant inhibitory effects on egg hatching, with low hatching percentages across all time intervals. Fluensulfone exhibited moderate inhibition, with slightly higher hatching percentages

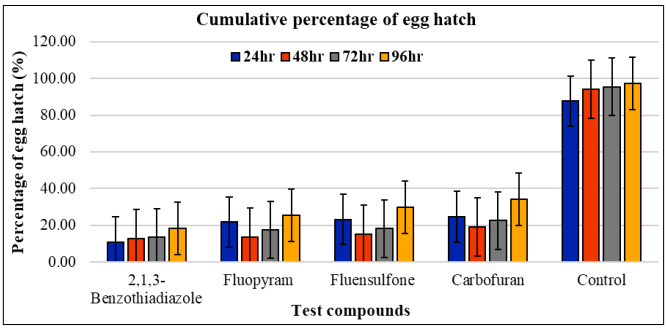

Fig. 1. Egg hatch percentage of Second stage juveniles (J2) against 2,1,3-Benzothiadiazole, Fluopyram, Fluensulfone and Carbofuran against rice root-knot nematode, *M. graminicola*. The graph illustrates the cumulative percentage of *M. graminicola* egg hatching over four-time intervals (24, 48, 72 and 96 h) in response to different test compounds, including 2,1,3-benzothiadiazole, fluopyram, fluensulfone, carbofuran and control (untreated). Each bar represents the mean cumulative percentage of egg hatch for the respective time point, while the error bars indicate the standard deviation (SD) of the mean across replicates.

than the first two compounds. Carbofuran displayed weaker inhibition, with a steady increase in egg hatching over time. In contrast, the control group, representing untreated eggs, showed a natural progression of egg hatching, reaching near 100% by 96 h. The error bars highlight the reproducibility of the observed trends, providing insights into the relative efficacy of the tested compounds in inhibiting egg hatch.The incidence of mortality rose in all concentrations of the fluopyram therapy as the incubation period progressed, with the highest juvenile mortality rate (91.26%) being noted 96 hours after inoculation followed by fluensulfone (86.25%), 2,1,3-Benzothiadiazole (74.14%) and carbofuran (67.41%). Few juvenile deaths (2.72%) and a small number of immobile juveniles (Fig. 2) were seen in the surfactant solution DMSO (100 ppm) used as the control. According to data on the ovicidal effects of test compounds on rice root-knot nematode egg masses, Fluopyram had the lowest cumulative percentage of egg hatching at 96 h after exposure, significantly lower than the untreated *M. graminicola* control (Fig. 2). Fluensulfone, 2,1,3 Benzothiadiazole and Carbofuran were the next-lowest cumulative percentages of egg hatching at the same time (Fig. 3).

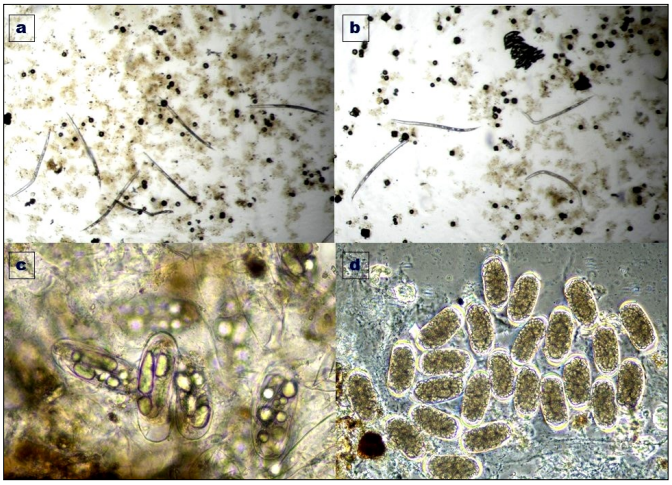

Fig. 2. Mortality and hatching assay of *M. graminicola* against Fluopyram under *in vitro*. The figure depicts the cumulative percentage of egg hatch and nematode mortality over time following exposure to fluopyram. The data illustrate the inhibitory effects of fluopyram on egg hatching and its nematicidal activity, measured as mortality rates. The findings demonstrate the potential of fluopyram as an effective nematicidal compound for controlling *M. graminicola*. **(a)** *M. graminicola* juveniles that died (straight) after being exposed to fluopyram **(b)** live juveniles (serpentine) of *M. graminicola* after exposure with DMSO (control) **(c)** Vacuolated (Deformed) eggs of *M. graminicola* after exposure to fluopyram **(d)** Eggs of *M. graminicola* remain normal after exposure with DMSO (control).

