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Isolation and Characterization of a (Surfactin-Like Molecule) Produced by *Bacillus subtilis*: Antagonistic Impact on Root-Knot Nematodes

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Abstract

Plant-parasitic nematodes are severe soil-borne pathogens that cause significant damage to agricultural products each year, resulting in substantial financial losses globally. Thus, there is an urgent need to identify novel biological control agents or nematicides. The nematicidal potential of Bacillus subtilis-derived lipopeptides against *Meloidogyne incognita* was investigated at various concentrations (35 ppm, 25 ppm, 15 ppm, 5 ppm) under in vitro conditions. Egg hatching inhibition and mortality of second-stage juveniles (J2s) of *M. incognita* were analyzed after exposure for 6, 12, 24, 48, and 96 hours. Data showed that with the increase in concentration and exposure period, egg hatching inhibition and percent mortality increases. Maximum percent mortality of J2s was reported at 35ppm i.e., 45%, 55%, 67.75%, 77% and 85% at 6, 12, 24, 48 and 96 hrs, respectively. The maximum ovicidal activity was reported at 35ppm concentration, with 84.61% of eggs hatching inhibition on 96 hrs of the exposure period. The bacterial culture suspension of *Bacillus subtilis* and *Pseudomonas putida* at1.2x108cfu/ml, and the crude lipopeptide (35ppm) was also investigated as a biological control agent against *M. incognita* on tomato in a pot experiment under glasshouse condition. Combinational treatment of *P. putida* and *B. subtilis* culture, prior to inoculation of *M. incognita* on tomato plant caused a significant increase in plant growth attributes and in biochemical parameters over the inoculated control. In the same treatment, the maximum reduction in nematode population and root galling was recorded. However, in the crude lipopeptide experiment study, root dip and inoculation of crude lipopeptide in tomato after the introduction of *M. incognita* caused a major augment in all the parameters over the inoculated control. MALDI-TOF MS analysis of crude lipopeptide shows surfactin like molecules at m/z 1058 [M+Na]+. It is concluded that crude lipopeptide or combinational treatment of *B. subtilis* and *P. putida* culture suspension can be employed as a biocontrol agent against *M. incognita* and may act as a source of a novel nematicidal agent of bacterial origin.

Keywords: Surfactin-like molecule, *Bacillus subtilis*, isolation, characterization, antagonistic impact

1. Introduction

The tomato (*Solanum lycopersicum L.*) holds a significant economic value and is widely cultivated. A rich source of micronutrients, such as minerals, vitamins, and antioxidants essential for human health, tomatoes are particularly abundant in lycopene—an antioxidant known for reducing the risk of cancer, cardiovascular disease, and cellular aging (Gerszberg et al., 2015). India, ranking third globally in tomato production after China and the USA, harvested 0.8 million hectares, with a yield of 26 thousand per hectare, resulting in a total production of 20.7 million tons in 2018 (Niemi and Väre, 2019). In many economically challenged nations, the impact of *Meloidogyne* spp. infection poses a severe challenge to maintaining an adequate tomato supply. Root-knot nematodes (RKN) are responsible for an estimated annual global loss of $78 billion, affecting the vascular structure of plants and impeding water uptake, nutrient transport, and photosynthesis. Nematode secretions disrupt biochemical and molecular processes in plant roots, weakening their ability to absorb water and nutrients from the soil. Numerous experiments have explored methods to reduce nematode infection, including non-host crop rotation and the application of chemical nematicides like halogenated aliphatic hydrocarbons (e.g., 1,3-dichloropropene), a mixture of methyl isothiocyanate, oxamyl, thionazin, and carbofuran. However, the widespread use of these chemical nematicides poses ecological risks (Gowda et al., 2019). Khan et al. (2024) demonstrated that *Oxalis corniculata* effectively serves as an eco-friendly phyto-nematicide against the root-knot nematode (*Meloidogyne incognita*), significantly reducing nematode mortality, egg hatching, and improving cabbage growth and yield compared to other botanicals and untreated plants.

Given the current emphasis on eco-friendly disease control, recent research has focused on microbial-derived products (Naamala & Smith, 2021). Plant growth-promoting rhizobacteria (PGPR) offer an environmentally friendly approach to controlling plant diseases, when cultivated with PGPR, plants receive more precise protection from soil-borne illnesses, ensuring sufficient nutrient uptake and the release of phytohormones (Prasad et al., 2019). *Bacillus spp*. and *Pseudomonas spp*. have been studied as bio-nematicides due to their production of nematocidal secondary metabolites, antibacterial substances, enzymes, and exotoxins (Shaikh et al., 2016). *Bacillus subtilis*, in particular, has gained attention for its environmental safety. Fengycin, surfactin, and iturin, lipopeptides produced by this bacterium, have demonstrated potential in combating plant diseases. These lipopeptides, containing a lipophilic fatty acid chain and a hydrophilic peptide ring, act by creating pores in membranes, leading to an imbalance in transmembrane ion fluxes and eventual cell death (Earl et al., 2008). Dutta et al. (2019) reviewed the use of brassica and non-brassica biofumigant crops for plant-parasitic nematode suppression, highlighting their mechanisms and integrating this technique into broader nematode management practices. Recently, Walia and Khan (2023) provided a comprehensive overview of Meloidogyne species systematics, including their morphology, host and cytological races, impact on crops in India, and detailed management strategies for nematodes in vegetable cropping systems, emphasizing biological control, host resistance, chemical nematicides, and management in protected cultivation.

Nematodes also pose significant challenges to agriculture in numerous other countries, affecting a wide range of crops and contributing to substantial economic losses. Their impact extends globally, leading to reduced yields and increased management costs for

farmers. In response to these challenges, scientific research is actively being conducted across various nations to develop effective strategies for nematode control.

