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SOIL AND AGRICULTURAL MICROBIOLOGY - RESEARCH PAPER

In vitro nematicidal and acaricidal effect of biosurfactants produced by *Bacillus* against the root-knot nematode *Nacobbus aberrans* and the dust mite *Tyrophagus putrescentiae*

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Abstract

In the present study, the nematicidal and acaricidal activity of three biosurfactants (BS) produced by strains of the *Bacillus* genus was evaluated. The BS produced by the *Bacillus* ROSS2 strain presented a mortality of 39.29% in juveniles (J2) of *Nacobbus aberrans* at a concentration of 30 mg/mL, this same strain is the one that presented the highest mortality in *Tyrophagus putrescentiae*, which was 57.97% at a concentration of 39 mg/mL. The BS were qualitatively identified by thin layer chromatography and are lipid in nature based on the retention factor (Rf). While the GC-MS analysis identified two main compounds that are 4,7-Methano-1H-indene-2,6-dicarboxylic acid, 3a,4,7,7a-tetrahydro-1, and Methyl 4-(pyrrol-1-yl)-1,2,5-oxadiazole-3-carboxylate1, which is the polar part indicated by the presence of dicarboxylic acid and carboxylate groups; while the non-polar portion can be interpreted as a hydrocarbon chain of variable length. Based on the present results, BS can be an alternative for the biocontrol of the root-knot nematode *N. aberrans* and the mite *T. putrescentiae*.

Keywords Biosurfactants · Phytonematodes · Dust mites · Sustainable alternatives · Agroecology

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Introduction

National and global agricultural pests caused by phytopathogenic nematodes and mites cause economic losses of 33 to 65% in economically important crops [1]. Among the phytopathogenic nematodes, there are extremely aggressive species, such as *Pratylenchus* spp., *Ditylenchus* spp. and *Psilenchus* spp., the root-knot *Meloidogyne* sp. and false root-knot *Nacobbus aberrans*. In Mexico, the false root-knot nematode *N. aberrans* causes important losses in the production of numerous crops, mainly potatoes (*Solanum tuberosum*), tomatoes (*Solanum lycopersicum*), chili peppers (*Capsicum annum*), and beans (*Phaseolus vulgaris*), to name a few. The nematode *N. aberrans* has a wide host range; it parasitizes 84 species of cultivated plants and weeds, belonging to 18 families [2].

Another organism that causes extensive damage and loss in food products of plant and animal origin is the dust mite *Tyrophagus putrescentiae*. Additionally, in the body they transport bacteria and fungi such as *Klebsiella* spp., *Staphylococcus*, and *Candida albicans* [3–7].

Studies relate the mite *T. putrescentiae* with the induction of anaphylactic reactions and asthmatic crisis, due to bronchospasm. The *T. putrescentiae* mite is classified as a vector of pathogenic bacteria of *Klebsiella* spp., *Candida albicans*, and the genus *Staphylococcus* [6, 7]. To control populations of pathogenic mites and parasitic nematodes, pesticides of synthetic origin are used, which cause damage to beneficial organisms to the soil, environment, and human health [8]. The chemicals used to control parasitic nematodes and pathogenic mites are Ivermectin, Chitosan (Poly-D-glucosamine equivalent to 78.3 g chitosan/l), Fenamiphos (ethyl 4-methylthio-m-tolyl isopropylphosphoramidate), Pyre-thrins: (Z)-(S)-2-methyl-4-oxo-3(penta-2,4dienyl)cyclopent-2-enyl(1R,3R)-2,2-dimethyl-3-(2methylprop-1-enyl) cyclopropanecarboxylate [9-11]. However, nematodes and mites have developed resistance to pesticides and anthelmintics, which has led to urgent interest in seeking sustainable actions to mitigate environmental impact.

Among the options is the use of secondary metabolites, which can be produced by different types of organisms and microorganisms. One of these alternatives is biosurfactants (BS), which have great potential for the biological control of phytopathogenic fungi.

The study of biosurfactants has increased considerably in the last 2 decades [12, 13]. The production of biosurfactants in freshwater and marine microorganisms has been reported [14, 15]. Patents on biosurfactants have since increased exponentially from around 250 in 2006 to more than 850 in 2019. However, the industrial production of these metabolites has a high cost in the bacterial culture, production, purification, and recovery processes. Biosurfactants have been reported to have antifungicidal, antibacterial, algicidal, antiviral, and zoosporicidal activity [16].