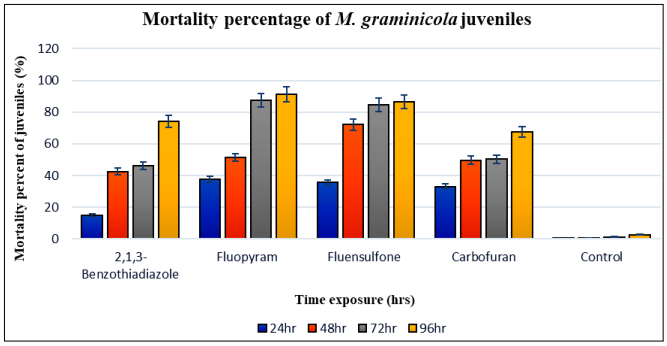

Fig. 3. Mortality percentage of Second stage juveniles (J2) against 2,1,3-Benzothiadiazole, Fluopyram, Fluensulfone and Carbofuran against rice root-knot nematode, *M. graminicola*. 2,1,3-benzothiadiazole, fluopyram, fluensulfone, carbofuran and control (untreated). Each bar represents the mean mortality percentage across replicates, with error bars indicating the standard deviation (SD).

Fluopyram and fluensulfone demonstrated the highest mortality percentages, with significant nematicidal activity evident as early as 24 h and increasing over time. Carbofuran showed moderate nematicidal effects, with lower mortality percentages compared to fluopyram and fluensulfone. 2,1,3-benzothiadiazole exhibited weaker but still notable nematicidal activity, with mortality percentages increasing gradually over the 96-h period. In contrast, the control group displayed negligible mortality, confirming the absence of external influences. These results highlight the superior efficacy of fluopyram and fluensulfone in juvenile mortality under *in-vitro* conditions, supporting their potential for managing *M. graminicola*.The significantly higher mortality observed with Fluopyram (91.26%) compared to the other compounds tested can be attributed to its unique mode of action and nematode-specific vulnerabilities. Fluopyram is a succinate dehydrogenase inhibitor (SDHI), which targets the mitochondrial electron transport chain, specifically the succinate dehydrogenase (complex II) enzyme. This inhibition disrupts energy production in nematodes by blocking the transfer of electrons from succinate to ubiquinone, a critical step in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. As a result, ATP synthesis is hindered, leading to energy depletion and eventual nematode mortality. Nematodes are particularly vulnerable to disruptions in mitochondrial respiration due to their high metabolic demands during activities such as hatching, movement and host penetration. Juvenile stages exhibit heightened metabolic activity, making them more susceptible to compounds like Fluopyram that target energy production pathways. In comparison, fluensulfone targets the nematode's cholinergic signaling pathways, essential for neuromuscular activity. Although effective, its mode of action does not directly disrupt energy metabolism, potentially explaining its slightly lower mortality rate (86.25%). The ovicidal effects observed further highlight Fluopyram's efficacy. Eggs of *M. graminicola* rely on mitochondrial function for embryonic development. The inhibition of succinate dehydrogenase in eggs likely interrupts this developmental process, leading to reduced hatching rates. The high binding affinity of Fluopyram to succinate dehydrogenase, as demonstrated in molecular docking studies (22), supports its superior nematicidal properties. Structural analyses of succinate dehydrogenase in nematodes reveal unique binding sites that Fluopyram exploits, enhancing its specificity and efficacy. In contrast, 2,1,3-Benzothiadiazole likely exerts its nematicidal effects through its role as a plant defense activator, inducing systemic acquired resistance in plants rather than directly targeting nematode physiological pathways. This indirect mode of action may contribute to its lower efficacy compared to Fluopyram. Similarly, Carbofuran, a carbamate insecticide, inhibits acetylcholinesterase but does not affect mitochondrial respiration, resulting in comparatively lower mortality rates. Fluopyram's superior efficacy is rooted in its precise targeting of nematode mitochondrial function, a critical vulnerability for energy-intensive stages of the nematode lifecycle. Future studies could focus on exploring the molecular variations in succinate dehydrogenase across nematode species to refine the use of SDHIs in integrated pest management strategies.

Plant growth characters

It was decided to combine the data from the two studies for analysis because there was no significant difference between the soil treatment and season. In these trials, no phytotoxicity of fluopyram, carbofuran, 2,1,3-benzothiadiazole, or fluensulfone was seen at the tested concentrations. Application of 2,1,3-benzothiadiazole significantly improved the plant growth parameters, including mean shoot length (51.33 cm), mean fresh shoot weight (7.96 g), mean shoot dry weight (4.23 g), mean root length (14.84 cm), mean fresh root weight (7.40 g) and mean dry root weight (2.18 g), followed by fluopyram, fluensulfone and carbofuran when compared to the untreated control (Fig. 4). Fig. 4A shows the highest mean shoot length in plants treated with 2,1,3-Benzothiadiazole and Fluensulfone, highlighting their effectiveness in mitigating nematode-induced stress. Fig. 4B presents root length results, with similar trends favoring 2,1,3-Benzothiadiazole and Fluensulfone treatments. Figures on shoot and dry shoot