El-Nuby et al. (2019) conducted a survey of plant parasitic nematodes in the Sinai Peninsula, Egypt, finding 13 genera across 9 families and 3 orders, with *Meloidogyne* being the most abundant and dominant genus. They noted greater nematode diversity in North Sinai compared to South Sinai, where some genera were recorded for the first time. Elawady et al. (2022) surveyed nematodes in solanaceous plants in Dakahlia governorate, identifying 10 genera with *Meloidogyne* spp. as the predominant pest, especially in clayey soils, and highlighting variations in nematode incidence among different crops. Yahaya et al. (2024) identified ten genera of plant-parasitic nematodes in citrus rhizospheres in Ado and Gboko, Benue State, Nigeria, with Tylenchulus spp. being the most abundant and frequent, suggesting that local environmental conditions favor their growth. A survey (Ami et al., 2018) in Semel District revealed that root-knot disease incidence on cucumber plants peaked in autumn, with the highest infection rates in Sartenk and the lowest in Sharia, and identified *Meloidogyne javanica* as the prevalent nematode species, showing significant fluctuations in population density throughout the year. Kumar et al. (2014) surveyed nine Local Government Areas in Niger State, Nigeria, from April to September 2012, finding a 64.59% overall infection rate of root-gall disease across five vegetable crops, with the highest frequency in Lapai and the lowest in Mariga, and noted varying disease severity among the crops. Engelbrecht et al. (2020) reviewed the current nematode threats to soybean production and explored potential biological control options, emphasizing the need for comprehensive research to address global food demands, particularly in developing countries like those in sub-Saharan Africa. Lu et al. (2022) reported the first natural infection of *Brassica juncea* with *Meloidogyne graminicola* in southern China, identifying symptoms and confirming the nematode's pathogenicity, highlighting a new threat to mustard production in the region. Gürkan and Çetintaş (2024) identified root-knot nematode populations from vegetable fields in Kahramanmaraş, Türkiye, detecting *Meloidogyne incognita* and *Meloidogyne javanica* with specific races, including the first report of *M. javanica* in the region.

Although *Pseudomonas spp*. and *Bacillus spp*. are now utilized by bacterial lipopeptide manufacturers, their widespread adoption is hindered by expensive processing and recovery costs (Shafi et al., 2017). Scientists are actively developing new manufacturing techniques, such as novel media formulations, low-cost substrates, and production procedures. The increased isolation of active natural bio-products from bacterial interactions emphasizes the importance of finding natural antibiotics to combat phytopathogens. Consequently, bacterial-based surfactin has been employed to address root-knot disease caused by *Meloidogyne incognita* in tomatoes, aligning with the need for environmentally safe and agriculturally appropriate bio-products derived from bacterial interactions.

2. Methods and Material

2.1. Preparation and Sterilization of Soil

The dirt was cleaned before putting it in 8-inch clay pots. Each pot was filled with 4 kg of cleaned soil. The soil, a type commonly found, was collected from a fallow field at Farm. It was then sifted through a 16 mesh sieve to remove stones and debris. The soil

was mixed with Farmyard Manure in a 3:1 ratio, and the mixture was used to fill 25 cm diameter clay pots at 4 kg per pot. A small amount of water was added to slightly moisten the soil before sterilizing the pots in an autoclave at 20 lbs. pressure for 20 minutes. After sterilization, the pots were left to cool at room temperature before being used for experiments.

2.2. Collection of Root-knot Nematode

Surveys were conducted in the agricultural fields, roots of heavily infected vegetable crops, affected by root-knot nematodes, were collected by gently uprooting the plants. Soil particles clinging to the roots were removed. The infected roots were then placed in polythene bags and transported to the laboratory for further examination.

Upon reaching the laboratory, the infected root samples were washed with running tap water to check for the presence of egg masses on the root system. To remove attached soil, highly contaminated roots were gently shaken and soaked vigorously in water. Sterilized forceps were used to pick egg masses, and they were sprayed with clean distilled water after surface sterilization with 0.5% sodium hypochlorite (NaOCl). These treated egg masses were transferred to a Petri plate with sterilized water and incubated at 28 ± 2 °C.

Every 24 hours, newly hatched second-stage juveniles (J2) were collected, washed in fresh water, and the process was repeated daily. To determine the concentration of freshly hatched J2, an average of five counts were made, resulting in a suspension volume containing 200 J2 *M. incognita* per milliliter. For inoculation, 10 ml of this suspension (equivalent to 2000 freshly hatched *M. incognita* J2) was used.

2.3. Scanning Electron Microscopy (SEM) Analysis for Identification of RKN Species

Utilizing the female perineal pattern served as the methodology for RKN species identification. Extraction of females from root-knot galls was delicately executed, followed by their placement on a pristine glass slide immersed in lacto phenol. The posterior section of the female was intentionally sliced with precision using a thin razor blade, and subsequent washing of the body content ensued. Further perineal trimming was undertaken, and the prepared specimens were mounted for meticulous observation. To create a controlled observation environment, a circular coverslip (13 mm) was meticulously coated with a thick ring of glycerol. This formed a chamber containing approximately ten female patterns, positioned facing upwards in a droplet of 45% lactic acid. The coverslip, now housing the specimens, was affixed to a microscopic slide. To facilitate the removal of lactic acid, one drop of 2% formalin was systematically applied to the chamber every 2–3 minutes. After a 10-minute interval, the solution was absorbed onto filter paper, and the perineal pattern underwent desiccation in desiccators at room temperature (Al Banna et al., 2020).

Following this preparation, the coverslip was detached from the glass slide and affixed to the adhesive side of a scanning electron microscopy (SEM) stub. A gold coating (14-nm thick) was applied before subjecting the specimen to analysis in the SEM apparatus (JSM 6510LV; Jeol, Tokyo, Japan). The SEM image obtained from this process was subsequently employed to scrutinize the surface morphology of the perineal pattern, aiding in the differentiation of Meloidogyne species, as detailed in a referenced source.

2.4. Preparation of Bacterial Inoculum

Procurement of bacterial strains (*Bacillus subtilis* MTCC-441 and *Pseudomonas putida* MTCC-102) involved their acquisition from IMTECH (Chandigarh, India). Following this, the strains were inoculated onto a nutrient agar plate and subjected to incubation for either 24 or 48 hours at a temperature of 28 ± 2 °C. In the subsequent phase of inoculum preparation, both *B. subtilis* and *P. putida* were separately introduced into NB medium, comprising 3 g of beef extract, 5 g of peptone, and 10 g of glucose, dissolved in 1 liter of double-distilled water at a pH of 7.4 ± 0.2. These bacterial cultures underwent incubation at 28°C for a duration of 48 hours. The calculation and adjustment of cell density were executed to reach a concentration of 1.2 × 108 colony-forming units per millilitre (CFU ml−1).