Some BS formed by rhamnolipids alter the zoospore membrane, causing the lysis of phytopathogenic Oomycetes ([17-19]) [7, 20, 21]. Hussain et al. [22] reported that the biosurfactant produced by *Bacillus subtilis* HussainT-AMU presents a 90% mortality of J2 of *M. incognita* at 24 h, as well as a decrease in the number of galls in the roots of tomato plants. Biosurfactants such as ranmolipids produced by *Pseudomonas* sp. EP-3, produce 80% mortality in *Myzus persicae* aphids through damage to the cuticle membrane [23] de Andrade et al. [8] reported that the biosurfactant produced by *Wickerhamomyces anomalus* has the ability to biocontrol *Aedes aegypti* larvae, a vector that causes the transmission of diseases of public health importance at the global and national levels, such as: dengue, zika, malaria, and chikungunya fever. The objective of this research was to evaluate the nematicidal effect on *N. aberrans* (J2) and the acaricidal effect on *T. putrescentiae* of BS produced by three strains of the genus *Bacillus.*

Materials and methods

Location

The present study was carried out at the National Center for Disciplinary Research in Animal Health and Safety (CENID-SAI) of INIFAP, located in Jiutepec, Morelos, Mexico, and at the Universidad Tecnológica de la Selva, located in Ocosingo, Chiapas, Mexico.

Isolation of microorganisms

The bacterial strains used in this study were isolated from a pilot-scale anaerobic reactor for the treatment of agro industrial waste in Tuxtla Gutierrez Chiapas, México. Bacterial strains were selected by culturing in blue methylene agar added with 2% waste cooking oil after an incubation period, bacteria showed whitish halo around colony growth as evidence of anionic biosurfactant production. The bacterial strains were stored on glycerol at −80 °C.

Nacobbus aberrans

The inoculum of *N. aberrans* was obtained from the galled roots of tomato plants (*Solanum lycopersicum*). They were collected at the Colegio de Postgraduados, Campus de Montecillo, State of Mexico, Mexico, the population was established from a single mass of eggs, which were extracted

according to the method described by Vrain [24] and incubated at 27 °C in Petri dishes with sterile distilled water, until hatching and obtaining second stage juveniles (J2) according to [25].

Tyrophagus putrescentiae

Populations of the *T. putrescentiae* mite were used, whose breeding stock is found in the CENID-SAI, INIFAP. The specimens were isolated in 2013 in the town of San Juan Tlacotenco, Morelos, Mexico. Currently, they are kept incubated in Petri dishes with 5% agar-agar at room temperature. The mites feed on *P. redivivus* (Goodey) as their main food source to facilitate their reproduction and increase their populations. New specimens are transferred every 2 weeks to plates with sterile agar in order to obtain monocultures. Mite cultures were kept at room temperature (28 ± 2 °C) under dark conditions [26].

Panagrellus redivivus

The nematodes were cultivated on oatmeal-water medium contained in plastic containers covered with a porous cloth to allow aeration and prevent the entry of insects. The medium was prepared according to the method used by De Lara et al. [27]. The containers were kept at room temperature 28 ± 2 °C [26]. The *P. redivivus* nematodes were provided by the (CENID-SAI, INIFAP).

Production and extraction of biosurfactants

Bacillus ROSS2, *Bacillus* ROSS4, and *Bacillus* ROSS 2214 strains were grown in liquid mineral salt medium with 2% soybean oil as the only carbon source, for the production of BS. It was incubated for 8 days at 28 °C, with orbital shaking at 250 rpm. After this period, the cultures were centrifuged at 3500 × g for 20 min. in a SOLBAT j-40 centrifuge. BS extraction was performed by acid precipitation, for which cell-free supernatants were acidified to pH 2 with 2N HCl for 8 h at 4 °C. It was centrifuged at 3500 × g for 20 min. the precipitate obtained was stored until use.

Sequencing of the 16S rRNA gene

Molecular identification of the three BS-producing strains was performed. Genomic DNA from each BS-producing bacterial strain was extracted using the ZR Fungal/Bacterial DNA Kit™. The 16S ribosomal gene was amplified using oligonucleotides rD1 and fD1 and the conditions described by Weisburg et al. [28]. The amplification products were purified from the gel using the GeneJET kit (Thermo Scientific) and sequenced at the Institute of Biotechnology of the National Autonomous University of Mexico (IBT, UNAM).