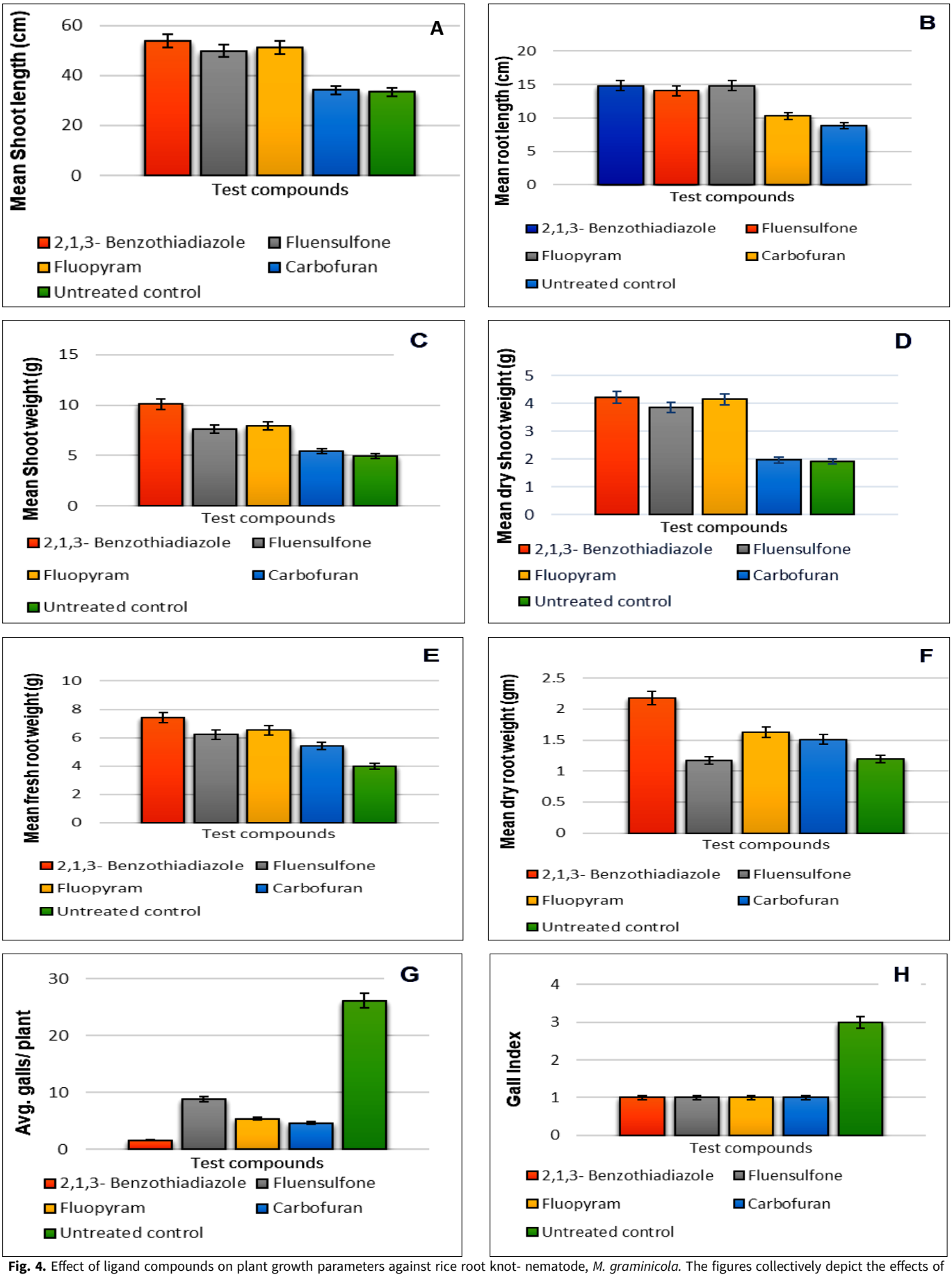

Fig. 4. Effect of ligand compounds on plant growth parameters against rice root knot- nematode, *M. graminicola.* The figures collectively depict the effects of various nematicidal compounds—2,1,3-Benzothiadiazole, Fluensulfone, Fluopyram Carbofuran—compared to an untreated control on plant growth and nematode stress indicators in *M. graminicola*-infected plants. Error bars in all graphs represent the standard deviation, reflecting variability in the data.

weights (4C & D) reveal the positive impact of these compounds on plant biomass, with the untreated control consistently showing the lowest values. The fresh and dry root weight graphs (Fig. 4E & F) further support the superior performance of 2,1,3-Benzothiadiazole and Fluensulfone in enhancing root development. The gall-index figures

(Fig. 4G & H) demonstrate a significant reduction in nematode infestation for treated plants, with the untreated control showing the highest gall counts and index. Test compound Fluopyram (2.42) was followed by 2, 1, 3 Benzothidiazole (3.61) in terms of its ability to dramatically lower the gall index of invading second stage juveniles of *M. graminicola*. Comparing the untreated control (26.12) to all test chemicals results in a significant reduction in gall index (Fig. 5).