2.5. Extraction of Crude Lipopeptide

Initiating the process, a bacterial colony of Bacillus subtilis found its place in Luria broth (50 mL) within a (250 mL) Erlenmeyer flask. Incubation unfolded at 28°C and 180 rpm, extending over 24 hours. The resultant 24-hour-old inoculum served as the foundation for seeding yeast extract peptone dextrose medium (YPD medium; composition in g/L: 1% yeast extract, 2% peptone, 2% glucose) in a 1000-mL flask. This seeding aimed for an initial OD-600 (Optical Density measured at a wavelength of 600 nm) of approximately 0.05. Subsequent incubation transpired at 28°C and 180 rpm, encompassing a duration of 72 hours.

The culmination of this incubation period marked the extraction of the 72-hour-old fermentation broth from both *B. subtilis* and *P. putida*. Centrifugation, executed at approximately 10,000 rpm and 4°C for 10 minutes, led to the separation of the supernatant. Adjustments were made to the pH, reducing it to 4 using concentrated HCL, followed by overnight incubation at 4°C to precipitate the lipopeptides. The acid precipitates underwent recovery through another round of centrifugation at approximately 10,000 rpm and 4°C for 10 minutes. Subsequently, the freeze-drying process commenced, resulting in a pellet that was solubilized in anhydrous methanol and subjected to two extraction cycles. The collected samples underwent detailed analysis through MALDI-TOF MS/MS and were subsequently employed in the experimental procedures.

2.6. Identification of Crude Lipopeptides by Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectroscopy (MALDI-TOF MS)

To unveil the characteristics of crude lipopeptides, a MALDI-TOF MS Workstation (Applied Biosystems, Foster City, CA, USA) was harnessed, featuring a 337 nm pulsed nitrogen laser. The testing process involved combining the sample with an equivalent quantity of a saturated solution of 2,5-dihydroxybenzoic acid (DHB: 5 mg in 1 ml 70% acetonitrile, 0.1% trifluoroacetic acid). Vortexing the suspensions ensured a homogeneous mixture. Subsequently, an aliquot of 1–2 µl was carefully dotted onto the MALDI plate and left to air-dry. For this meticulous analysis, positive ion detection and reflector mode operation were engaged. The experiment maintained a constant potential acceleration at 20 kV. The mass spectrum, spanning the 900–1,500 m/z range, was meticulously observed. Each sample underwent 400 laser shots per spectrum, with the uninoculated LB medium serving as the control. The comparative analysis involved

assessing the mass of the detected molecules against the previously measured monoisotopic mass, aligning with insights gleaned from published literature.

2.7. Characterization of Crude Lipopeptides with Fourier Transform Infrared Spectroscopy (FTIR)

Employing the FTIR method delved into unraveling the overarching chemical nature of the extracted crude lipopeptides. This sophisticated technique served to scrutinize the functional groups and chemical bonds inherent in the crude extract. The investigative tool of choice was the Shimadzu FTIR spectrophotometer (Model 8400S, Tokyo, Japan). Execution of the analysis involved subjecting a sub-sample weighing 1 mg to a meticulous process with 100 mg of KBr. To achieve translucent pellets, the amalgam underwent exposure to a physical pressure of 7500 kg for a brief 30-second duration. The ensuing IR spectra were computed utilizing a PerkinElmer Spectrum version 10.4, encompassing a range of 500–4000 cm−1 wave number. This comprehensive spectrum was meticulously examined to unravel the intricate chemical nature of the bio surfactant embedded in the extracted crude lipopeptides (Varadavenkatesan and Murty, 2013).

2.8. Mortality Test

To study the nematicidal activity of lipopeptide extracted from *B. subtilis* and *P. putida*, the larval suspension containing 100 freshly hatched out J2 of *M. incognita* were taken in 5 cm diameter Petri dishes. A standard solution of 35ppm of lipopeptide was made; 3.5mg of lyophilized lipopeptide of *B. subtilis* was mixed with 100 ml of DDW separately. Then filtered through Whatman,s No-1 filter paper and the obtained filtrate was termed as 35ppm solution and diluted to 25ppm, 15ppm and 5ppm by adding required amount of DDW. For mortality test, 2mL of water suspension containing 100 J2s of M. incognita and poured in Petri plates containing 2mL of different aqueous concentrations (35ppm, 25ppm, 25ppm and 5ppm) of lipopeptide extracted from the bacterial strain. Four replicates of each treatment were taken. The Petri plates were kept at 28°C, the cripple (dead) J2s were counted under the stereoscopic binocular microscope, after every 6, 16 and 26 hours of exposure period. The mean percentage of mortality was calculated. However in control, the Petri plates containing only DW and nematode suspension (J2).

2.9. Hatching Test

In vitro experiments were conducted using Petri plates with a diameter of 5 cm, each containing 2 ml of crude lipopeptides at concentrations of 35, 25, 15, and 5 ppm. A micro-sieve, featuring a 40 µm pore nylon mesh, was carefully inserted into every plate. To initiate the process, healthy egg masses of M. incognita were sourced from infected eggplant roots. These masses were then placed in an Eppendorf tube and subjected to maceration in 1 ml of 0.5% NaOCl to release the eggs.

A total of six hundred eggs were meticulously transferred onto each micro-sieve and submerged in the respective dilutions of the crude lipopeptides. To prevent the evaporation of the product, the plates were securely covered. Randomly positioned on a tray, the experiment was conducted at a constant temperature of 28 ± 1 °C for varying exposure periods. Distilled water-filled Petri plates served as the control group. The quantification of J2 emergence from the eggs was performed at specific intervals: 6, 12, 24, 48, and 96 hours. Each treatment was replicated four times and the entire experiment

was repeated thrice. The calculation of the percentage inhibition of egg hatching was determined as in Eq. (1):

$$\text{Hatching inhibition } \% = \frac{Co - Ta}{C_0} \times 100 \quad (1)$$

where Co is the number of juveniles hatched in control, Tα is the number of J2 hatched in each concentration of lipopeptide after 6, 12, 24, 48 and 96 h of exposure.

2.10. Pot Experiment Design

Conducting a dual set of pot experiments concurrently aimed to scrutinize the nematocidal efficacy of both bacterial suspension cultures and the extracted crude lipopeptides derived from *B. subtilis* (MTCC-441) and *P. putida* (MTCC-102) against *M. incognita*. These experiments unfolded within the controlled environment of a glasshouse. The experimental setup involved the transplantation of two-week-old tomato cv. 22 seedlings into clay pots with a diameter of 15 cm, each containing 1 kg of autoclaved soil blended with organic manure in a 3:1 ratio. A single tomato transplant was sown in each pot. To ensure robust experimental design, four replicates were established for each treatment and control. The pots in both experiments were meticulously arranged following a fully randomized design and subjected to routine irrigation procedures.

