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Iron(II) chloride enhances the biocontrol activity of *Pseudomonas fluorescens* against root-knot nematodes

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Abstract. *Pradana AP, Hoesain M, Asyiah IN, Adiwena M, Angelina Y, Putri D, Masnilah R, Dewi RR. 2025. Iron(II) chloride enhances the biocontrol activity of* Pseudomonas fluorescens *against root-knot nematodes. Biodiversitas 26: 4243-4256.* Root-knot nematodes (*Meloidogyne incognita*) account for over 15% of global crop losses, highlighting the urgent need for sustainable biocontrol solutions. Microbial-based strategies, particularly those employing *Pseudomonas fluorescens*, present environmentally responsible alternatives to chemical nematicides while enhancing soil microbial diversity. The aim of this study was to evuluate the systematic assessment of Iron(II) Chloride ($FeCl_2$) supplementation for improving the nematicidal efficacy of *P. fluorescens*. Five treatment combinations of $FeCl_2$ (0, 50, and 75 ppm) and pH (6 and 8) were evaluated in vitro. The results showed that at 24 hours, J2 mortality reached 16.8% in the 75 ppm $FeCl_2$ treatments (pH 6 and 8), significantly exceeding the control (4.4%, $p<0.05$). By 96 hours, $FeCl_2$-fortified cultures achieved 55.6-56.4% J2 mortality, compared to 35.0% in the control ($p<0.05$). At 168 hours, maximum J2 mortality of 97.0% was recorded with 75 ppm $FeCl_2$ at pH 8, nearly three times higher than the control ($p<0.01$). Similarly, inhibition of egg hatching was markedly enhanced; after 168 hours, 82.8% of eggs remained unhatched in the 50 ppm $FeCl_2$ at pH 6 treatment, versus 29.8% in the control ($p<0.01$). Gas Chromatography-Mass Spectrometry analysis revealed a significant increase in bioactive fatty acid derivatives, such as methyl linoleate, in $FeCl_2$-supplemented cultures, supporting a mechanism of nematode membrane disruption. Collectively, these findings demonstrated that $FeCl_2$ supplementation significantly enhances the nematicidal activity of *P. fluorescens*, offering a novel and effective strategy for sustainable management of root-knot nematodes. This approach offers considerable potential for integration into environmentally compatible crop protection strategies, contributing to the development of sustainable nematode management practices in agricultural systems.

Keywords: $FeCl_2$, *Meloidogyne incognita,* methyl linoleate, *Pseudomonas fluorescens*, secondary metabolites

INTRODUCTION

Meloidogyne incognita, commonly known as the root-knot nematode, is one of the most economically destructive plant-parasitic nematodes globally (Subedi et al. 2020), causing significant yield losses in crops, such as tomato (Asyiah et al. 2021) and kenaf (Parnidi et al. 2021) due to root gall formation, impaired nutrient and water uptake, stunting, and chlorosis (Tapia-Vázquez et al. 2022). Conventional nematode management relies primarily on chemical nematicides; however, their extensive use faces issues like resistance development, environmental contamination, regulatory restrictions, and risks to non-target organisms (Fuller et al. 2008; Tiwari 2024). Biological control, especially using beneficial rhizobacteria such as *Pseudomonas fluorescens*, has therefore become an attractive alternative within integrated pest management programs due to its demonstrated efficacy and environmental sustainability (Noureldeen et al. 2021; Gowda et al. 2022).

Beyond functioning as a biocontrol agent, *P. fluorescens* also shapes soil microbial diversity and ecological functions (Tomar et al. 2024; Amichot et al. 2025). Within the soil microbiota, it contributes to nutrient cycling, organic matter decomposition, and the suppression of soil-borne diseases (Dignam et al. 2019; Benaissa 2024). The presence of such beneficial microbes is closely tied to agroecosystem health and resilience, as greater microbial diversity supports soil fertility, plant productivity, and stability against pests, pathogens, and environmental stress (Wei et al. 2024). Incorporating microbe-based pest management into crop systems not only improves plant protection but also conserves belowground biodiversity and essential ecosystem services (Finckh et al. 2018).

P. fluorescens suppresses nematodes through direct antagonism, competition in the rhizosphere, and induction of systemic resistance in host plants (El-Nagdi et al. 2022). In vitro studies show up to 45% reduction in *M. incognita* egg hatch and 15% increase in juvenile mortality within three days (Das et al. 2021), while greenhouse and field trials report 25-35% lower root galling and 20-35% higher plant vigor and yield compared to controls (Noureldeen et al. 2021; Ayub et al. 2024). Beyond nematode suppression, it also promotes plant growth, nutrient uptake, and root development, reinforcing its role as a multifunctional biocontrol agent (Mohanty et al. 2021).

Despite promising results, the biocontrol efficacy of *P. fluorescens* can vary widely depending on bacterial physiological condition and the cultural environment. Although many studies have demonstrated its broad antagonistic activities, there remains a significant gap in systematically optimizing culture conditions to maximize both consistency and strength of nematode suppression. In particular, targeted manipulation of micronutrient availability —especially iron—during cultivation has not been thoroughly investigated, particularly regarding its measurable effects on biocontrol efficacy (Adiwena et al. 2023).

Iron is essential for bacterial metabolism and biocontrol, with optimal $FeCl_2$ supplementation in *P. fluorescens* enhancing siderophore production, rhizosphere competitiveness, and systemic resistance in plants to suppress nematodes (Mayneris-Perxachs et al. 2022). $FeCl_2$ enrichment can increase growth rates by 20-30% in vitro (Abo-Zaid et al. 2020; Adiwena et al. 2023), while iron also regulates metabolite production, though excessive levels may disrupt biocontrol pathways (Ratledge and Dover 2000). Its role in siderophore production and ISR is central to biocontrol (Kraemer 2004; Saranraj et al. 2022), as siderophores enhance competitive fitness and pathogen suppression in the rhizosphere (Timofeeva et al. 2022; Schalk 2025). Iron also modulates ISR pathways that trigger plant immunity and reduce nematode susceptibility, indicating that fortification could improve the nematicidal efficacy of *P. fluorescens* (Liu et al. 2021; Schalk 2025).

Iron availability influences siderophore and secondary metabolite production in *P. fluorescens*, directly affecting biocontrol effectiveness (Gaonkar 2015). By chelating iron, it deprives nematodes of nutrients and can induce systemic resistance in host plants (Proença et al. 2019). Under optimal iron supply, *P. fluorescens* produces nematicidal compounds such as 2,4-diacetylphloroglucinol, hydrogen cyanide, phenazines, and cyclic lipopeptides that suppress nematodes (Kloepper et al. 1980), though excess iron can reduce siderophore production and competitiveness. Supplementation with $FeCl_2$ can enhance growth and metabolite output, improving biocontrol against *M. incognita* (Proença et al. 2019; Mayneris-Perxachs et al. 2022). However, the specific effects of $FeCl_2$ on nematode suppression and metabolite shifts remain unclear, and this study aims to evaluate different $FeCl_2$ concentrations on juvenile mortality, egg hatching, and metabolite profiles of *P. fluorescens* using GC-MS.

MATERIALS AND METHODS

Study duration and location

The study was conducted from July 2024 to December 2024. All experiments were performed at the Plant Protection Technology Laboratory, Faculty of Agriculture, Universitas Jember, Jember, Indonesia.

Bacterial isolate source and renewal

The bacterial isolate (*P. flouorescens* isolate PF-TGL) was obtained from the Plant Disease and Pest Observation Laboratory, Department of Agriculture and Food Security of East Java Province, Indonesia. The isolate was re-cultured on Nutrient Agar (NA) plates (HiMedia, India) with a diameter of 9 cm.