Fig 5. Effect of test nematicidal compounds on rice plant against rice root-knot nematode *M. graminicola* at 28 days after transplanting. The figure illustrates the comparative efficacy of various nematicidal treatments on plant health and nematode population reduction, highlighting significant differences in growth parameters and nematode infestation (gall index) levels. (**A**) 20 µL fluopyram 34 SC (0.5 L a.i. ha^{-1}) treated rice plant (**B**) 1 g of fluensulfone 2G (22.2 to 25.6 Kg/ha) treated rice plant (**C**) 2,1,3-benzothiadiazole (50 mg/kg) treated rice plant (**D**) 25 mg carbofuran 10G (2

Molecular docking study

The potential targets of *M. graminicola*'s seven receptor proteins were evaluated against the ligand molecules 2,1,3 -benzothiadiazole, fluopyram, fluensulfone and carbofuran in the current study. SWISS-MODEL was used to model MgGPP using a template protein with 46.59 percent identity, 87 percent coverage and a 0.56 GMQE score that had been previously characterised using electron microscopy (PDB ID: 7C7S.1.A). When the template for MgM0237 (PDB ID: 1JDN.1. A) was experimentally solved using the X-ray crystallography technique, it had a GMQE score of 0.55, 57.18 percent identity and 78 percent coverage. Although neither MgPDI1 nor MgPDI2 have any homologs or templates in the SWISS-MODEL database, Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/) was used for modelling. MgPDI1 had 96 percent coverage and 63 percent identity (Fold Library ID: C6W9ZD), compared to MgPDI2, which had 86 percent coverage, 100 percent confidence and 64 percent identity. Metalloendopeptidase's target protein sequence (101-138 amino acids) was found using the UniProt ID A0A8S9Z7T8. Modeller v. 9.24 was used to resemble the target proteins MgPEL1 and Venom Allergen-Like Protein. The potential protein sequences were found using the NCBI and UNIPROT databases. The molecular and biological functions of the query sequences were searched and annotated using the BLAST service. The Ramachandran Plot analysis revealed that the target MgGPP has 92.3% of its residues in the core region and 7.7% in other permitted regions. In the MgM0237 targets, the percentages of preferred and additionally preferred residues are 92.3 and 7.7, respectively. Target MgPDI1 and MgPDI2 had 90.8% and 93.8 % of their residues in the most favourable region, 8.1% and 5.4% in additional permitted regions and 1.2 and 0.3% in other permitted regions, respectively. Target MgPEL1 had 0.3% residues in favourable regions, 6.6% residues in permitted regions and 93.0% residues in the most favourable region, or core region. The most favourable regions accounted for 89.7% of metalloendopeptidase residues, whereas permitted regions accounted for 7.6%. Sequence similarity analysis was carried out utilising the BLASTP tool for the nematode target proteins as a query against the rice genome proteins in order to find any related proteins in rice. Neither a single hit nor any similar sequences were discovered during the similarity search. Molecular Docking and Virtual Screening of Modelled protein structures were docked with various compounds to determine their mode of binding (Table 1). 2,1,3-Benzothiadiazole's binding affinity with the target, MgGPP, was -4.5 kcal/mol (H-bonds: LEU150, ALA141 and SER154); for MgM0237, it was -3.6 kcal/mol (H-bonds: ASP31 and SER20) (Table 2) and for MgPDI1, it was -5.0 kcal/mol (H-bonds: LYS220, SER MgPDI2 gains 4.9 kcal/mol (H-bonds: VAL445), while MgPEL1 gains 4.5 kcal/mol (H-bonds: TYR44). Venom allergen protein has a binding affinity of -4.1 kcal/mol (H-bonds: ASN89 and GLY136) and metalloendopeptidase has a binding affinity of -4.9 kcal/mol (H-bonds: ASN23) (Table 1, Fig. 6). Hydrogen bonds were present in every complex, demonstrating the stability and potency of 2,1,3-Benzothiadiazole's affinity for *M. gramini-*

Table 1. Binding affinity values of 2,1,3- Benzothiadiazole, Fluopyram, Fluensulfone and Carbofuran with H-bonds and its virulent targets

Targets	Binding affinity (kcal/mol) of small molecules on different targets			
	2,1,3- Benzothiadiazole	Fluopyram	Fluensulfone	Carbofuran
MgGPP	- 4.5	- 7.3	- 5.1	- 5.7
MgM0237	- 3.6	- 5.9	- 4.4	- 4.9
MgPDI1	- 5.0	- 7.9	- 5.6	- 6.2
MgPDI2	- 4.9	- 7.6	- 6.9	- 6.5
MgPEL1	- 4.5	- 8.3	- 5.1	-6.4
Venom Allergen Protein	- 4.1	- 6.9	- 5.6	- 5.4
Metalloendo peptidase	-4.9	-7.4	-4.7	-5.2