The sequences obtained were deposited in the GenBank of the National Center for Biotechnology Information (NCBI) with the access numbers: (1) ROSS2 (ON740901), (2) ROSS4 (ON740900), and (3) ROSS2214 (ON740899). 16S rDNA sequences were compared to 16S rDNA genes from the GenBank database using BlastN. The phylogenetic analysis of the matrix was carried out with the Kimura 2-parameter model with 1000 bootstrap in the seaview 4.6.1 program [29].

Thin-layer chromatography characterization of biosurfactant produced by bacterial strains

BS extracted from cell-free supernatants (CFS) were concentrated and analyzed by thin layer chromatography (TLC) on silica gel plates (G60; Merck, Germany). Chromatograms were developed with chloroform: methanol: acetic acid (65:15:2, v/v) as mobile phase. Lipids detection was performed by exposure to iodine vapor, saccharides detection by exposure to the diphenylamine reagent. The reagents were sprayed, and the plates were heated at 105 °C for 10 to 20 min until the appearance of respective colors [30, 31]. The *Rf* values were calculated, which are the mean distance traveled by the biosurfactant, divided by the distance traveled by the solvent from the origin.

Biosurfactants characterization by GC-MS analysis

Analysis of gas-mass chromatography was performed on a Thermo Scientific brand TRACE GC with an ITQ900 ion trap mass detector (Thermo Electron Corporation, Milan, Italy). The carrier gas was helium with a flow of 1 mL/min. The column used was TRACE-5MS (30 m, 0.25 µm of film, and 0.25 mm internal diameter). The GC was equipped with a split-splitless injector that was held at 270 °C. In method the oven was programed as follows: initially 50 °C by 1 min, then the temperature was ramped in three steps: from 50 to 300 °C at 7 °C/ min and finally held at 300 °C by 5 min, giving a total chromatographic time of 56 min. The mass spectra were acquired by electron impact ionization at 70 eV and the detector was set in TIC/Scan (Total Ion Current) mode from 50 to 650 m/z with 0.2 scan s-1 (Dwell) of scanning rate. The transfer line and ion source temperatures were held at 270 and 200 °C respectively. The raw data were processed using Xcalibur™ software version 4.0 (Thermo Scientific, USA).

In vitro evaluation of biosurfactants against *Nacobbus aberrans* (J2)

The BS produced by the *Bacillus* ROSS2, *Bacillus* ROSS4, and *Bacillus* ROSS 2214 strains at different concentrations (10, 20, and 30 mg/mL) were evaluated. A control group (organic nematicide: Nematrol Plus at 6 mg/mL) was used. For the in vitro tests, 96-well microtiter plates were used,

in each well 50 µL of distilled water containing 100 J2 and 50 µL of each of the treatments (*Bacillus* ROSS2, *Bacillus* ROSS4, and *Bacillus* ROSS 2214) were mixed, each one of the control and treatment groups consisted of four repetitions, respectively. Subsequently, they were incubated in a humid chamber at 28 °C; after 72 h, the mortality of the J2 was evaluated, adding 10 µL of NaOH at 1N to stimulate the larvae and the response to the chemical stimulus was observed under a 4X microscope, those nematodes that did not present motility were considered dead [32].

The percentage results of the different bioassays and mortality experiments were estimated based on the following formula [33]:

% Mortality = (treated group-negative control)/ (100-negative control) ×100

In vitro evaluation of biosurfactants against the dust mite *Tyrophagus putrescentiae*

BS obtained from *Bacillus* ROSS2, *Bacillus* ROSS4, and *Bacillus* ROSS 2214 were evaluated against *T. putrescentiae*. For each BS, the following concentrations were evaluated: 39, 40 and 60 mg/mL, with four repetitions respectively, in the same way, two control groups were established: (1) Ivermectin® at 5 mg/mL and (2) distilled water as a negative control. The in vitro test was performed in 24-well plates, ten mites with 50 µL of BS were added to each well. Subsequently, they were incubated in a humid chamber at 28 °C; after 72 h, the readings were made with a stereoscope, the mites without activity were considered dead.