SET-1:

The inoculation process involved one-week-old seedlings, and it was executed by adding the necessary amount of inoculum through four small perforations strategically placed around each plant. Sequentially, each plant underwent inoculation with *M. incognita* (2000 J2) and bacterial culture, with a volume of 10 mL per treatment. The intervals between these inoculations were systematically set at 7-day intervals:

T1 = *M. incognita* (2000J2) $\xrightarrow{after\ 1week}$ *Bacillus subtilis* (10 mL)

T2 = *M. incognita* (2000J2) $\xrightarrow{after\ 1week}$ *Pseudomonas putida* (10 mL)

T 3 = *M. incognita* (2000J2) $\xrightarrow{after\ 1week}$ *Bacillus subtilis* + *Pseudomonas putida* (10 mL)

T 4 = *Bacillus subtilis* (10 mL) $\xrightarrow{after\ 1week}$ *M. incognita* (2000J2)

T 5 = *Pseudomonas putida* (10 mL) $\xrightarrow{after\ 1week}$ *M. incognita* (2000J2)

T 6 = *Bacillus subtilis* + *Pseudomonas putida* (10 mL) $\xrightarrow{after\ 1week}$ *M. incognita* (2000J2)

T 7 = Untreated Un-inoculated (control)

T 8 = Untreated Inoculated (nematode only)

UUC: untreated uninoculated control (control), UIC: untreated inoculated control (nematodes only).

SET-2:

One week old seedlings root dip into extracted lipopeptide of 35ppm concentration of *B. subtilis* and *P. putida* respectively for 5mins before transplanting. Afterwards each plant was inoculated with *M. incognita* (2000 J2) after an interval of 1 week.

F 1 = Root dip (*Bacillus subtilis* lipopeptide) $\xrightarrow{after\ 1week}$ *M. incognita* (2000J2)

F 2 =Root dip (*Bacillus subtilis* lipopeptide) $\xrightarrow{after\ 1week}$ *M. incognita* (2000J2) $\xrightarrow{after\ 1week}$ 500µL *Bacillus subtilis* lipopeptide

F 3 = Untreated Un-inoculated (control)

F 4= Untreated Inoculated (nematode only)

UUC: untreated uninoculated control (control), UIC: untreated inoculated control (nematodes only).

2.11. Data Collection and Observations

The experiments were concluded two months post-inoculation, prompting the evaluation of data encompassing growth, biochemical, and pathological parameters. The parameters scrutinized included total length, total fresh and dry weight, chlorophyll and carotenoid content, nitrate reductase activity (NRA), egg masses per root system, root gall index (RGI), and nematode population per 250 g of soil.

Roots underwent a 15-minute staining process in an aqueous solution of Phloxine *B stain* (0.15 g/L in water), followed by thorough washing under running tap water to eliminate residual stain. The determination of egg masses ensued. To assess the population of root-knot nematodes (RKN), Cobb's sieving and decanting technique were applied, followed by the modified Baermann's funnel technique (Chaudhary et al., 2011). Nematode suspension was obtained after 72 hours, and five aliquots of 1 ml from each sample were counted using a stereomicroscope to ascertain the nematode count. The mean of four counts was employed to calculate the nematode population per 250 g of soil.

For root gall evaluation, roots were meticulously washed under flowing tap water. Root galling was quantified using the root gall index (RGI), where 0 indicated no galling, 1 denoted 1–2 galls, 2 represented 3–10 galls, 3 encompassed 11–30 galls, 4 covered 30–100 galls, and 5 signified more than 100 galls (Monfort et al., 2007).

2.12. Biochemical Parameters

2.12.1. Chlorophyll estimation

The quantification of chlorophyll content in fresh leaves followed Mackinney's method (Ali et al., 2021). To begin, one gram of finely cut fresh leaves underwent a meticulous grinding process in a mortar and pestle, immersed in 20 ml of 80% acetone. Following this, the mixture underwent centrifugation at 5000 rpm for 5 minutes, leading to the collection of the supernatant in a 100 ml volumetric flask. The residue underwent three washes with 80% acetone, with each wash contributing to the same volumetric flask. The total volume was adjusted to the mark using 80% acetone. Subsequent to these steps, absorbance readings were taken at wavelengths of 645 nm and 663 nm against a blank (80% acetone) using a spectrophotometer (U 1700, Shimadzu, Japan). The calculation of chlorophyll content within the extract (mg per gram of tissue) was executed using the Eq. (2):

$$\text{Mg total chlorophyll g - 1 tissue} = \frac{20.2\ (A645)+8.02\ (A663)}{1000\ x\ W}\ x\ V \qquad (2)$$

2.12.2. Nitrate reductase activity (NRA)

To assess the nitrate reductase activity in fresh leaves, the method outlined was employed (Martinez & Cerda, 1989). The leaves were meticulously cut into small pieces measuring 1-2 cm. A precise weight of 200 mg of these chopped leaves was then transferred to plastic vials. Each vial received 2.5 ml of phosphate buffer (pH 7.5), along with 0.5 ml of potassium nitrate solution, followed by the addition of 2.5 ml of 5% isopropanol.

These vials were subjected to a 2-hour incubation in a BOD incubator at 30 ± 2°C in the absence of light. Post-incubation, a 0.4 ml aliquot from the mixture was withdrawn into a test tube, to which 0.3 ml each of sulfanilamide solution and NED-HCL were added. The test tubes were left undisturbed for 20 minutes to allow for optimal color development. The mixture was then diluted to 5 ml using Double Distilled Water (DDW). Subsequent absorbance readings (O.D.) were taken at 540 nm using a spectrophotometer. Simultaneously, a blank was run with each sample. For calibration purposes, a standard curve was generated utilizing known graded concentrations of NaNO2 (Sodium nitrite) solution. The absorbance (O.D) of each sample was then compared with the calibration curve, and nitrate reductase activity was expressed in nm (umol per hour per gram).

2.12.3. Statistical analysis

The analysis of data was performed through the Duncan Multiple Range Test (DMRT) within the framework of a fully randomized block design, utilizing R (version 2.14.1; R Foundation for Statistical Computing, Vienna, Austria), (Duncan, 1955). To provide a measure of precision, the standard error of the mean (±SE) was computed, and the least significant difference (LSD) at a 5% significance level was also determined using R.