Phenotypic and biochemical characterization of bacterial isolates

Gram reaction was assessed via a 3% KOH test, where the formation of mucilage indicated a Gram-negative phenotype (Wiratno et al. 2019). Phosphate solubilization was evaluated on Pikovskaya medium by observing a clear zone around colonies (Mardhiana et al. 2017). Fluorescence was checked on King's B medium under UV light (Meliani et al. 2017). Proteolytic activity was determined on skim milk agar, with clear zones indicating proteolysis (Munif et al. 2021).

Preparation of bacterial treatments

Nutrient Broth (NB) was prepared in 100 mL aliquots and supplemented with Iron(II) Chloride ($FeCl_2$) (Merck, Germany), with the pH adjusted according to experimental requirements. Five treatment groups were established: standard NB as the control (without $FeCl_2$), NB supplemented with 50 ppm $FeCl_2$ at pH 6, NB with 50 ppm $FeCl_2$ at pH 8, NB with 75 ppm $FeCl_2$ at pH 6, and NB with 75 ppm $FeCl_2$ at pH 8. The $FeCl_2$ concentrations (50 ppm and 75 ppm) were chosen according to the findings of Adiwena et al. (2023) which, indicated that these levels promote optimal bacterial proliferation and enhance nematicidal efficacy in related *Bacillus* species, without adversely affecting bacterial viability. Each medium was aseptically inoculated with *P. fluorescens* isolate in a laminar airflow cabinet and incubated on a rotary shaker at 45 rpm for seven days. Following incubation, actively growing bacterial cultures were used directly as treatment agents in subsequent assays.

In vitro nematicidal assay against *Meloidogyne incognita*

The nematicidal efficacy of *P. fluorescens* cultures grown in different fortified media was evaluated in vitro against *M. incognita* eggs and second-stage juveniles (J2). The *M. incognita* inoculum originated from the established collection of the Plant Protection Laboratory, Faculty of Agriculture, Universitas Jember, where the culture was maintained under controlled conditions and its purity was regularly verified. The five treatments consisted of: (i) *P. fluorescens* grown in standard Nutrient Broth (NB) as the control (without $FeCl_2$), (ii) *P. fluorescens* grown in NB supplemented with 50 ppm $FeCl_2$ at pH 6, (iii) *P. fluorescens* grown in NB supplemented with 50 ppm $FeCl_2$ at pH 8, (iv) *P. fluorescens* grown in NB supplemented with 75 ppm $FeCl_2$ at pH 6, and (v) *P. fluorescens* grown in NB supplemented with 75 ppm $FeCl_2$ at pH 8. For each assay, 100 eggs or 100 J2 were placed in test dishes containing 5 mL of the respective bacterial culture. The experimental design was a Completely Randomized Design (CRD) with five treatments, each replicated five times. $FeCl_2$ was incorporated directly into the nutrient broth prior to bacterial inoculation, and all media were prepared and incubated with *P. fluorescens* for 7 days before use in the nematode assays. No $FeCl_2$ was added at the time of nematode inoculation; only the resulting bacterial cultures were applied to the test dishes. Observations were made every 24 hours for 168 hours. For egg assays, the number of unhatched eggs was recorded, while juvenile assays measured the number of immobile or dead J2 (Pradana et al. 2025).

GC-MS analysis of culture filtrates

To complement the bioassay data and explore potential modes of action, culture filtrates from three representative treatments were subjected to Gas Chromatography-Mass Spectrometry (GC-MS). The selected samples comprised: (i) the non-fortified control culture, (ii) the culture that produced the highest egg-hatch inhibition in vitro, and (iii) the culture that achieved the greatest J2 mortality. Each culture was first clarified by passage through a 0.14 µm syringe filter. A 1 µL aliquot of the filtered supernatant was injected into the GC-MS system in split mode.

Helium served as the carrier gas at a constant pressure of 64.1 kPa. The injector temperature was maintained at 250°C and the mass-selective detector at 280°C. Chromatographic separation was achieved on a Restek Rtx®-50 capillary column (30 m × 0.25 mm × 0.5 µm). The oven program was set to an initial temperature of 80°C with a 2-min hold, followed by a ramp to 280°C. Mass spectra were acquired and matched against the Wiley9.LIB library for compound identification (Asyiah et al. 2025; Pradana et al. 2025).

Statistical analysis

All data were tabulated and subjected to arcsine transformation prior to statistical analysis. One-way ANOVA was conducted to determine the significance of treatment effects, followed by Tukey's HSD test at a 95% confidence level to separate means, using DSAASTAT version 1.101 software. Data visualization was performed in Python using the Matplotlib library (Pradana et al. 2023, 2025).

RESULTS AND DISCUSSION

Characteristicts of bacterial isolate

Based on the characterization results, *P. fluorescens* isolate used in this study was identified as a Gram-negative bacterium. The isolate exhibited phosphate-solubilizing ability, as evidenced by the formation of a clear halo around the bacterial colonies on Pikovskaya's Agar, indicating the dissolution of insoluble phosphate compounds. Furthermore, when grown on King's B medium, it displayed fluorescence under Ultraviolet (UV) light, a well-known characteristic of *P. fluorescens*. The proteolytic activity result showed a distinct clear zone was formed around the bacterial growth, indicating the production and secretion of protease enzymes capable of hydrolyzing milk proteins. The detailed characteristics are presented in Table 1.

Table 1. Characterization of *Pseudomonas fluorescens* isolate

Observations	**Results**
Gram	Negative
Phosphate solubilization activity	+
Fluorescence activity	+
Proteolytic activity	+

Note: +: Indicates the presence of the characteristic

Pseudomonas fluorescens is a Gram-negative bacteria and shows fluorescence under UV light on King's B medium as a sign of siderophore production (Suman et al. 2018). Its phosphate-solubilizing capacity enhances plant nutrition, indirectly fortifying defenses against *M. incognita* by promoting healthier roots (Billah et al. 2019). Additionally, proteolytic activity observed on skim milk agar points to the secretion of protease enzymes capable of degrading vital nematode proteins, thus impairing egg hatching and juvenile mobility (Siddiqui et al. 2005). Consequently, these biochemical traits underscore the isolate's potential as a biocontrol agent against *M. incognita*.

Nematicidal activity of *Pseudomonas fluorescens* against *Meloidogyne incognita* second-stage juveniles and eggs

The nematicidal activity of *P. fluorescens* cultured under varying media conditions exhibited distinct efficacy patterns. Supplementation with $FeCl_2$ and pH adjustment generally exerted a positive effect, as indicated by increased mortality of *M. incognita* second-stage juveniles (J2). At 24 hours post-treatment, the control group displayed a mortality rate of only 4.4%, which was statistically significantly lower than all other treatments. At this time point, the highest J2 mortalities (16.8%) were observed in cultures grown in media supplemented with 75 ppm $FeCl_2$ at both pH 6 and pH 8. This trend remained consistent throughout subsequent observation periods. At 96 hours post-treatment, the mean J2 mortality in the control reached 35%, which was significantly lower than that observed in the treated groups. In contrast, *P. fluorescens* grown in media containing 75 ppm $FeCl_2$ at pH 6 and pH 8 resulted in mean J2 mortalities of 55.6% and 56.4%, respectively. By the end of observation period (168 hours post-treatment), the most pronounced effect was observed in the treatment with *P. fluorescens* cultured in 75 ppm $FeCl_2$ at pH 8, which achieved J2 mortality rate of 97% (Figure 1).