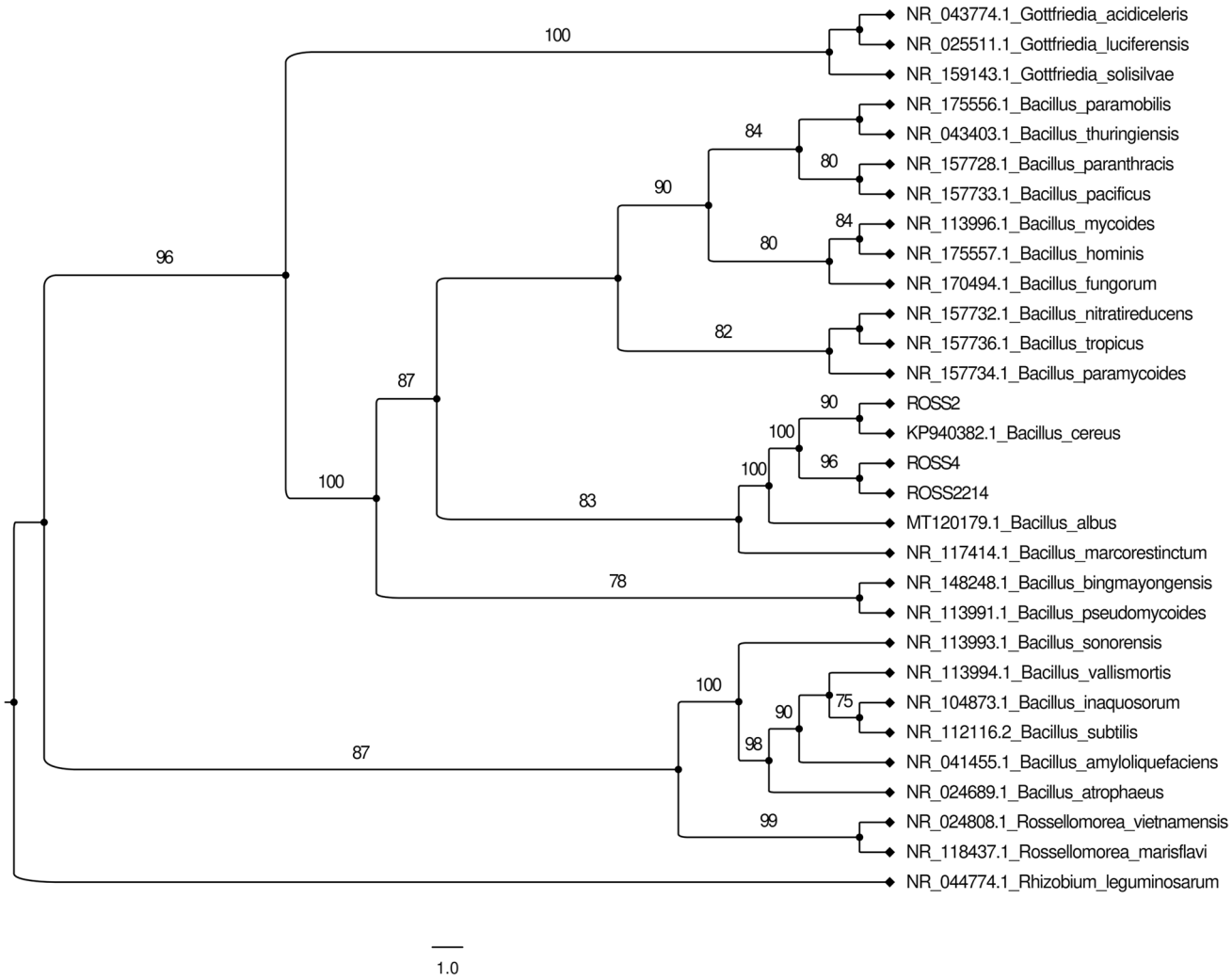

Fig. 1 Sequence-based phylogenetic analysis of the 16S rDNA gene of isolates capable of producing biosurfactants. Accession numbers of reference sequences are shown before the species name

Statistical analysis

Mortality data for nematodes *N. aberrans* (J2) and mites *T. putrescentiae* were subjected to normalization using the square root of arcsine transformation and analyzed as a completely randomized design. The means were compared a posteriori using a Tukey test, considering a value of statistical significance of $\alpha = 0.05$. The tests were analyzed using R software version 4.1.1 [34].

Results

Identification molecular

The 16S ribosomal gene sequences of the three strains were analyzed by the Blast algorithm, where it was observed that the ROSS2, ROSS4, and ROSS2214 strains have a high similarity with species of the *Bacillus* genus (Fig. 1). The phylogenetic tree was constructed using fragments of the 16S ribosomal gene sequences to confirm the identity of the isolates at the genus level. The cladogram shows that ROSS2, ROSS4, and ROSS2214 strains are probably related to *Bacillus cereus* and *Bacillus albus* (Fig. 1).