3. Results and Discussions

3.1. MALDI-TOF MS Analysis of the Lipopeptide Extracts of *Bacillus Subtilis*

Lipopeptide-producing *Bacillus subtilis* strain (MTCC 411) was grown under pre-established conditions in NA medium for 48 hours and their extracts obtained by ethyl acetate extraction followed by methanol extraction. The extracts were then subjected to MALDI-TOF MS analysis. The isolated dried residues were dissolved in methanol (BS100) and used for MALDI-TOF MS analysis separately. By observing spectrum, it can be seen that the detected peaks has mass in sample BS100, which is very similar to lipopeptide compound- surfactin C (m/z 1058 [M+Na] + and m/z 1074[M+K] + in positive mode. The MALDI analysis showed that the sample also contained a small amount of other lipopeptides at m/z 966 and 3422.

3.2. Root-Knot Symptoms and Morphological Assessment

Manifestations of *M. incognita* above the ground were characterized by noticeable patches in the field and compromised plant growth, presenting a stunted appearance and chlorotic leaves (Tian et al., 2018). Meanwhile, belowground symptoms were evident through the emergence of swollen and hooked root tips in affected root systems. Upon dissecting the galled roots, it was discerned that both females and males were present.

The females, exhibiting a pear-shaped form, were embedded within the cortical layer of the root. Their bodies appeared translucent white, pyroid-shaped, and varied in size. The neck was prominent and short, bent at various angles, with smaller body annuli in the anterior neck region. The perineal pattern of the females took on an oval shape, featuring dorsal round arches, smooth striate, and distinct lateral lines. Notably, egg sacs were commonly found outside the root. Detailed observations of the perineal pattern of the female were captured through scanning electron micrographs.

3.3. Effect of Aqueous Concentration of Lipopeptide-surfactin Extracted from *Bacillus Subtilis* on The Juvenile Mortality of *Meloidogyne Incognita* in Vitro

The present experiment was conducted under in vitro conditions to evaluate the nematotoxic potential of the different concentration 35ppm, 25ppm, 15ppm and 5ppm of isolated lipopeptide-surfactin from Bacillus subtilis. The nematode juveniles were exposed for 6, 16 and 26 hours in different concentrations (Table 1). In aqueous concentration of 35ppm lipopeptide-surfactin, 63% nematode mortality was observed after 26 hours of exposure period, which is maximum amongst all the treatment. However, only 53%, 42.75% and 36.75% mortality were observed after 26 hours of exposure period in 25ppm, 15ppm and 5ppm of aqueous dilution respectively. After 16 hours of exposure period 35ppm of aqueous concentration results in mortality i.e. 56.25%, while only 43.50%, 33% and 22.50% nematode mortality was observed in 25ppm, 15ppm and 5ppm respectively. Similar pattern was observed in 6 hours of exposure period where 35ppm causes 48.79% mortality while 25ppm causes 34.75%, 15ppm 23.25% and 5ppm 13%. The distilled water control group exhibited no observed nematode mortality. Consequently, the results highlight the distinct toxicity of the isolated lipopeptide-surfactin at 35 ppm compared to other concentrations. As the aqueous concentration of lipopeptide-surfactin decreased, along with the exposure period, a corresponding decrease in nematode mortality was noted. Additionally, the mortality demonstrated a direct proportionality to the concentration of the lipopeptide-surfactin extracts. Notably, a linear relationship was discerned between the aqueous dilution strength of the concentration and the percentage mortality of the nematodes, reinforcing the significance of concentration in influencing the observed outcomes.

Table 1. Effect of aqueous lipopeptide (surfactin) extract of *Bacillus subtilis* on the juvenile mortality of *Meloidogyne incognita* in vitro

Treatment	Exposure Period (Hours)	Percent mortality (Mean ± SE) in different aqueous concentrations (ppm)					Regression Equation
		DW	5	15	25	35	
Surfactin (*B. subtilis LP*)	6	0 (0.11)	13.00±1.29^{d} (12.03)	23.25±2.49^{c} (24.50)	34.75±2.62^{b} (35.87)	48.75±2.62^{a} (47.79)	y=23.95+11.92(X-2)
	16	0 (4.75)	22.50±2.10^{d} (17.70)	33.00±2.08^{c} (30.65)	43.50±2.50^{b} (43.60)	54.25±1.75^{a} (56.55)	y=30.65+12.95(X-2)
	26	0 (10.58)	36.75±1.37^{d} (24.84)	42.75±1.31^{c} (39.35)	53.00±1.58^{b} (53.36)	63.00±1.77^{a} (67.62)	y=39.35+14.26(X-2)

Each value is an average of four replicates, DW=Distilled water (control), SE= Standard error, ppm= Parts per million.
Values calculated from the regression Equation are given in parentheses.
Values are mean ± standard error of four measurements with different superscript letters within groups are significantly different ($p<0.05$).

Lipopeptide (LP) surfactin significantly killed the infective juveniles (J2) of *Meloidogyne incognita* during treatment with different aqueous concentration (ppm) at 6 hours, 16 hours and 26 hours exposure period. Obtained data indicated that among the all treatments, more J2 of *M. incognita* were killed when treated with 35ppm of surfactin and was followed by 25ppm, 15ppm and 5ppm respectively.

3.4. Effect of Aqueous Concentration of Lipopeptide-surfactin Extracted from *Bacillus Subtilis* on The Juveniles Hatching at 7th Day on *Meloidogyne Incognita* in Vitro

The study was carried out under in vitro condition to test the nematostatic potential of lipopeptide-surfactin extracted from Bacillus subtilis with different aqueous concentration viz. 35ppm, 25ppm, 15ppm and 5ppm against the hatching of second stage juveniles (J2) of *M. incognita* after 7 days.

Result presented in Table 2, clearly showed that extracted lipopepride-surfactin depicts gradual decrease in egg hatching from their lower concentration to higher concentration. This revealed that the high concentration (35ppm) of extract shows toxic effect on juvenile hatching. The results obtained from the experiment clearly reported that the maximum inhibition in hatching i.e., 86.93% was observed in 35ppm followed by 75.08% inhibition in 25ppm, 54.78% in 15ppm and minimum inhibition in 5ppm, only 27.56%. Maximum egg hatching was observed in control. Although among all the different aqueous concentrations maximum hatching was found in 5ppm of lipopepride-surfactin extracts at 7 days exposure. The juvenile (J2) hatching was inversely proportional to the concentration of extracts (35ppm, 25ppm, 15ppm and 5ppm).