Table 2. H-bonds and its virulent targets of ligand molecules

Targets	H – bonds			
	2,1,3- Benzo-thiadiazole	Fluopyram	Fluensul-fone	Carbofuran
MgGPP	LEU150, ALA141, SER154	ARG126, TYR 117	GLY119, ASP187	ALA116
MgM0237	ASP31, SER20	THR21, SER20, SER17	SER24	PHE12
MgPDI1	LYS220, SER254, THR257	LYS426, GLU501	ARG246, THR257, LYS220	LYS220, SER254, GLU252
MgPDI2	VAL445	MET337, GLU444, VAL450	GLN451, VAL445, VAL450	VAL450, GLN451
MgPEL1	TYR44	ARG133, TRP105, TYR44	ARG46	TRP21
Venom Allergen Protein	ASN89, GLY136	HIS81	ASN174, GLU88, HIS137	GLU88
Metalloen-dopeptidase	ASN23	THR49	THR49	GLU51

The two types of hydrogen bonds formed between docked complexes are those with the backbone and side-chain of amino acid residues. Other types of contacts were visible in the docked complex, including hydrophobic interactions, van der waals, pi-pi, alkyl and pi-alkyl.

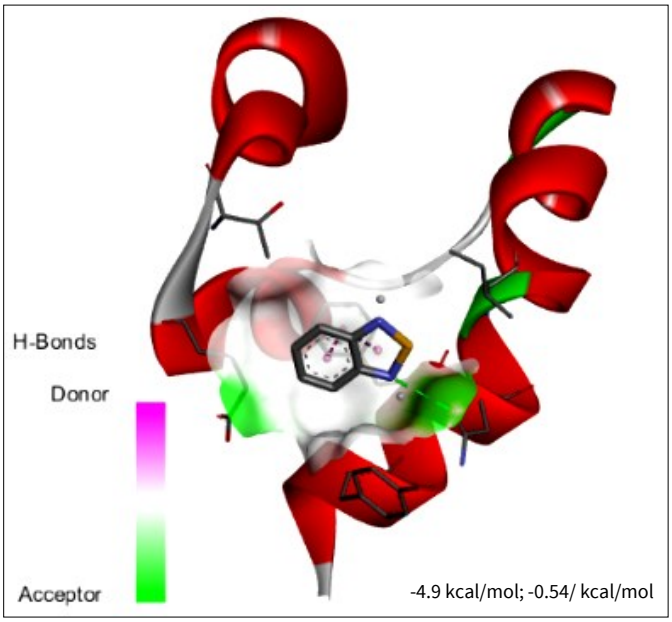

Fig. 6. Binding affinity and Ligand efficiency (kcal/mol) of 2,1,3 Benzothiadiazole against Metallopeptidase.

cola targets. Compound Fluopyram had the binding affinity of -7.9 kcal/mol for MgPDI1 (H-bonds: LYS426, GLU501), -7.6 kcal/mol for MgPDI2(H-bonds: MET337, GLU444, VAL450, GLN451), -8.3 kcal/mol for MgPEL1 (H-Bonds: ARG133, TRP105, TYR44) and -7.4 kcal/mol for Metalloendopeptidase (H-Bonds: THR49) (Table 1).The binding affinity value of Fluensulfone was -5.1 kcal/mol (H-bonds: GLY119, ASP187) with the target MgGPP, -4.4 kcal/mol (H-bonds: SER24) (Table 2) for MgM0237, -5.6 kcal/mol (H-bonds: ARG246, THR257, LYS220) for MgPDI1, -6.9 kcal/mol (H-bonds: GLN451, VAL445, VAL450)for MgPDI2, -5.1 kcal/mol (H-bonds: ARG46) for MgPEL1, -5.6 kcal/mol (Fig. 7 and Table 1) (H-bonds: ASN174, GLU88, HIS137) for venom allergen like protein (Fig. 8 and Table 1 & 2) and - 4.7 kcal/mol

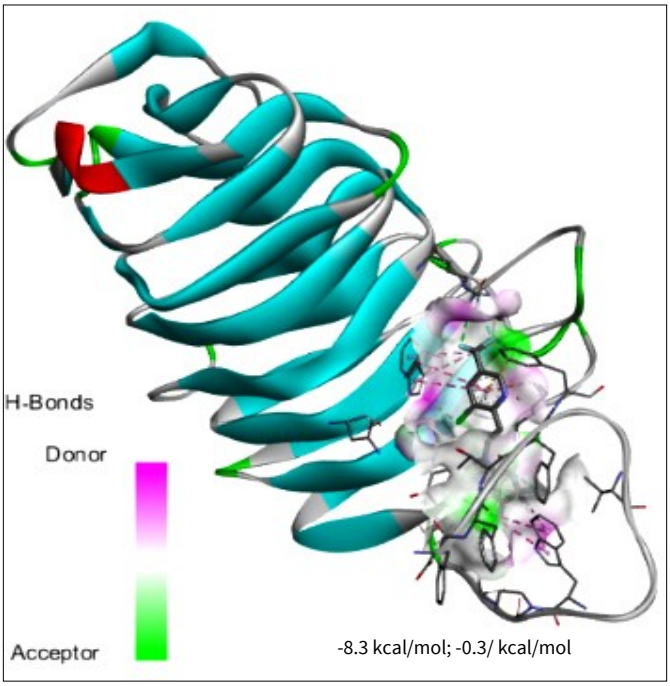

Fig.7. Binding affinity and Ligand efficiency (kcal/mol) of Fluopyram against MgPEL1.