In vitro evaluation of biosurfactants against *Nacobbus aberrans*

The results of the in vitro evaluation against *N. aberrans* (J2) are shown in Table 1. They present a range of 16.66% *Bacillus* (ROSS2214) to 39.29% *Bacillus* (ROSS2) from a concentration of 10 to 30 mg/mL, it is important to point out that the activity of BS depends on the concentration of the strains evaluated. We can also observe that at a concentration of 30 mg/mL, the BS produced by *Bacillus* (ROSS2) presented a J2 mortality of 39.29%, while the BS from *Bacillus* ROSS4 and *Bacillus* (ROSS2214) presented a mortality of 34.06 and 25.83%, respectively. As can be seen in Table 1, at concentrations of 10 and 20 mg/mL the BS of the three *Bacillus* strains were lower than the concentration of 30 mg/mL.

In vitro evaluation of biosurfactants against *T. putrescentiae*

In the in vitro evaluation against the dust mite *T. putrescentiae*, the concentration that presented the greatest activity in the three *Bacillus* strains was selected, in the bioassay against the nematode *N. aberrans* and the concentration of these strains was increased to 39, 40 and 60mg/mL. Table 2 shows that the BS produced by the *Bacillus* ROSS2 strain at a concentration of 39 mg/mL, a mortality of 57.97% was obtained, the BS of the *Bacillus* ROSS2214 strain at a concentration of 40 mg/mL was a mortality of 16.60% was obtained and the BS produced by the *Bacillus* ROSS4 strain at a concentration of 60 mg/mL a mortality of 33.25% was obtained.

Table 1 In vitro evaluation of the biosurfactants produced by the *Bacillus* strains at different concentrations against the root-knot nematode *Nacobbus aberrans* (J2)

Strains	Concentration mg/mL	% Mortality
Bacillus ROSS2	30	39.29 ± 18.36^{b}
Bacillus ROSS4		34.06 ± 10.92^{bc}
Bacillus ROSS2214		25.83 ± 4.38^{cd}
Bacillus ROSS2	20	33.35 ± 12.27^{bc}
Bacillus ROSS4		30.16 ± 8.95^{bcd}
Bacillus ROSS2214		25.43 ± 5.46^{cd}
Bacillus ROSS2	10	20.08 ± 5.71^{cd}
Bacillus ROSS4		16.76 ± 8.01^{d}
Bacillus ROSS2214		16.66 ± 11.02^{d}
Water	----	2.54 ± 0.77^{e}
Nematrol plus	6	90.24 ± 6.72^{a}

$n = 4$. Average with different letters indicates that there are significant differences (Tukey, $P < 0.05$)

Thin-layer chromatography characterization of biosurfactant produced by bacterial strains

TLC is a study method for the qualitative determination biochemical composition of BS. TLC analysis of BS extracted from cell free supernatants of the four bacterial strains cultured in medium with soybean oil 2 % as only carbon source is shown in Fig. 2, spots detected have the same mobility for a positive lipids detection [35, 36], no pink spots were detected when the TLC plate was treated with diphenylamine, indicating lack of carbohydrates.

In Table 3, reported data on *Rf* are calculated for each BS extracted. The *Rf* value from BS produced by bacterial strain 4 is similar to *Rf* values (0.18, 0.39) reported by Kuyukina et al. [37], indicating presence of neutral and polar lipids in the crude biosurfactants composition produced by

Table 2 In vitro evaluation of biosurfactants produced by *Bacillus* strains against the dust mite *T. putrescentiae*

Strains	Concentration (mg/mL)	% Mortality
Bacillus ROSS2	39	57.95 ± 7.25^{a}
Bacillus ROSS4	60	33.25 ± 23.50^{ab}
Bacillus ROSS2214	40	16.60 ± 16.16^{b}
Water	----	2.20 ± 4.40^{b}
Ivermectin	5	95.0 ± 10^{a}

$n = 4$. Average with different letters indicates that there are significant differences (Tukey, $P < 0.05$)

