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ORIGINAL ARTICLE

# *Bacillus halotolerans* strain LYSX1-induced systemic resistance against the root-knot nematode *Meloidogyne javanica* in tomato

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## Abstract

**Purpose** Determination of the nematicidal potential and mode of action of bacteria isolated from tobacco rhizosphere soil against the root-knot nematode *Meloidogyne javanica* in tomato plants.

**Methods** Antagonistic bacteria were isolated from rhizosphere soil of tobacco infested with root-knot nematodes. Culture filtrate was used to examine nematicidal activity and ovicidal action of bacterial strains. Biocontrol of *M. javanica* and growth of treated tomato plants were assessed in pot experiments. To clarify whether secondary metabolites of bacteria in tomato roots induced systemic resistance to *M. javanica*, bacterial culture supernatants and second-stage juvenile nematodes were applied to spatially separated tomato roots using a split-root system. Bacterial strains were identified by 16S rDNA and *gyrB* gene sequencing and phylogenetic analysis.

**Results** Of the 15 bacterial strains isolated, four (LYSX1, LYSX2, LYSX3, and LYSX4) demonstrated nematicidal activity against second-stage juveniles of *M. javanica*, and strain LYSX1 showed the greatest antagonistic activity; there was dose-dependent variability in nematicidal activity and inhibition of egg mass hatching by strain LYSX1. In vivo application of LYSX1 to tomato seedlings decreased the number of egg masses and galls and increased the root and shoot fresh weight. Treatment of half of the split-root system with LYSX1 reduced nematode penetration to the other half by 41.64%. Strain LYSX1 was identified as *Bacillus halotolerans*.

**Conclusion** *Bacillus halotolerans* LYSX1 is a potential microbe for the sustainable biocontrol of root-knot nematodes through induced systemic resistance in tomato.

**Keywords** Biocontrol · *Meloidogyne javanica* · *Bacillus halotolerans* LYSX1 · Culture filtrates

## Introduction

Root-knot nematodes (RKNs), *Meloidogyne* spp., are sedentary endoparasites that cause extensive damage to a wide variety of economically important crops in tropical, subtropical, and temperate zones (Kayani et al. 2013) and cause annual global yield losses of more than US$400 million (Huang et al. 2014). Control of RKNs is problematic, due to their wide range of hosts and their adaptability to soil conditions. Synthetic broad-spectrum pesticides are commonly used to reduce RKN parasitism; however, many have been withdrawn or banned in some countries, because of their potential deleterious effects on environmental safety and human health following prolonged use (Nicol et al. 2011). In contrast, biological control is considered a safe and eco-friendly alternative to hazardous chemical pesticides, where microorganisms have been recommended for the management of RKNs in sustainable agricultural production systems (Hallman et al. 2009).

*Bacillus* spp. bacteria may be sustainable alternatives in the management of plant-parasitic nematodes (Li et al. 2015; Castaneda-Alvarez and Aballay 2016; Engelbrecht et al. 2018). For example, *B. subtilis* may suppress nematode host recognition through the production of toxins (Siddiqui and Mahmood 1999), and pesticidal crystal proteins (Cry proteins) formed by *B. thuringiensis* during the stationary phase have nematicidal effects against RKNs (Peng et al. 2011; Bravo et al. 2012) due to the degradation of the nematode cuticle by extracellular bacteria enzymes (Niu et al. 2007; Wei et al. 2010), and. *B. cereus* has been shown to be toxic to second-

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stage juvenile (J2s) of *M. javanica*, as a result of the ammonia and nitrite production (Oka et al. 1993). Rhizobacteria-mediated-induced systemic resistance (ISR) in plants may affect nematode activity (Hasky-Günther et al. 1998; Reitz et al. 2000; Siddiqui and Shaukat 2002; Niknam and Dhawan 2003; Adam et al. 2014), and while *B. halotolerans* has been shown to manage fungal plant diseases (Sagredo-Beltrán et al. 2018; Slama et al. 2019), its nematicidal effects remain unclear. Hence, the objectives of this study were to (1) isolate and identify *Bacillus* spp. from rhizosphere soils of tobacco, (2) evaluate the in vitro and in vivo antagonistic efficacy of *B. halotolerans* strain LYSX1 against the RKN *M. javanica*, and (3) examine the antagonistic mode of action of strain LYSX1 against *M. javanica*.