(H-bonds: THR49) for the target protein Metalloendopeptidase (Fig. 8, Table 1). A 3D and 2D docked complex for each target's H-bond donor and acceptor groups has been created (Fig. S1). The binding affinity value of Carbofuran was -5.7 kcal/mol (H-bonds: ALA116) with the target MgGPP (Fig. 9 and Table 1 & 2), -4.9 kcal/mol (H-bonds: PHE12) for MgM0237, -6.2 kcal/mol (H-bonds: LYS220, SER254, GLU252, THR257) for MgPDI1, -6.5 kcal/mol (H-bonds: VAL450, GLN451) for MgPDI2, -6.4 kcal/mol (H-bonds: TRP21) for MgPEL1, -5.4 kcal/mol (H-bonds: GLU88) for venom allergen like protein and -5.4 kcal/mol (H-bonds: GLU51) for the target protein Metalloendopeptidase. The side-chain and backbone of the residues on the binding site create hydrogen bonds with the docked complexes (Fig. 9 & Table 1).

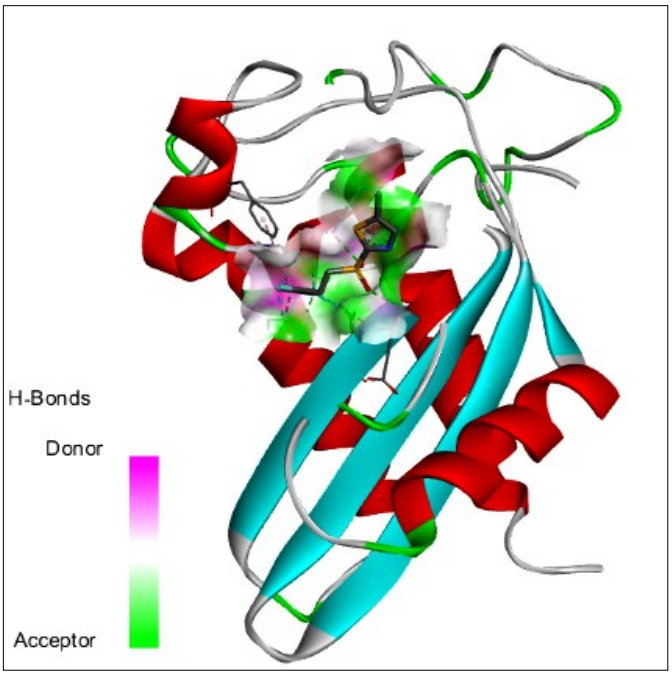

Fig. 8. Binding affinity and ligand efficiency (kcal/mol) of fluensulfone against Venom Allergen Like Protein.

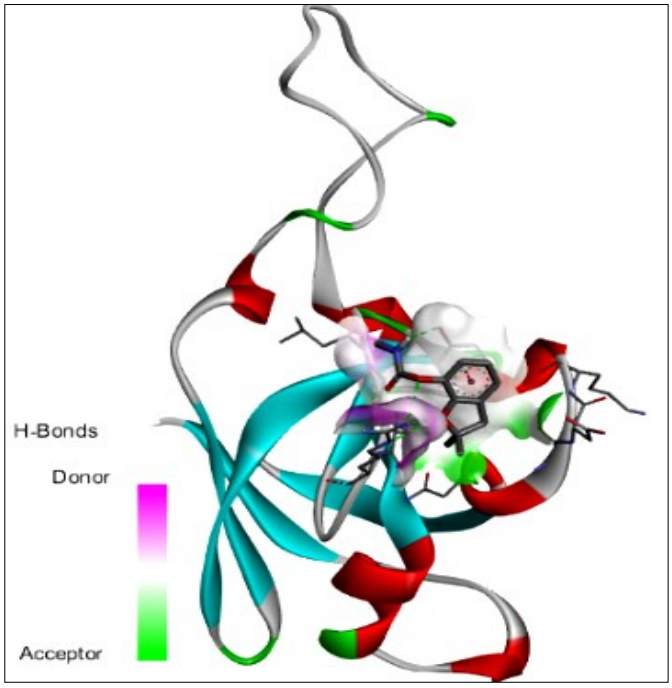

Fig. 9. Binding affinity and ligand efficiency (kcal/mol) of Carbofuran against MgGPP.