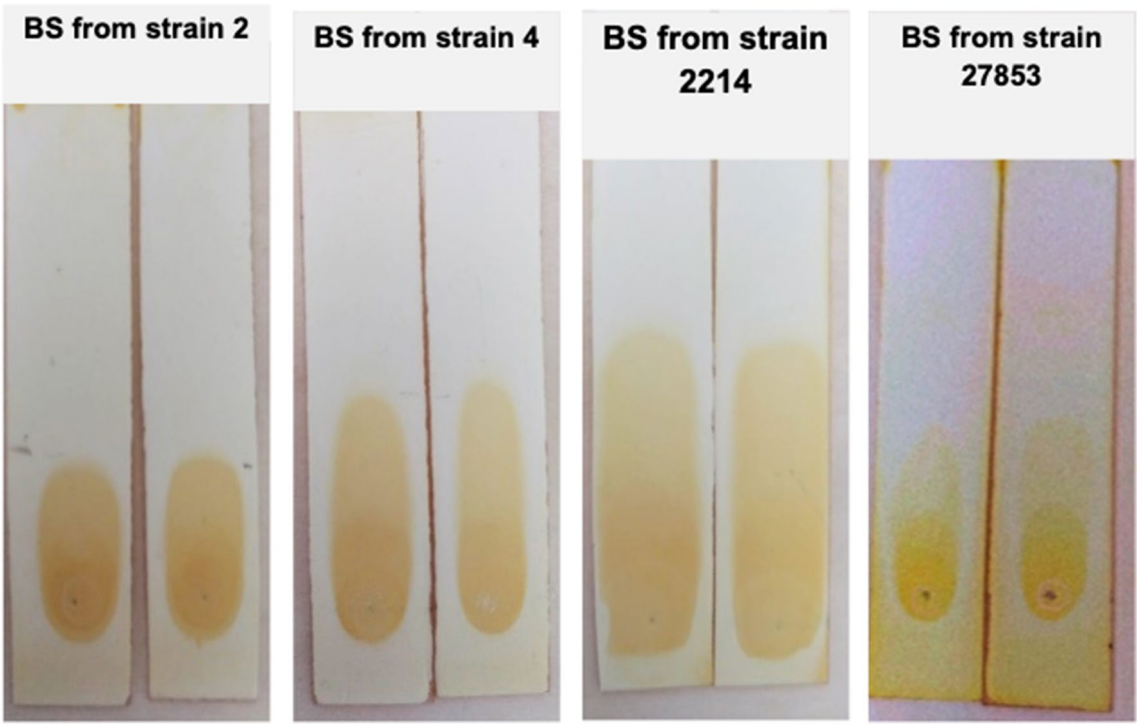

Fig. 2 TLC analysis of biosurfactants produced by *Bacillus* strains ROSS2, ROSS 4, ROSS 2214, and *P. aeruginosa* ATCC27853 as positive control

Rhodococcus ruber. The *Rf* = 0.26 correspond to di-rhamnolipid, while the *Rf* = 0.47 comes from phosphatidylethanolamines [38].

Biosurfactant characterization by GC-MS

The biosurfactants produced by the ROSS2, ROSS4, and ROSS2214 strains were extracted and analyzed by GC-MS. In Fig. 3, chromatograms of crude biosurfactant representative for each strain are presented. Two main signals with high abundance were detected at 7.6- and 10.4-min RT.

The isoform chemical of crude biosurfactants extracted from the cultures after 18 days of incubation is reported in Table 4, which revealed the presence of two metabolites having retention times at 10.4 and 7.5 min, molecular weight 220 and 193. The metabolite Methyl 4-(pyrrol-1-yl)-1,2,5-oxadiazole-3-carboxylate, was found only in BS produced by bacterial strain ROSS2.

Discussion

In the present study, the nematicidal and acaricidal activity of BS produced by the *Bacillus* ROSS2, *Bacillus* ROSS4, and *Bacillus* ROSS2214 strains was evaluated. The strains were identified by 16S ribosomal gene sequence and are related to *B. cereus* and *B. albus* species (Fig. 1). The BS produced by the strains are of a lipid nature, this can be established based on the results of the retardation factor (*Rf*) obtained in thin layer chromatography, this reference value corresponds to lipids, as reported by Ibrahim et al. [35]; Ismail et al. [36]. Tuleva et al. [39] reported the production of a BS by the *B. cereus* 28BN strain whose part of its structure contains a rhamnose. Durval et al. [40] also reported a BS of a lipopeptide chemical nature, produced by the *B. cereus* UCP1615 strain. The different biosurfactants that have been reported are mainly related to bioremediation processes.