The varied antagonistic effects observed among *P. fluorescens* treatments can be attributed to the influence of iron concentration and pH on bacterial physiology and biocontrol efficacy (Wandersman and Delepelaire 2004). Iron availability is known to regulate multiple cellular mechanisms in *P. fluorescens*, including growth, stress tolerance, and the expression of genes involved in nematode suppression. The superior nematicidal activity exhibited by bacterial cells cultured at higher $FeCl_2$ concentrations (75 ppm) and alkaline pH (pH 8) may be associated with enhanced bacterial proliferation, increased metabolic activity, or the upregulation of biocontrol-related pathways. These findings are consistent with previous reports indicating that iron fortification and pH optimization can improve the antagonistic potential of *P. fluorescens* against plant-parasitic nematodes (Wahla et al. 2012).

Recent studies have highlighted the key role of iron (Fe^{2+}) in modulating biocontrol efficacy through siderophore-mediated antagonism. Iron supplementation enhances siderophore production in *Pseudomonas* spp., which can sequester iron, making it less available to competing organisms such as nematodes and pathogens, thus indirectly weakening their viability (Timofeeva et al. 2022; Schalk 2025). Abo-Zaid et al. (2020) specifically reported increased siderophore production by *P. fluorescens* under iron-rich conditions, suggesting that iron fortification may enhance nematicidal activity through intensified competition for iron. Furthermore, Liu et al. (2021) indicated that elevated iron levels alter rhizosphere microbial composition and activity, potentially promoting antagonistic bacteria and suppressing nematode populations. These mechanistic insights suggest that the improved biocontrol observed in this study may result not only from direct toxicity of bacterial metabolites but also from enhanced iron-mediated competitive interactions within the microbial community.

Modification of the culture medium for *P. fluorescens* also positively influenced its ability to inhibit the hatching of *M. incognita* eggs. At 24 hours post-treatment, the percentage of unhatched eggs in the control group was 92.6%, which was not statistically different from the proportions observed in treatments where *P. fluorescens* was cultured with 50 ppm $FeCl_2$ at pH 8 (94.4%), 75 ppm $FeCl_2$ at pH 6 (93.6%), and 75 ppm $FeCl_2$ at pH 8 (94.4%). At 96 hours post-treatment, a slight shift in this pattern was observed, but the differences remained statistically insignificant, except for the treatment with 50 ppm $FeCl_2$ at pH 6, which maintained a notably higher proportion of unhatched eggs (90.8%). By the end of the observation period (168 hours post-treatment), the percentage of unhatched eggs in the control had declined to 29.8%, which was not significantly different from most other treatments. However, the 50 ppm $FeCl_2$ at pH 6 treatment continued to exhibit a substantially higher percentage of unhatched eggs at 82.8% (Figure 2).

The enhanced inhibition of egg hatching observed in certain treatments compared to effects on second-stage juveniles likely reflects differences in nematode developmental stage sensitivity. Nematode eggs are protected by robust chitin and lipid layers, necessitating the production of bacterial metabolites or enzymes capable of penetrating or disrupting these barriers (Lee et al. 2014). In contrast, the second-stage juveniles may be more susceptible to direct toxic effects. The sustained high proportion of unhatched eggs observed in the 50 ppm $FeCl_2$ at pH 6 treatment suggests that *P. fluorescens* cultured under these conditions may induce the production or secretion of specific bioactive compounds or enzymes that more effectively compromise egg membranes, thereby enhancing ovicidal activity (Kavitha et al. 2012).

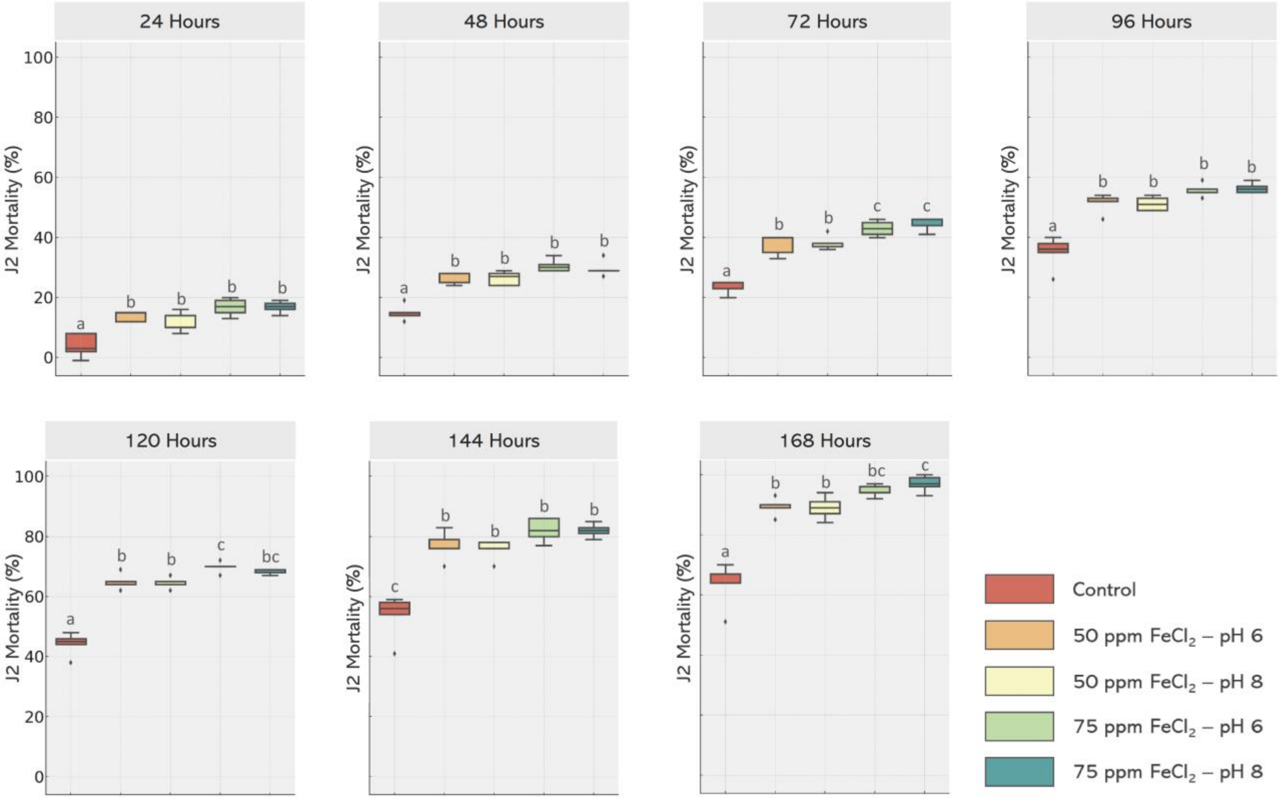


Figure 1. Effect of *Pseudomonas fluorescens* cells cultured under different media conditions on the mortality of *Meloidogyne incognita* second-stage juveniles (J2). Identical letters above the boxplots at the same time point indicate no statistically significant difference, as determined by Tukey's HSD post-hoc test at a 95% confidence level