Discussion

In this investigation, we demonstrated the sensitivity of the test compounds 2,1,3-benzothiadiazole, flupyram, fluensulfone and carbofuran to the rice root-knot nematode, *M. graminicola*. Due to its strong nematicidal activity against *M. graminicola* during the tests, fluopyram dramatically decreased the galling index and assisted plant development metrics. The *in vitro* testing and the findings of the experiment with two biological repetitions agreed, showing that fluopyram was a potent nematicide that could be used to control *M. graminicola* in rice. In the current investigation, we carried out thorough chemoprofiling of nematicidal ligands to identify their potential interactions with *M. graminicola* target sites. Therefore, for the control of root-knot nematodes, pesticides with a lower risk to the environment and public health are preferred. Fluopyram is one such pesticide that is now being evaluated for its capability to control plant-parasitic nematodes (23). In addition to evaluating the efficacy of the tested compounds, potential synergistic effects when used in combination should be considered. Synergistic interactions between nematicidal compounds could enhance effectiveness, potentially reducing the required dosage of each compound and mitigating environmental impact. For instance, fluopy -ram's strong nematicidal activity could complement other compounds such as 2,1,3-benzothiadiazole or fluensulfone, which may target different biochemical pathways in *M. graminicola*. Future studies could focus on combination treatments to investigate these interactions, measuring metrics like galling index reduction, nematode mortality rates and improvements in plant growth under co-application. Furthermore, molecular docking and *in silico* studies could identify whether these compounds interact with distinct or overlapping target sites, providing a mechanistic basis for any observed synergy. Such insights would contribute to the development of more effective integrated nematode management strategies for rice cultivation.Crop rotation is not always effective and cultural practices require a greater knowledge of nematode biology in order to achieve satisfactory results. Consequently, application of nematicides is the primary approach in managing root-knot nematodes (24). To our knowledge, this is the first report of the nematicidal activity of fluopyram against *M. graminicola* in rice production in India. This is in agreement with the results of a previous study by (2, 25), who reported fluopyram could be used to control *M. incognita* and increase tomato yield. Yield increase associated with application of fluopyram may have been a result of nematode control (2) or perhaps management of fungal pathogens, especially soil borne root pathogens such as *Fusarium* spp. (26). Fluopyram at 320 g ha^{-1} was effective in suppressing infection of plant roots by *M. incognita*, suggesting that fluopyram may act by disrupting the chemoreception and interfering the activity of nematodes to penetrate roots of host plants (27). SDH-inhibiting fungicides, such as boscalid, flutolanil, penthiopyrad, fluxapyroxad and solatenol, do not have nematicidal effects on *M. incognita*. Fluopyram is the only SDH-inhibiting fungicide with nematicidal activities against *M. incognita* (28). This implies that fluopyram may work in a different way than just targeting nematodes' succinate dehydrogenase (SDH) (3). The nematicidal impact of fluopyram on *M. incognita* after a 24-hour exposure period was reversed in the study (29) after J2s of *M. incognita* were submerged in water, which was correlated with our current findings. The use of fluopyram in agricultural production raises certain problems, though. The environmental risk of fluopyram is one thing to be worried about. Fluopyram had a negative impact on the microorganisms, according to a study done to ascertain its impacts on a soil microbial community. Following the application of fluopyram, significant changes in microbial community structure and functional diversity were found, with the effects remaining after 90 days (30). Both fluopyram and fluensulfone are chemical nematicides that, while effective in controlling plant-parasitic nematodes like *M. graminicola*, may have unintended consequences on soil health. Several studies have indicated that chemical pesticides, including fluopyram and fluensulfone, can disrupt soil microbial populations, potentially leading to changes in the structure and function of microbial communities essential for soil fertility and plant health. These disruptions could impact nutrient cycling, organic matter decomposition and the balance of beneficial microbes, thus affecting overall soil ecosystem stability (31). For example, fluopyram, classified as a fungicide with nematicidal properties, has been shown to influence microbial diversity in soil environments. It may inhibit the growth of certain beneficial microorganisms crucial for maintaining a healthy soil microbiome. Similarly, fluensulfone, while targeting nematodes, could also impact soil microbial communities, leading to reductions in microbial populations essential for soil processes. In light of these concerns, it is important to highlight the need for sustainable use of these chemicals. Strategies such as integrated pest management (IPM), which combines chemical treatments with biological control, crop rotation and soil health practices, can help mitigate the negative environmental