Table 3 *Rf* calculated by thin layer chromatography (TLC) of the biosurfactants produced by the *Bacillus* strains

BS produced from bacterial strain	R_f
Bacillus ROSS2	0.29
Bacillus ROSS2	0.26
Bacillus ROSS4	0.36
Bacillus ROSS4	0.39
Bacillus ROSS2214	0.46
Bacillus ROSS2214	0.51
P. aeruginosa ATCC27853	0.45
P. aeruginosa ATCC 27853	0.42

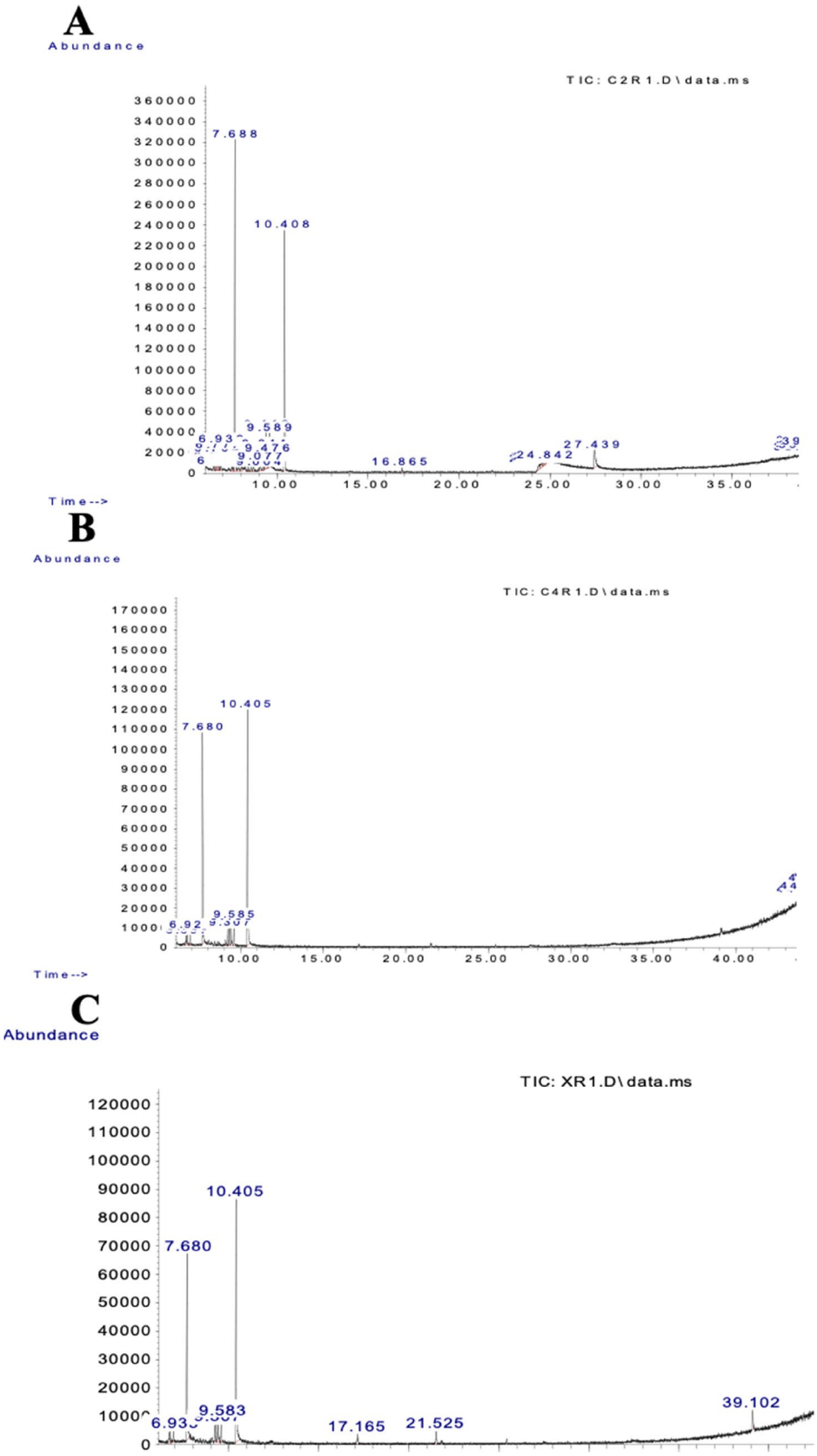

Fig. 3 GC-MS chromatogram of the biosurfactants extracted from the three strains of *Bacillus*

Table 4 Isoforms detected by GC-MS of crude biosurfactants extracted from bacterial strains ROSS2, ROSS4 and ROSS2214