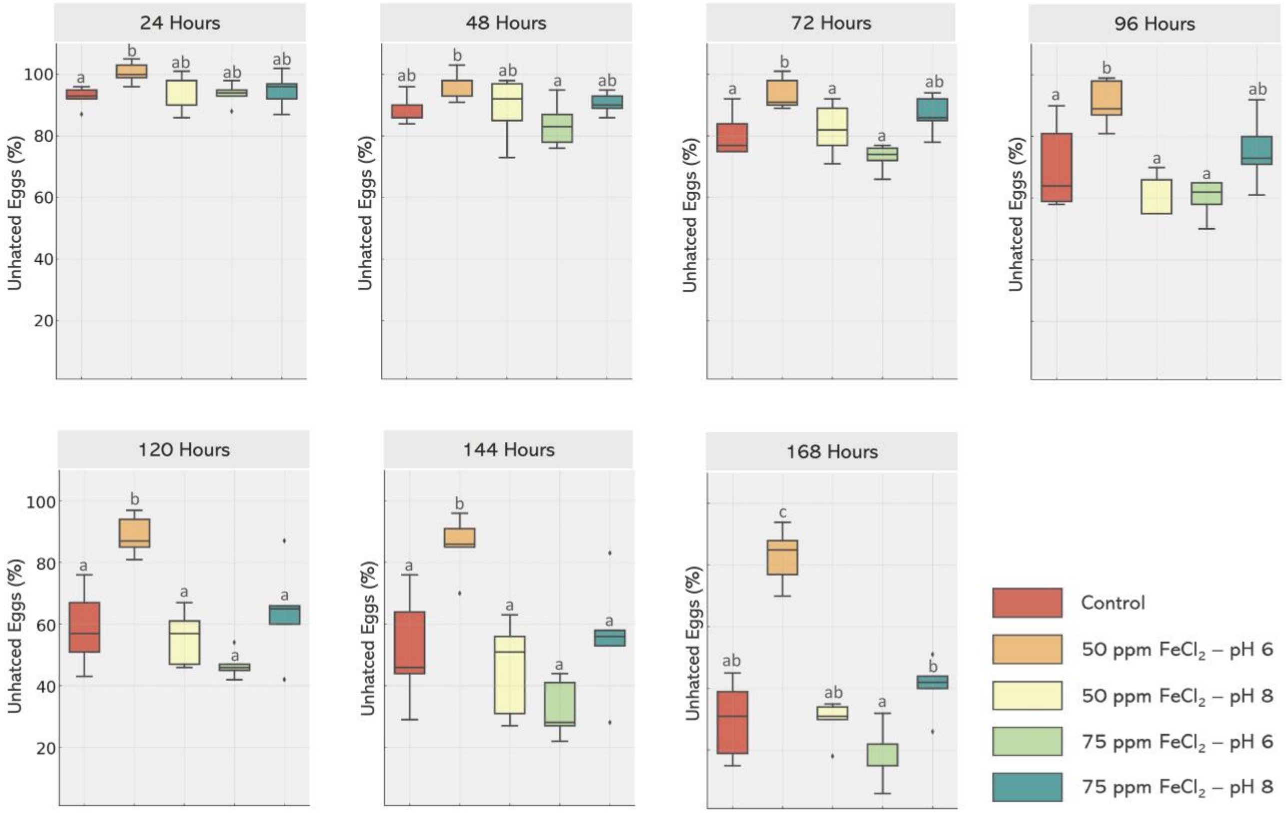


Figure 2. Effect of *Pseudomonas fluorescens* cells cultured under different media conditions on the inhibition of *Meloidogyne incognita* egg hatching. Identical letters above the boxplots at the same time point indicate no statistically significant difference, as determined by Tukey's HSD post-hoc test at a 95% confidence level

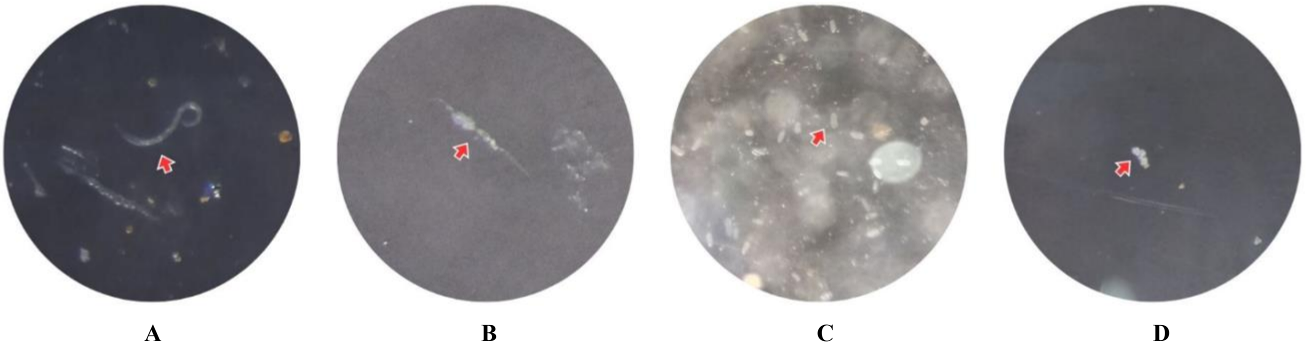


Figure 3. Effects of *Pseudomonas fluorescens* cells cultured under different media conditions on *Meloidogyne incognita* second-stage juveniles (J2) and eggs. A. Normal J2 with intact body structure, B. J2 treated with *Pseudomonas fluorescens* cells showing body deformation and internal fluid leakage, C. Normal egg with intact structure, D. Egg treated with *Pseudomonas fluorescens* cells exhibiting structural damage and leakage

In this study, the highest mortality (97%) of *M. incognita* second-stage juveniles was observed following treatment with *P. fluorescens* cells cultured in NB medium fortified with 75 ppm $FeCl_2$ at pH 8. This effect was primarily manifested as nematode lysis, evidenced by internal fluid leakage and marked body deformation. Similarly, the highest inhibition of egg hatching was achieved with *P. fluorescens* cells cultured in NB medium supplemented with 50 ppm $FeCl_2$ at pH 6, where 82.8% of eggs remained unhatched due to egg lysis, leakage, and structural disintegration (Figure 3). These findings are in accordance with previous research, which has demonstrated that bacterial cells can disrupt nematode cell walls through lytic mechanisms (Dihingia et al. 2017) and induce egg collapse (Lee et al. 2014), likely as a result of bioactive compounds such as proteases, chitinases, and other toxic agents that compromise the integrity of nematode juveniles and egg cell walls (Abd El-Aal et al. 2021).

It should be emphasized that this study was specifically designed to assess the direct nematicidal effects of *P. fluorescens* cultured under $FeCl_2$-fortified conditions in vitro. Assessments of plant growth promotion or indirect effects on plant health were not within the scope of the current investigation. Thus, the observed reductions in *M. incognita* juvenile and egg viability can be attributed to the direct activity of the bacterial cells and their metabolites produced under the tested conditions. Further research, particularly under greenhouse or field conditions, will be needed to determine whether $FeCl_2$ fortification of *P. fluorescens* cultures also confers benefits for plant growth and health.

Secondary metabolic profile of *Pseudomonas fluorescens*

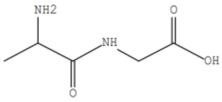
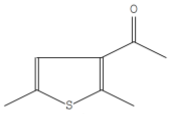
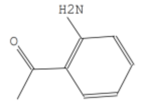
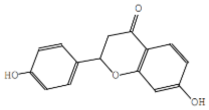
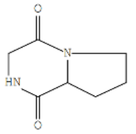
To elucidate the biochemical basis underlying the observed nematicidal and ovicidal activities, secondary metabolite profiling of *P. fluorescens* was conducted. Results of in vitro assays revealed that the highest *M. incognita* J2 mortality was achieved with *P. fluorescens* cells cultured in NB medium fortified with 75 ppm $FeCl_2$ at pH 8, while the most effective inhibition of egg hatching occurred with cells grown in NB medium supplemented with 50 ppm $FeCl_2$ at pH 6. These specific treatments were selected for metabolite analysis and compared with the profile of *P. fluorescens* cultured in standard NB medium under unamended conditions.

The GC-MS chromatogram of the unfortified NB culture showed multiple peaks, indicating the presence of a diverse mixture of secondary metabolites (Figure 4). Identified compounds included amino acid derivatives, such as Dl-Alanylglycine, sulfur-containing heterocyclic molecules (e.g., 3-Acetyl-2,5-dimethylthiophene), aromatic compounds such as Ethanone, 1-(2-aminophenyl)-, as well as flavonoid derivatives and cyclic urea compounds. This broad metabolic repertoire highlights the capability of *P. fluorescens* to produce a wide range of secondary metabolites with potential biological activity (Table 2).