impacts while still controlling nematode populations effectively. To ensure minimal impact on soil health, future studies should focus on evaluating the long-term effects of fluopyram and fluensulfone on soil microbial communities, as well as identifying alternative or complementary approaches for nematode control that are less harmful to the environment. On the other hand, fluopyram application did not endanger the soil, as reported by (32), who stated that no fluopyram residues were found 40 days after application. However, the main criticism of fluopyram use lies in the expensive labor needed for its application. While fluopyram application does not require extensive equipment, hand soil drenching demands significant labor, as free flooding with irrigation is not feasible. Seed treatment could provide a potential solution to this issue. Fluopyram applied as a seed treatment might pass into or through the roots and be present at concentrations high enough to control nematodes in soybean (28, 33). However, further research is required to evaluate the effectiveness of seed treatments with fluopyram on nematode motility, hatching and the control of *M. graminicola*. Moreover, an appropriate fluopyram application rate for drip irrigation needs to be determined. While fluopyram has shown promising nematicidal activity, with a high mortality rate and strong binding affinity to effector proteins of *M. graminicola*, potential environmental risks must also be considered. Previous studies have indicated that fluopyram and fluensulfone might influence soil microbial communities, which are crucial for soil health and ecosystem balance. For instance, alterations in microbial diversity or function could have downstream effects on nutrient cycling and plant health. Therefore, it is essential to assess the long-term impacts of fluopyram and fluensulfone on soil microbiota through rigorous field and laboratory studies to ensure their sustainable use in nematode management.

The overall predictive results suggest that the nematicidal compound fluopyram is a suitable candidate for managing *M. graminicola*. It demonstrated a high rate of nematode mortality and strong binding affinity to effector proteins, which are critical for nematode pathogenicity. However, a comparison with other nematicides highlights several key aspects that need consideration, particularly regarding cost, efficacy and scalability for agricultural use. In terms of efficacy, fluopyram has shown promising results, often comparable to other commonly used nematicides such as fluensulfone and fosthiazate. While fluensulfone has been effective in reducing nematode populations, its mode of action primarily disrupts nematode mobility and feeding. Fluopyram, on the other hand, targets mitochondrial respiration, providing a different mechanism that might complement integrated nematode management strategies. From a cost perspective, fluopyram is often criticized for its high labor requirements when applied as a soil drench. In comparison, other nematicides such as oxamyl are more cost-effective in application but may require repeated treatments due to shorter residual activity. Fluopyram's potential use as a seed treatment or in drip irrigation systems could improve its cost-effecti veness and reduce labor inputs, making it more competitive with other options. Regarding scalability, fluopyram's compatibility with seed treatment and irrigation systems offers significant advantages for large-scale agricultural use. This scalability contrasts with nematicides that rely on traditional broadcasting or soil fumigation, which can be labor-intensive and environmentally challenging. However, fluopyram's environmental impact on soil microbial communities and its persistence in various soil types must be thoroughly investigated to ensure its safe integration into agricultural practices. Overall, fluopyram's unique mode of action, high efficacy and potential for innovative application methods position it as a promising nematicidal candidate. Nonetheless, further comparative studies on long-term cost-effectiveness, environmental impact and large-scale applicability are needed to establish its role in sustainable nematode management.

Conclusion

The present study employs analytical and molecular modeling tools to investigate the nematicidal activity of potential compounds and their interactions with target site proteins of *M. graminicola*. Among the four nematicidal ligands screened *in vitro*, fluopyram and 2,1,3-benzothiadiazole were identified as the most effective for immobilizing and killing nematodes. *In silico* analysis revealed a higher binding affinity of fluopyram and fluensulfone to the selected target proteins. Predictions of pharmacokinetics, bioavailability, drug-likeness and medicinal chemistry friendliness indicated that fluopyram could serve as a lead compound for managing rice root-knot nematodes. The findings on 2,1,3-benzothiadiazole's effects on hatching will be validated through wet lab studies and used for nematicidal product development. To ensure the effective deployment of these compounds in rice cultivation, specific recommendations are proposed. Fluopyram should be applied at the seedling stage through soil drenching or as a seed treatment, with seed treatment offering a cost-effective and labor-efficient alternative by providing systemic protection during early root development. Drip irrigation could also be employed for the even distribution of fluopyram around the root zone. For 2,1,3-benzothiadiazole, application during the vegetative growth phase, coinciding with peak nematode hatching, is suggested. This compound could also be delivered through foliar sprays, which may translocate to roots for additional protection. Integrating these compounds into an Integrated Pest Management (IPM) framework can enhance their effectiveness and sustainability. Combining chemical treatments with crop rotation can reduce nematode populations in subsequent seasons, while deploying resistant rice varieties provides a complementary layer of protection. Incorporating biological control agents, such as *P. lilacinus* or *Pochonia chlamydosporia*, can further target nematode eggs and juveniles, reducing reliance on chemical inputs. To address potential impacts on soil microbial communities, the use of organic amendments like compost or neem cake is recommended alongside these nematicides to maintain soil health. These strategies, coupled with further research to optimize application rates

and assess long-term sustainability, will support the practical and environmentally responsible use of fluopyram and 2,1,3-benzothiadiazole in rice nematode management programs.

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Authors' contributions

PV carried out the experiments and wrote the original draft. AA carried out the molecular docking and simulation PG carried out the *in vivo* assay. TS supplied the necessary resources and interpretation of data. PS provided the necessary resources and interpretation of data. SM performed the statistical analysis. LR validation and review of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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