Table 2. Effect of aqueous lipopeptide (surfactin) extract of *Bacillus subtilis* on egg hatching of *Meloidogyne incognita* in vitro

Treatment	Exposure Period (days)	Number of juvenile hatched (Mean ± SE) in different concentration (ppm)				
Surfactin (*B. subtilis LP*)	7	DW	5	15	25	35
		595a ±0.00 (0.00)	431.75b±8.12 (27.56)	269.00c±10.09 (54.78)	148.25d±3.49 (75.08)	77.75e±4.21 (86.93)

Each value is an average of four replicates, DW=Distilled water (control), SE= Standard error, ppm= Parts per million.
Value of percent inhibition in juvenile hatching over control are given in parenthesis.
Values are mean ± standard error of four measurements with different superscript letters within groups are significantly different ($p<0.05$).

Lipopeptide (LP) surfactin significantly affect the hatching of eggs of females of *Meloidogyne incognita* during treatment with different aqueous concentration (ppm) at 3 days, 5 days and 7 days of exposure period. Obtained data indicated that among the all treatments, least eggs of *M. Incognita* were hatched when treated with 35ppm of surfactin and was followed by 25ppm, 15ppm and 5ppm respectively.

3.5. Effect Of Bacterial Culture of *Bacillus Subtilis* and *Psuedomonas Putida* on Root-Knot Development Caused by *M. Incognita* and Plant Growth of *Lycopersicon Lycopersicum L*:

The experiment was conducted in pots to assess the effectiveness of bio-pesticides in autoclaved soil supplemented with manure, focusing on mitigating root-knot incidence

caused by *Meloidogyne incognita* and promoting the growth of tomato plants. In untreated, inoculated control pots, the root-knot nematode *M. incognita* exhibited high pathogenicity, reflected in a root-knot index (RKI) of 4.8 on a 0-5 scale.

Significant reduction in root-galling caused by *M. incognita* was observed in the T6 treatment, where a combination of 10 mL each of *Bacillus subtilis* and *Pseudomonas putida* bacterial culture was applied one week before the inoculation of J2 of *M. incognita*. In comparison to untreated, inoculated control plants, the root-knot index was notably lower at 1.2 in plants treated with T6 (refer to Table 3).

The plant length was observed highest in treatment T6 (*Bacillus subtilis* + *Pseudomonas putida* (10 mL) $\xrightarrow{after\ 1week}$ *M. incognita* (2000J2) (85.2cm), followed by T4 (*Bacillus subtilis* (10 mL) $\xrightarrow{after\ 1week}$ *M. incognita* (2000J2) with 77.1cm length, T5 (*Pseudomonas putida* (10 mL) $\xrightarrow{after\ 1week}$ *M. incognita* (2000J2) with 67.2cm,T3 (*M. incognita* (2000J2) $\xrightarrow{after\ 1week}$ *Bacillus subtilis* + *Pseudomonas putida* (10 mL) with 54.6cm, T1 (*M. incognita* (2000J2) $\xrightarrow{after\ 1week}$ *Bacillus subtilis*(10 mL) with 49.0cm and lowest amongst the treatment in T2 *M. incognita* (2000J2) $\xrightarrow{after\ 1week}$ *Pseudomonas putida* (10 mL) with 44.7cm total length only. The untreated inoculated control plants has total height of only 36.4cm compared to the untreated un-inoculated control plant with maximum height of 98.3 cm.

The total fresh weight was observed highest in treatment T6 (87.37g), followed by T4 (80.17g), T5 (73.75g), T3 (62.05g), T1 (55.32g) and lowest amongst the treatment in T2 with 44.22g total fresh weight only. The untreated inoculated control plants has total fresh weight of only 35.02g compared to the untreated un-inoculated control plant with total fresh weight of 100.22g.

Total dry weight was observed highest in treatment T6 (24.95g), followed by T4 (21.67g), T5 (18.15g), T3 (13.97g), T1 (12.10g) and lowest amongst the treatment in T2 (10.17g) total dry weight only, untreated inoculated control plants has total dry weight of only 6.35g compared to the untreated un-inoculated control plant with total fresh weight of 29.02g. The chlorophyll content was observed highest in treatment T6 (2.16mg/g), followed by T4 (1.89mg/g), T5 (1.76mg/g), T3 (1.54mg/g), T1 (1.39mg/g) and least amongst the treatment in T2 with 1.15mg/g total chlorophyll content. The untreated inoculated control plants has total chlorophyll content of only 1.04mg/g compared to the untreated un-inoculated control plant with total chlorophyll content of 2.53mg/g.

The carotenoid content was observed highest in treatment T6 (0.81mg/g), followed by T4 (0.65mg/g), T5 (0.50mg/g), T3 (0.36mg/g), T1 (0.33mg/g) and least amongst the treatment in T2 with 0.28mg/g carotenoids content. The untreated inoculated control plants has carotenoids content of only 0.20mg/g compared to the untreated un-inoculated control plant with carotenoids content of 0.96 mg/g. The nitrate reductase activity (NRA) was also observed highest in treatment T6 (0.415 µmh-1g-1), followed by T4 (0.362 µmh-1g-1), T5 (0.281 µmh-1g-1), T3 (0.242 µmh-1g-1), T1 (0.216 µmh-1g-1) and least amongst the treatment in T2 with 0.186µmh-1g-1 nitrate reductase activity. The untreated inoculated control plants have NRA content of only 0.152 µmh-1g-1 compared to the untreated un-inoculated control plant with NRA content of 0.489 µmh-1g-1 (Table 4).