Secondary metabolite analysis of *P. fluorescens* cells cultured in NB medium fortified with 75 ppm $FeCl_2$ at pH 8 revealed a notable increase in both metabolite diversity and complexity compared to cells grown in standard NB medium. GC-MS chromatographic analysis identified 15 distinct peaks in the fortified medium (Figure 5), indicating enhanced metabolic activity under these conditions. The metabolites detected several fatty acid derivatives, including methyl esters of hexadecanoic acid, 9-octadecenoic acid, and (Z,Z)-9,12-octadecadienoic acid, along with pentadecanoic acid and unsaturated alcohols such as 9,12-octadecadien-1-ol. These compounds suggest active lipid biosynthesis, which may contribute to the increased membrane-associated or signaling activity observed.

Furthermore, iron-enriched, alkaline culture conditions promoted the synthesis of various heterocyclic and aromatic compounds, such as piperidinone, 1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane, and aromatic esters like 1,2-benzenedicarboxylic acid, dioctyl ester. Terpenoid derivatives and cyclic alcohols, including 7-octen-3-ol, 2,6,6-trimethyl-, and 3a,6,9a-tetramethyldodecahydronaphtho[2,1-B]furan-2-ol, were also present. This shift from simpler aromatic and peptide derivatives to more complex fatty acid, cyclic, and terpenoid compounds illustrates how iron enrichment and pH optimization can significantly modulate the metabolic output of *P. fluorescens*. Such changes in the secondary metabolite profile may help explain the enhanced biocontrol efficacy observed in bacterial cells grown under these conditions (Table 3).

Table 2. Metabolite profile of *Pseudomonas fluorescens* grown in standard NB medium

Peak	Retention time	Concentrations (%)	Molecular weight (g mol^{-1})	Compound name	Formula	CAS registry number	Chemical structures
1	1.587	59.14	146	Dl-Alanylglycin	$C_5H_{10}N_2O_3$	1188-01-8	
2	2.049	35.44	154	3-Acetyl-2,5-Dimethylthiophene	$C_8H_{10}OS$	2530-10-1	
3	2.670	0.58	135	Ethanone, 1-(2-Aminophenyl)- (CAS)	C_8H_9NO	551-93-9	
4	7.259	1.58	256	4H-1-Benzopyran-4-One, 2,3-Dihydro-7-Hydroxy-2-(4-Hydroxyphenyl)- (CAS)	$C_{15}H_{12}O_4$	69097-97-8	
5	9.480	3.26	154	1,4-Diaza-2,5-Dioxobicyclo[4.3.0]Nonane	$C_7H_{10}N_2O_2$	19179-12-5	
Total		100					

Note: The compound identification was performed by comparing the mass spectra from the chromatogram with the Wiley9.LIB mass spectral database

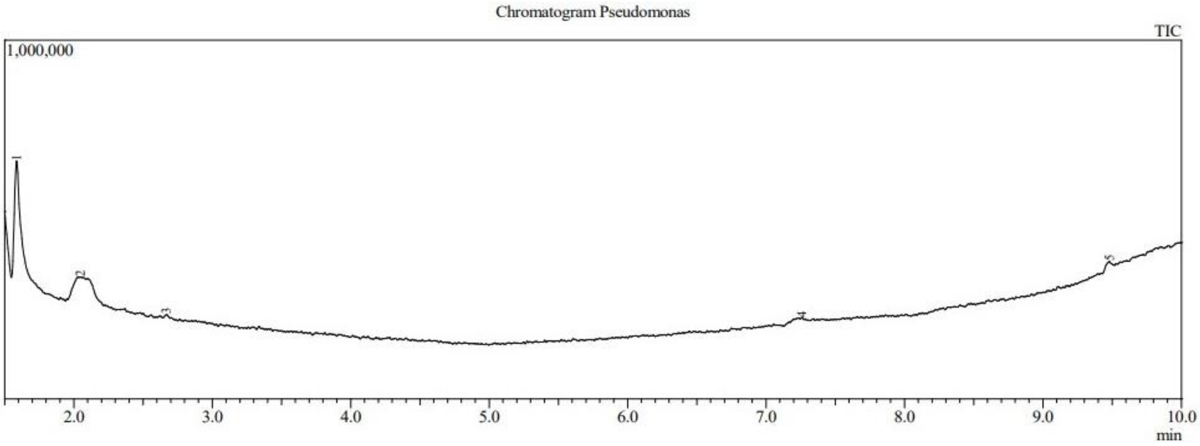


Figure 4. GC-MS chromatogram of secondary metabolites produced by *Pseudomonas fluorescens* in standard NB medium

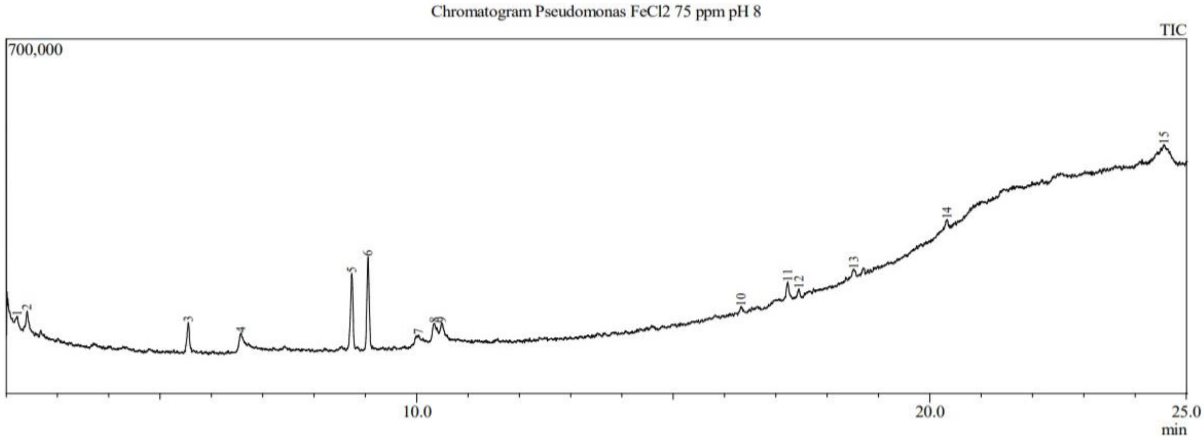


Figure 5. GC-MS chromatogram of secondary metabolites produced by *Pseudomonas fluorescens* in NB fortified with 75 ppm $FeCl_2$ at pH 8 medium