Chemical name	RT (min)	MW	ROSS2	ROSS4	ROSS2214
4,7-Methano-1H-indene-2,6-dicarboxylic acid, 3a,4,7,7a-tetrahydro-[1]	7.68 and 10.4	220.074	Present	Present	Present
Methyl 4-(pyrrol-1-yl)-1,2,5-oxadiazole-3-carboxylate[1]	7.5	193.049	Present	Absent	Absent

[1]National Center for Biotechnology Information (2022). PubChem Compound Summary for CID 296887. Retrieved June 24, 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/296887

These metabolites have also been reported with antifungal and antibacterial, as an alternative against pathogens resistant to antibiotics and as well as in the protection of seeds against the phytopathogenic fungi *Botrytis cinerea* and *Fusarium verticillioides*. The mechanism used by biosurfactants for bactericidal activity is related to damaging the lipids of the cell membrane, which leads to the formation of pores. It has also been reported that they reduce bacterial growth in the exponential phase, affecting cell division [41-43].

The strains of *Bacillus* ROSS2, *Bacillus* ROSS4, and *Bacillus* ROSS2214 presented nematicidal activity against *N. aberrans* (J2), it is worth mentioning that this is the first report of BS with nematicidal activity against this species of false root-knot nematode. A similar effect of a biosurfactant produced by *Bacillus subtilis* HussainT-AMU was reported by Hussain et al. [22] where 90% mortality of *M. incognita* J2 was obtained at 24 h, as well as a decrease in the number of galls in the roots of tomato plants. Similar effects have been reported from biosurfactants in organisms other than nematodes. In another study conducted by Fazaeli et al. [20], it was reported that the BS produced by *Staphylococcus epidermidis* presented mortality of 63.3% against larvae of the flour beetle, *Tribolium castaneum*; the same effect was observed with ochrosin BS produced by the strain *Ochrobactrum* sp. BS-206 against *T. castaneum*, *Callosobruchus chinensis*, *and Sitophilus oryzae* was reported by Kumar et al. [44]. Franco et al. [21], also reported the production of a BS of a glycolipid nature with activity against larvae of the *A. aegypti* mosquito. Meanwhile, Geetha et al. [45] tested "surfactin" produced by *B. subtilis* against pupae of the *Anopheles stephensi* mosquito, as well as against *Culex quinquefasciatus* larvae.

As the different activities of BS against other pests have been mentioned, it had not been reported against plant pathogenic nematodes, for this reason it is important to highlight that the present investigation represents the first study on the notification of BS with activity against the false root-knot nematode *N. aberrans*, which represents an important alternative for sustainable control.

Another activity that was detected in the BS produced by these strains of the *Bacillus* genus is against mites. There is a report of this activity in the patent called composition comprising recombinant *Bacillus* cells and lipopeptide [46]. The BS produced by *Bacillus* strains are lipopetidic in nature and are not of high molecular weight. However, the chemical structure of the GC-MS analysis identifies the majority presence of polar portions, indicated by the dicarboxylic acid and carboxylate groups; while the non-polar portion can be interpreted as a hydrocarbon chain of variable length. With these GC-MS data we can say that possibly the biosurfactant produced by these strains of the *Bacillus* genus is a neutral or polar lipopeptide. Lipopeptides are biosurfactants with strong biocontrol properties with potential in agriculture, since they inhibit a wide range of fungal plant pathogens [47].

The best-known BS produced by species of the *Bacillus* genus are lipopeptides such as surfactin, Iturin, krustakin, lichenysin, bacillomycin, and fengycin [47]. According to Nawazish et al. [48], low-cost substrates or carbon sources are used for the production of lipopeptides, such as vegetable oils and agro-industrial residues. This study notifies the first report of BS with nematicidal activity against the nematode *N. aberrans*, for this reason BS represent a sustainable control alternative against pests of agricultural importance at the national and global levels.

Conclusion

The BS produced by the *Bacillus* ROSS2 strains presented a nematicidal effect against *N. aberrans* (J2), at a concentration of 30 mg/mL. The same strain also had acaricidal activity against *T. putrescentiae*. The nematicidal and acaricidal activity of this strain may be related to the production of biosurfactants. The use of this bacterial strain has potential in sustainable management for the biocontrol of the false root-knot nematode *N. aberrans* and the mite *T. putrescentiae.*

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Declarations

Conflict of interest The authors declare no competing interests.

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