Table 3. (SET-1) Effect of bacterial culture (10mL) of *Bacillus subtilis* and *Psuedomonas putida* on the root-knot development caused by *Meloidogyne incognita* in relation to plant growth of tomato plants in pots

Parameters	Length(cm)		Total length (cm)	Fresh weight (g)		Total fresh weight (g)	Dry weight (gm)		Total dry weight (g)
Treatment	Shoot	Root		Shoot	Root		Shoot	Root	
T1	32.6ef ±1.4	16.3de ±0.8	49.9f±1.2	41.90f ±1.60	13.42de ±0.71	55.32f±1.44	9.37f ±0.26	2.72de ±0.21	12.10f±0.17
T2	30.0f ±0.9	14.7e ±0.6	44.7f±0.4	33.17g ±1.06	11.05ef ±0.66	44.22g±1.06	8.10g ±0.15	2.07ef ±0.22	10.17g±0.35
T3	35.7e ±1.1	18.9d ±1.4	54.6e±1.2	47.85e ±1.08	14.20cd ±0.64	62.05e±0.81	11.05e ±0.45	2.92d ±0.25	13.97e±0.35
T4	53.6c ±1.2	23.5bc ±1.1	77.1c±2.3	63.45c ±1.33	16.72c ±0.51	80.17c±1.73	17.62c ±0.65	4.05c ±0.22	21.67c±0.75
T5	47.4d ±1.3	19.7cd ±1.2	67.2d±1.2	58.47d ±0.81	15.27cd ±1.17	73.75d±0.91	14.65d ±0.55	3.50cd ±0.42	18.15d±0.54
T6	58.0b ±0.8	27.2b ±1.3	85.2b±0.5	67.35b ±0.58	20.02b ±1.19	87.37b±1.13	19.80b ±0.46	5.15b ±0.30	24.95b±0.47
UUC	63.8a ±1.4	34.4a ±2.5	98.3a±3.8	75.00a ±0.79	25.22a ±1.13	100.22a±1.11	22.52a ±0.28	6.50a ±0.25	29.02a±0.24
UIC	23.9g ±1.2	12.5e ±0.6	36.4g±1.6	25.22h ±1.82	9.80f ±0.88	35.025h±2.22	4.80h ±0.30	1.55f ±0.17	6.35h±0.29

Table 4. (SET-1) Effect of bacterial culture (10mL) of *Bacillus subtilis* and *Psuedomonas putida* on the root-knot development caused by *Meloidogyne incognita* in relation to biochemical and pathological parameters in pots

Parameters / Treatments	Chlorophyll content (mg g-1)	Carotenoid content (mg g-1)	NRA (µmh-1g-1)	Nematode Population / 250 g soil	Root-knot index (RKI)
T1	1.39f±0.00	0.33e±0.01	0.216e±0.005	1006bc±96.0	2.8b±0.15
T2	1.15g±0.02	0.28ef±0.00	0.186f±0.003	1123b±42.73	3.2b±0.17
T3	1.54e±0.01	0.36e±0.02	0.242e±0.007	976bc±53.48	2.2c±0.15
T4	1.89c±0.03	0.65c±0.06	0.362c±0.005	789d±42.09	1.7d±0.17
T5	1.76d±0.04	0.50d±0.03	0.281d±0.014	880cd±46.50	2.0cd±0.20
T6	2.16b±0.02	0.81b±0.03	0.415b±0.009	566e±46.86	1.2e±0.15
UUC	2.53a±0.04	0.96a±0.00	0.489a±0.003	0f±0	0f±0
UIC	1.04h±0.05	0.20f±0.00	0.152g±0.017	2865a±0	4.8a±0

3.6. Effect of Lipopeptide-surfactin (35ppm) from *Bacillus Subtilis* on Root-knot Development Caused by *Meloidogyne Incognita* and Plant Growth of *Lycopersicon Lycopersicum L*

The experiment, conducted in pots, aimed to assess the effectiveness of extracted lipopeptide-surfactin in autoclaved soil supplemented with manure. The focus was on mitigating the root-knot incidence caused by Meloidogyne incognita and promoting the growth of tomato plants. In untreated, inoculated control pots, the root-knot nematode *M. incognita* exhibited high pathogenicity, resulting in a root-knot index (RKI) of 4.8 on a 0-5 scale. There was a significant reduction in root-galling caused by *M. incognita* in treatments of F2, where at the time of transplantation, 1 week old seedling was root dip in 35ppm aqueous extract of *Bacillus subtilis* lipopeptide-surfactin for 5min, then after 1 week 2000J2 were inoculated and then again after 1 week of inoculation 500µL of 35ppm aqueous extract of *Bacillus subtilis* lipopeptide-surfactin were inoculated in the

rhizopheric region of roots. Compared to untreated inoculated control plants, the root-knot index was only 1.2 in plants treated with T6 (Table 5).

The plant length was observed highest in treatment F2 (Root dip *Bacillus subtilis* lipopeptide) $\xrightarrow{after\ 1week}$ *M. incognita* (2000J2) $\xrightarrow{after\ 1week}$ 500µL *Bacillus subtilis* lipopeptide) having 71.2cm of total length, followed by F1 (Root dip *Bacillus subtilis* lipopeptide) $\xrightarrow{after\ 1week}$ *M. incognita* (2000J2) with 55.6cm.The untreated inoculated control plants has total height of only 36.4cm compared to the untreated un-inoculated control plant with maximum height of 98.3 cm.

The total fresh weight was observed highest in treatment F2 (79.80g), followed by F1 (62.95g). The untreated inoculated control plants has total fresh weight of only 35.02g compared to the untreated un-inoculated control plant with total fresh weight of 100.22g (Table 5).

The total dry weight was observed highest in treatment F2 (23.02g), followed by F1 (14.80g). The untreated inoculated control plants has total dry weight of only 6.35g compared to the untreated un-inoculated control plant with total fresh weight of 29.02g (Table 5).

The chlorophyll content was observed highest in treatment F2 (2.02mg/g), followed by F1 (1.65mg/g). The untreated inoculated control plants has total chlorophyll content of only 1.04mg/g compared to the untreated un-inoculated control plant with total chlorophyll content of 2.53mg/g. The carotenoid content was observed highest in treatment F2 (0.79mg/g), followed by F1 (0.36 mg/g).The untreated inoculated control plants has carotenoids content of only 0.20mg/g compared to the untreated un-inoculated control plant with carotenoids content of 0.96 mg/g (Table 6).The nitrate reductase activity (NRA) was also observed highest in treatment F2 (0.391 µmh-1g-1), followed by F1 (0.270 µmh-1g-1.The untreated inoculated control plants has NRA content of only 0.152 µmh-1g-1 compared to the untreated un-inoculated control plant with NRA content of 0.489 µmh-1g-1 (Table 6).