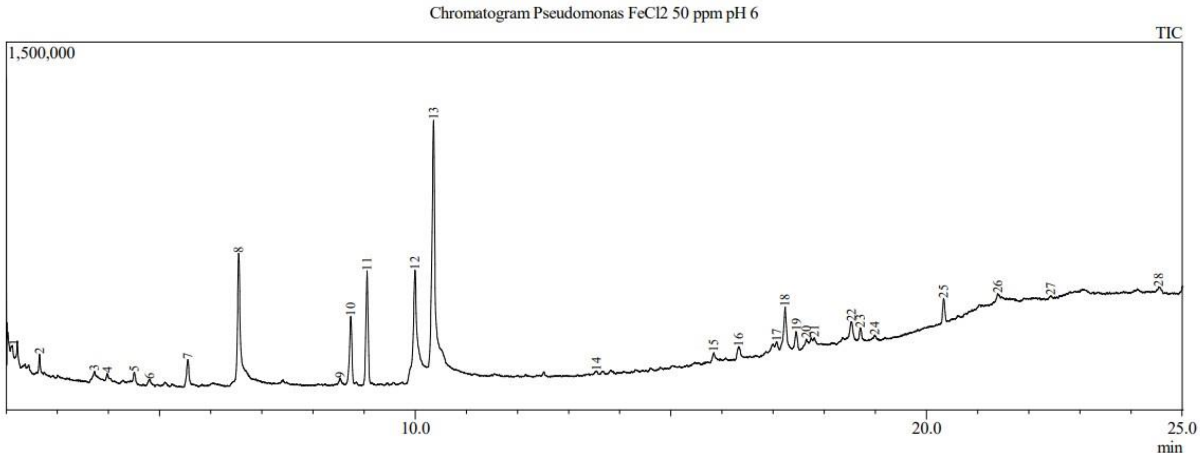
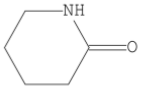
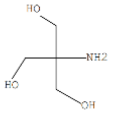
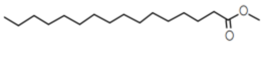
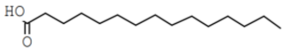
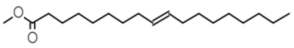
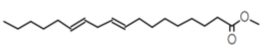
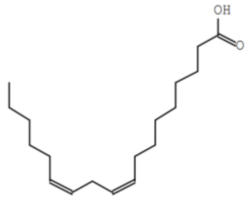
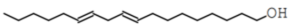
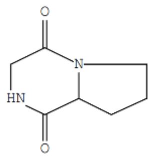
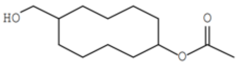
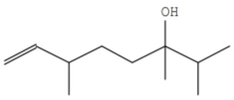
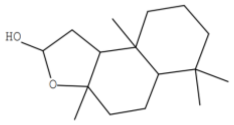
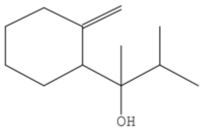
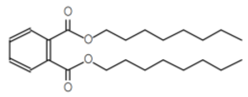
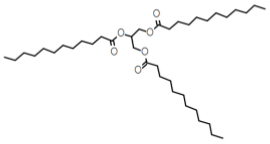


Figure 6. GC-MS chromatogram of secondary metabolites produced by *Pseudomonas fluorescens* in standard NB fortified with 50 ppm $FeCl_2$ at pH 6 medium

Table 3. Metabolite profile of *Pseudomonas fluorescens* grown in NB fortified with 75 ppm $FeCl_2$ at pH 8 medium

Peak	Retention time	Concentrations (%)	Molecular weight (g mol^{-1})	Compound name	Formula	CAS registry number	Chemical structures
1	2.215	1.79	99	Piperidinone (CAS)	C_5H_9NO	27154-43-4	
2	4.406	4.62	121	1,3-Propanediol, 2-amino-2-(hydroxymethyl)- (CAS)	$C_4H_{11}NO_3$	77-86-1	
3	5.92	6.75	270	Hexadecanoic acid, methyl ester (CAS)	$C_{17}H_{34}O_2$	112-39-0	
4	6.153	6.92	242	Pentadecanoic acid (CAS)	$C_{15}H_{30}O_2$	1002-84-2	
5	9.876	17.98	296	9-Octadecenoic acid, methyl ester (CAS)	$C_{19}H_{36}O_2$	2462-84-2	
6	9.952	18.84	294	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (CAS)	$C_{19}H_{34}O_2$	112-63-0	
7	10.845	3.74	280	9,12-Octadecadienoic acid (Z,Z)-(CAS)	$C_{18}H_{32}O_2$	60-33-3	
8	10.878	5.53	266	9,12-Octadecadien-1-ol	$C_{18}H_{34}O$	1577-52-2	
9	11.743	3.02	154	1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane	$C_7H_{10}N_2O_2$	19179-12-5	
10	17.231	4.52	228	Acetic acid, 6-hydroxymethyl-cyclodecyl ester	$C_{13}H_{24}O_3$	0-00-0	
11	17.45	3.6	170	7-Octen-3-ol, 2,3,6-trimethyl-	$C_{11}H_{22}O$	118989-21-2	
12	18.553	3.02	252	3a,6,6,9a-Tetramethyldodecahydronaphtho[2,1-B]Furan-2-Ol	$C_{16}H_{28}O_2$	52811-62-8	
13	18.72	3.6	182	3-Methyl-2-(2-methylene-cyclohexyl)-butan-2-ol	$C_{12}H_{22}O$	0-00-0	

14	18.915	3.23	390	1,2-Benzenedicarboxylic acid, dioctyl ester	$C_{24}H_{38}O_4$	117-84-0	
15	24.563	19.24	638	Decanoic acid, 1,2,3-propanetriyl ester (CAS)	$C_{39}H_{74}O_6$	538-24-9	
Total		100					

Note: The compound identification was performed by comparing the mass spectra from the chromatogram with the Wiley9.LIB mass spectral database

Secondary metabolite profiling of *P. fluorescens* cells cultured in NB medium supplemented with 50 ppm $FeCl_2$ at pH 6 revealed a notably diverse and complex metabolite profile. GC-MS analysis detected 28 distinct peaks in the chromatogram (Figure 6), reflecting a substantial increase in metabolic diversity compared to cells grown in standard NB medium. Among the major metabolites identified were several fatty acid derivatives, including dodecanoic acid, tetradecanoic acid, pentadecanoic acid, hexadecanoic acid methyl ester, and 9-octadecenoic acid methyl ester (E-isomer), many of which closely resemble those found in cultures grown with 75 ppm $FeCl_2$ at pH 8. These findings suggest that iron fortification under acidic conditions similarly activates common lipid metabolic pathways, although the specific metabolites and their relative abundances may differ between treatments.

In addition to these shared lipid derivatives, the 50 ppm $FeCl_2$ at pH 6 treatment was characterized by the production of unique aromatic compounds, such as benzeneacetic acid, as well as flavonoid-related metabolites like 5-hydroxy-3,7-dimethoxy-2-phenyl-4H-1-benzopyran-4-one, and nucleoside derivatives including xanthosine. Furthermore, ester derivatives such as ethyl linoleate and methyl ricinoleate were also detected. This expanded metabolite profile underscores the influence of iron and pH on the secondary metabolic output of *P. fluorescens*, providing insights into the mechanisms underlying the enhanced ovicidal activity observed in bacterial cells cultured under these conditions (Table 4).

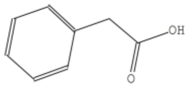
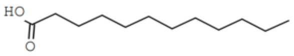
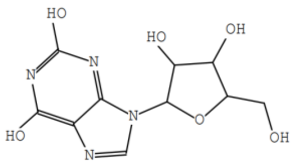
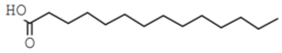
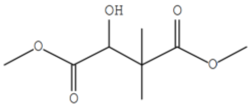
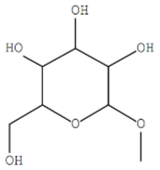
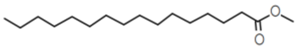
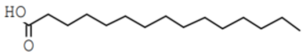
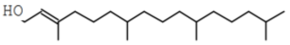
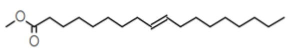
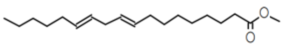
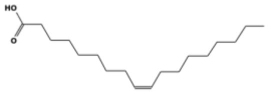
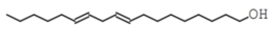
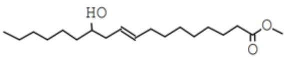
In this study, the enhanced nematicidal and ovicidal efficacy of *P. fluorescens* cells cultured under fortified conditions was investigated, and secondary metabolite profiling was performed to elucidate potential mechanisms underlying this improved activity. The metabolite profile of *P. fluorescens* cells grown in NB medium fortified with 75 ppm $FeCl_2$ at pH 8 revealed several bioactive compounds potentially associated with the observed nematicidal activity against *M. incognita*. The detection of linoleyl alcohol (9,12-octadecadien-1-ol) is particularly significant, as Kuo et al. (2020) previously demonstrated that this compound is produced by *Arthrobotrys* spp. exerts nematicidal effects through oxidative mechanisms affecting the POD-2 enzyme involved in lipid metabolism. A similar disruption in lipid metabolism and membrane integrity may explain the nematicidal effects observed in *M. incognita* juveniles treated with *P. fluorescens* in this study.