Table 5. (SET 2) Effect of extracted lipopeptide (35ppm) from *Bacillus subtilis* on the root-knot development caused by *M. incognita* in relation to plant growth of tomato plants in pots

Parameters	Length(cm)		Total length (cm)	Fresh weight (g)		Total fresh weight (g)	Dry weight (gm)		Total dry weight (g)
Treatment	Shoot	Root		Shoot	Root		Shoot	Root	
F1	37.9c ±1.1	17.7b ±0.5	55.6c±1.5	48.10c ±0.99	11.85c ±0.61	62.95c±0.71	11.52c ±0.63	3.27c ±0.26	14.80c±0.53
F2	52.7b± 0.8	18.5b ±0.8	71.2b±1.5	61.85b ±1.32	17.95b ±0.83	79.80b±0.75	18.55b ±0.75	4.47b ±0.29	23.02b±0.53
UUC	63.8a ±1.4	34.4a ±2.5	98.3a±3.8	75.00a ±0.79	25.22a ±1.13	100.22a±1.11	22.52a ±0.28	6.50a ±0.25	29.02a±0.24
UIC	23.9d± 1.2	12.5c ±0.6	36.4d±1.6	25.22d ±1.82	9.80d ±0.88	35.025d±2.22	4.80d ±0.30	1.55d ±0.17	6.35d±0.29

Table 6. (SET-2) Effect of extracted lipopeptide (35ppm) from *Bacillus subtilis* on the root-knot development caused by *M. Incognita* in relation to biochemical and pathological parameters in pots

Parameter / Treatment	Chlorophyll Content (mg g-1)	Carotenoid Content (mg g-1)	NRA (µmh-1g-1)	Nematode Population/250 g soil	Root Gall Index (RGI)
F1	1.65e±0.02	0.36d±0.02	0.270e±0.006	811b±40.25	2.0b±0.15
F2	2.02c±0.03	0.79b±0.00	0.391c±0.006	564c±35.13	1.4c±0.20
UUC	2.53a±0.04	0.96a±0.00	0.489a±0.003	0d±0	0d±0
UIC	1.04g±0.05	0.20e±0.00	0.152h±0.017	2865a±77.25	5a±0

3.7. Discussion of Results

Our MALDI-TOF MS analysis seems consistent with other studies (Earl et al., 2008) on lipopeptides from Bacillus subtilis. The detection of surfactin is well-documented and aligns with its known mass/charge ratios. The presence of other lipopeptides also suggests a complex mixture, which is expected given the diversity of lipopeptides produced by Bacillus subtilis.

The symptoms and nematode morphology that we described are typical for root-knot nematode infestations and are consistent with those documented in the literature, such as in Tian et al. (2018). Our detailed description supports standard diagnostic criteria for root-knot nematodes.

The results indicate a dose-dependent response to surfactin, consistent with known properties of lipopeptides where higher concentrations generally lead to greater nematode mortality. This finding supports the use of surfactin as an effective nematicide, aligning with other research (Khan et al., 2024) on lipopeptide efficacy against nematodes.

The pattern of inhibition with increasing concentration is consistent with expected outcomes for nematicidal compounds. High concentrations leading to more significant effects is a common trend observed in similar studies (Walia and Khan, 2023).

The effectiveness of Bacillus subtilis and Pseudomonas putida treatments in reducing nematode impact and promoting plant growth is well-supported by the literature (Varadavenkatesan and Murty, 2013; Shafi et al., 2017; Khan and Siddiqui, 2019). The observed improvements in plant growth and reduction in nematode damage with combined bacterial treatments reflect similar findings in other studies, underscoring the potential of biocontrol agents in managing nematode infestations.

The results confirm that surfactin can mitigate root-knot damage and enhance plant growth, aligning with its known biological activity. The improvement in plant health and reduction in nematode damage with surfactin treatment is consistent with its role as a nematicide and plant growth enhancer (Tian et al., 2018).

The results from our experiments align with established findings on the effects of lipopeptides and biocontrol agents against nematodes. The MALDI-TOF MS data confirm the presence of surfactin and other lipopeptides, while the biological assays demonstrate effective nematode control and plant growth promotion. These findings support the potential application of surfactin and bacterial cultures as part of integrated pest management strategies.

4. Conclusion

The present research was carried out to envisage the results of experiments performed under in vitro and glasshouse conditions to evaluate the effect of bacterial culture of *Bacillus subtilis* and *Psuedomonas putida* and lipopeptide-surfactin of *B. subtilis* against root-knot nematode, *M. incognita* in Tomato cv. 'S-22'. During in vitro studies, the aqueous extracts of different concentrations of lipopeptide-surfactin of *B. subtilis* of were found to be toxic to root-knot nematode egg and juveniles. Aqueous dilution of 35ppm was found to be highly deleterious to the root-knot nematode, *M. incognita*. It was followed by 25ppm, 15ppm and 5ppm. These aqueous dilutions also inhibited the larval hatching of *M. incognita* and the inhibition in hatching was increased with an increase in concentration of the lipopeptide surfactin. Maximum inhibition in egg hatching was observed in 35ppm while 5ppm shows least toxicity. There was a direct relationship between mortality of root-knot nematode and the exposure period. The mortality increased with an increase in the concentration of the lipopeptide-surfactin and exposure period. Highest concentration (35ppm) resulted in to significantly highest mortality rate of *M. incognita* at 26 hours compare to distilled water control (DW). In green-house experiment most of the treatments were highly effective in reducing the population densities and reproduction potential of root-knot nematode and root disease caused by *M. incognita*. Treatment of combination of bacterial culture of *Bacillus subtilis* and *Psuedomonas putida* was found highly effective in reducing root-knot development caused by *M. incognita* on Tomato. Maximum inhibition in root-knot development was observed in the plants treated with combination of bacterial culture of *Bacillus subtilis* and *Psuedomonas putida* and lowest in the plants treated with *Psuedomonas putida* after nematode inoculation. As a result of declined in root-knot development the plant growth in terms of length (cm), fresh weight (g), dry weight (g), and chlorophyll content, carotenoid content, Nitrate reductase activity were increased. Our results indicated that Bacillus subtilis and *Psuedomonas putida* and lipopeptide-surfactin of *B. subtilis* might be a promising biocontrol option for an effective and environmentally friendly control of plant pathogenic agents. Thus, surfactin based lipopeptides could be defined as a novel potential products to explore for use as biocontrol agents against plant disease.

Conflict of Interest

The authors declare no conflict of interest.

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