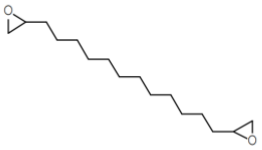
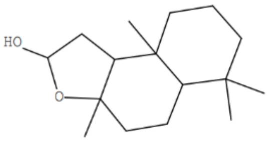
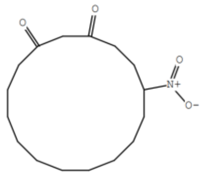
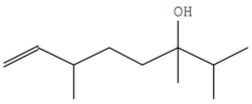
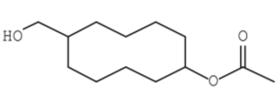
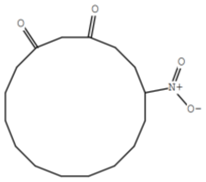
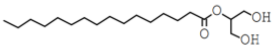
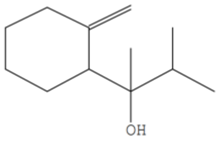
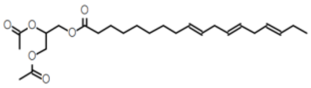
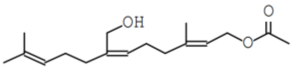
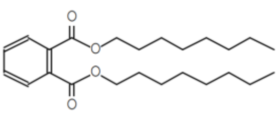
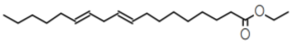
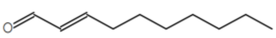
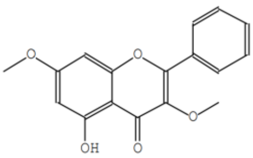
Additionally, methyl linoleate (9,12-octadecadienoic acid methyl ester), another key compound identified, is known for its rapid nematotoxic effects. Panda et al. (2020) reported that linoleic acid derivatives impair nematode motility and survival, suggesting a comparable mode of action for methyl linoleate produced by *P. fluorescens*, potentially disrupting juvenile lipid membranes or interfering with signaling pathways.

In the ovicidal assays, treatments using *P. fluorescens* cells cultured in NB medium fortified with 50 ppm $FeCl_2$ at pH 6 exhibited distinct metabolites correlated with egg-hatching inhibition. GC-MS analysis identified thiophene derivatives such as 3-acetyl-2,5-dimethylthiophene, recognized for nematicidal properties (Ibrahim et al. 2022). Moreover, E-2-decenal, commonly known as 2-decenal (E-isomer), was also detected. Ntalli et al. (2016) previously reported that this compound exhibited potent nematicidal activity, with concentrations as low as 1 μg/mL causing up to 50% inhibition of egg hatching. This compound is known to interfere with cell division within nematode eggs, causing shrinkage of the larvae and structural damage to the eggshell. Dodecanoic acid (lauric acid), another metabolite detected, has also been documented for its nematicidal activity. Patidar et al. (2024) reported that a 50% concentration of lauric acid significantly inhibited egg hatching by approximately 89.3%, demonstrating efficacy both in vitro and in vivo.

Furthermore, methyl oleate (9-octadecenoic acid methyl ester) was identified, adding another layer to the nematicidal potential of the secondary metabolites. As described by Asyiah et al. (2025), methyl oleate exhibits antimicrobial and nematicidal properties by interacting directly with nematode lipid membranes, increasing permeability and leading to cellular lysis. Collectively, these findings indicate that the diverse fatty acid esters and alcohols produced by *P. fluorescens* under fortified conditions play a critical role in its nematicidal capability. These metabolites likely act synergistically to disrupt nematode membranes and metabolic processes.

Table 4. Metabolite profile of *Pseudomonas fluorescens* grown in NB fortified with 50 ppm $FeCl_2$ at pH 6 medium

Peak	Retention time	Concentrations (%)	Molecular weight (g mol^{-1})	Compound name	Formula	CAS registry number	Chemical structures
1	2.1	0.22	136	Benzeneacetic Acid (CAS)	$C_8H_8O_2$	103-82-2	
2	2.651	0.95	200	Dodecanoic Acid (CAS)	$C_{12}H_{24}O_2$	143-07-7	
3	3.726	0.88	284	Xanthosine (CAS)	$C_{10}H_{12}N_4O_6$	146-80-5	
4	3.974	0.46	228	Tetradecanoic Acid (CAS)	$C_{14}H_{28}O_2$	544-63-8	
5	4.505	0.86	190	Butanedioic Acid, 3-Hydroxy-2,2-Dimethyl-, Dimethyl Ester	$C_8H_{14}O_5$	37887-82-4	
6	4.805	0.6	194	.beta.-D-Glucopyranoside, methyl (CAS)	$C_7H_{14}O_6$	709-50-2	
7	5.552	2.15	270	Hexadecanoic Acid, Methyl Ester (CAS)	$C_{17}H_{34}O_2$	112-39-0	
8	6.547	12.14	242	Pentadecanoic Acid (CAS)	$C_{15}H_{30}O_2$	1002-84-2	
9	8.531	0.59	296	2-Hexadecen-1-Ol, 3,7,11,15-Tetramethyl-	$C_{20}H_{40}O$	150-86-7	
10	8.739	5.71	296	9-Octadecenoic acid, methyl ester, (E)- (CAS)	$C_{19}H_{36}O_2$	1937-62-8	
11	9.059	8.36	294	9,12-Octadecadienoic Acid (Z,Z)-, Methyl Ester (CAS)	$C_{19}H_{34}O_2$	112-63-0	
12	10.995	1.42	282	Heptadecene-(8)-Carbonic Acid-(1)	$C_{18}H_{34}O_2$	0-00-0	
13	10.359	31.57	266	9,12-Octadecadien-1-Ol (CAS)	$C_{18}H_{34}O$	1577-52-2	
14	13.537	15.92	312	Methyl Ester of Ricinoleic Acid	$C_{19}H_{36}O_3$	141-24-2	

15	15.841	0.95	254	1,2-15,16-Diepoxyhexadecane	$C_{16}H_{30}O_2$	0-00-0	
16	16.333	1.34	252	3a,6,6,9a-Tetramethyldodecahydronaphtho [2,1-B]Furan-2-Ol	$C_{16}H_{28}O_2$	52811-62-8	
17	17.067	1.14	297	6-Nitro-Cylohexadecane-1,3-Dione	$C_{16}H_{27}NO_4$	86572-48-7	
18	17.235	0.56	170	7-Octen-3-Ol, 2,3,6-Trimethyl-	$C_{11}H_{22}O$	118989-21-2	
19	17.735	0.71	288	Acetic Acid, 6-Hydroxymethyl-Cyclodecyl Ester	$C_{13}H_{24}O_3$	0-00-0	
20	17.933	0.96	297	6-Nitro-Cylohexadecane-1,3-Dione	$C_{16}H_{27}NO_4$	86572-48-7	
21	17.65	0.08	330	Hexadecanoic Acid, 2-Hydroxy-1-(Hydroxymethyl)Ethyl Ester (CAS)	$C_{19}H_{38}O_4$	23470-00-0	
22	18.405	0.28	182	3-Methyl-2-(2-Methylene-Cyclohexyl)-Butan-2-Ol	$C_{12}H_{22}O$	0-00-0	
23	18.737	1.02	436	1-Linolensaeure-Sn-Glycerylester-2,3-Diacetat	$C_{25}H_{40}O_6$	0-00-0	
24	18.701	1.08	280	2,6,10-Dodecatriene, 12-Acetoxy-6-Hydroxymethyl-2,10-Dimethyl-, (E,E,E)-	$C_{17}H_{28}O_3$	0-00-0	
25	20.1	0.12	390	1,2-Benzenedicarboxylic Acid, Dioctyl Ester (CAS)	$C_{24}H_{38}O_4$	117-84-0	
26	20.335	1.02	308	Ethyl Linoleate	$C_{20}H_{36}O_2$	544-35-4	
27	22.427	0.59	154	E-2-Decenal	$C_{10}H_{18}O$	0-00-0	
28	24.54	0.5	298	5-Hydroxy-3,7-Dimethoxy-2-Phenyl-4H-1-Benzopyran-4-One	$C_{17}H_{14}O_5$	0-00-0	
Total		100					

Note: The compound identification was performed by comparing the mass spectra from the chromatogram with the Wiley9.LIB mass spectral database

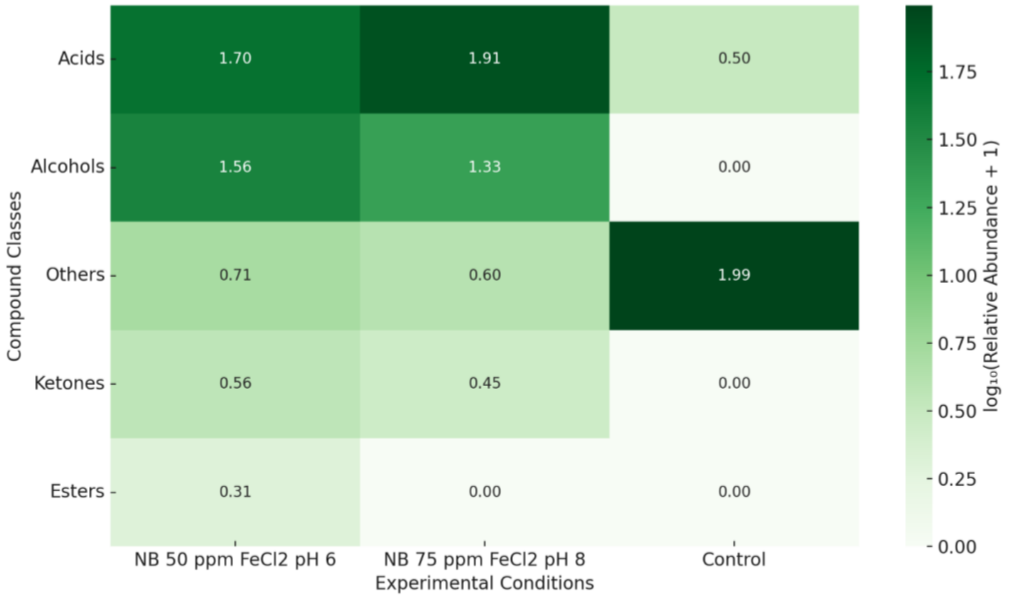


Figure 7. Heatmap of compound classes identified by GC-MS analysis of metabolites associated with *Pseudomonas fluorescens* cells cultured in NB medium fortified with $FeCl_2$ and under standard conditions. Data were log-transformed [$\log_{10}$(Relative Abundance + 1)] for improved visualization

The efficacy of *P. fluorescens* cells in suppressing *M. incognita* was closely linked to the culture conditions, as reflected in their distinct metabolite profiles. Heatmap visualization of compound classes [$\log_{10}$(Relative Abundance + 1)] (Figure 7) demonstrated that cells cultured in NB medium fortified with 75 ppm $FeCl_2$ at pH 8 were associated with an increased presence of acids and alcohols, compound groups known to contribute to nematicidal activity. In contrast, cells grown under standard conditions exhibited a metabolite distribution dominated by the “others” category, with relatively low levels of acids and alcohols. These findings suggest that optimizing the growth environment of *P. fluorescens* cells can enhance their nematicidal potential by favoring the production of more bioactive compounds.

This differential distribution reflects a treatment-induced modulation of bacterial metabolism. The increased abundance of acids and alcohols observed in cultures fortified with $FeCl_2$ is particularly noteworthy, as these compound classes are known to disrupt nematode cuticles and eggshells, contributing to cytotoxic effects (Zhang et al. 2012; Ye et al. 2022). Such metabolic changes in the bacterial cells likely underlie the enhanced nematicidal and ovicidal activities observed against *M. incognita*.

The improved biocontrol activity achieved through $FeCl_2$-fortified *P. fluorescens* cultures has broader implications for rhizosphere ecology and the dynamics of soil microbial communities. By selectively enhancing beneficial bacterial populations, iron supplementation may shift the microbial balance toward a more functionally diverse and disease-suppressive state (Aguado-Norese et al. 2025). Such enrichment of the rhizosphere with antagonistic and growth-promoting microbes contributes to nutrient cycling, organic matter turnover, and the suppression of plant pathogens, supporting overall soil health and agroecosystem resilience (Lurthy et al. 2021; Bhat et al. 2024).

Furthermore, iron-mediated microbial interactions in the rhizosphere are known to shape both competition and cooperation among microbial taxa, influencing the structure and stability of microbial networks. Promoting the abundance and activity of beneficial microbes such as *P. fluorescens* through targeted nutrient management not only enhances pest suppression but also helps maintain the ecological balance of soil communities. Importantly, these strategies offer a sustainable alternative to chemical nematicides, reducing risks to non-target organisms and preserving belowground biodiversity essential for long-term agricultural productivity (Lemanceau et al. 2009; Philippot et al. 2024).

In addition to the enhanced production of nematicidal metabolites, $FeCl_2$ supplementation may further potentiate the biocontrol efficacy of *P. fluorescens* by improving its colonization and persistence in the rhizosphere, as well as stimulating Induced Systemic Resistance (ISR) in host plants. Improved rhizosphere colonization increases the bacterium’s ability to compete for space and resources, enabling more effective suppression of *M. incognita* populations and promoting long-term soil disease suppressiveness. Thus, $FeCl_2$ fortification may enhance biocontrol not only through direct nematicidal activity but also by supporting beneficial rhizosphere interactions and the activation of plant defense responses.

In conclusion, fortification of nutrient broth medium with $FeCl_2$ significantly enhanced the nematicidal efficacy of *P. fluorescens* cells, with cultures grown in NB medium supplemented with 75 ppm $FeCl_2$ at pH 8 achieving a second-stage juvenile (J2) mortality rate of 96.8% after 168 hours, and those with 50 ppm $FeCl_2$ at pH 6 resulting in 84.4% inhibition of egg hatching. GC-MS analysis revealed that these optimized conditions facilitated the production of bioactive metabolites such as methyl linoleate, methyl oleate, and linoleyl alcohol, which were possibly responsible for compromising nematode membrane integrity and thus enhancing biocontrol activity. These findings not only highlight the promise of $FeCl_2$-fortified *P. fluorescens* as a biological control agent against *M. incognita*, but also support the potential for developing microbial-based strategies that reduce reliance on synthetic nematicides, enhance agroecosystem biodiversity, and promote sustainable agriculture. However, several important considerations must be addressed before large-scale field application can be recommended. Future research should therefore focus on multi-season field trials across diverse cropping systems, as well as molecular studies to further elucidate the mechanisms underlying enhanced nematicidal activity, such as the expression profiles of biocontrol-related genes and interactions with plant immune responses.

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