## Materials and methods

### Preparation of M. javanica

*M. javanica* were isolated from Hainan Province and maintained as described by Wang et al. (2007). Egg masses collected from infested tomato roots were sterilized using 3% hydrogen peroxide solution for 5 min and rinsed three times with sterile distilled water prior to use. J2s were separated using a Baermann funnel technique and sterilized using the method described by Gray (1984).

### Bacteria strains and growth conditions

Antagonistic bacteria were isolated from the rhizosphere soil of tobacco infested with RKN in Songxian County, Henan Province, using the dilution-plate method, and were identified according to growth cultural characters on Luria broth (LB) solid medium (Sui 2015). *Escherichia coli* strain DH5α, which was stored in our laboratory, was used as the host for all plasmids. LB was used as the culture medium for *E. coli* strains, and Landy culture medium was used to ferment isolated bacteria (Landy et al. 1948). When required, media were supplemented with 100 μg/mL ampicillin.

### In vitro antagonism of bacterial isolates against M. javanica

Isolated strains of bacteria were cultured separately in Landy culture medium at 37 °C, and then placed in a shaker at 200 rpm for 48 h. Fermented cultures were centrifuged at 12,000 rpm for 15 min at 4 °C, and supernatants were sterilized through 0.22-μm filters; these were designated as undiluted standard culture filtrate (100% concentration) that was subsequently diluted, with sterile distilled water, to 50 and 25% concentration to test nematicidal activity using methods described by Xia et al. (2011). The number of dead nematodes in each treatment was counted under a light microscope after 12, 24, 36, and 48 h. Landy medium without bacteria and sterile distilled water served as controls, and the experiment was repeated three times with three replicates per trial.

Ovicidal action of bacterial strains was assessed using a slightly modified procedure from that described by Terefe et al. (2009), where six equal-sized sterilized egg masses were randomly selected and transferred, using sterile forceps, to a 6-cm-diameter watch crystal containing 1 mL of 100%, 50%, or 25% concentration of LYSX1 supernatant. Egg masses kept in sterile water were used as a control and incubated at 25 °C for up to 14 days in hatching chambers, and hatch solution was replaced every 2 days. Nematode suspension was poured into a counting dish, and the number of juveniles was counted using a dissecting microscope. Tests were performed in triplicate and repeated three times.

### In vivo antagonism of strain LYSX1 against M. javanica

Biocontrol efficiency against *M. javanica* reproduction and host plant growth of strain LYSX1, which elicited high levels of mortality in J2s and hatching inhibition of egg masses, was assessed in a pot experiment. Tomato seeds (*Lycopersicon esculentum* cv. Sufen) were sterilized using 1% sodium hypochlorite solution for 5 min and rinsed several times with distilled water, and then treated seeds were germinated in bowl-growing tray containing autoclaved sandy loamy soil (1:1 v/v). One week after germination, single seedlings were transplanted into 10-cm diameter plastic pots, and 40 days later, 25 mL cell-free culture filtrates at 100%, 50%, and 25% concentration were added as a soil drench around the root system of each plant; control plants were treated with tap water. After 24 h, 1000 newly-hatched *M. javanica* per plant were inoculated into three × 3-cm deep holes. The experiment was repeated three times and comprised five replicates of each treatment that were arranged in a randomized complete block design in a glasshouse. Plants were watered daily and fertilized weekly with a compound fertilizer. The experiment was terminated 50 days after nematode inoculation, and fresh weight of shoots and roots, numbers of galls, and egg masses per root system were recorded.

### Induced systemic resistance to M. javanica

To clarify whether secondary metabolites of LYSX1 in tomato roots induce systemic resistance to *M. javanica*, supernatants and J2s were applied to spatially separated tomato roots using a split-root approach (Reitz et al. 2000). We transplanted 7-day-old tomato seedlings, which had been cultured as described above, into an upper pot, so that roots grew through two openings in the base into the two pots below. After

40 days, one of the two lower pots was inoculated with 25 mL of 100% concentration of LYSX1 supernatant. The same amount of tap water served as a control treatment. After 48 h, the untreated lowest root system pot was inoculated with 1000 *M. javanica* J2s. Each treatment was replicated five times and arranged in a randomized complete block design, and the experiment was repeated three times. The experiment was terminated 15 days after nematode inoculation, and nematode invasion was determined using the method described by Siddiqui and Shaukat (2002).

### Identification of LYSX1

Morphological and physiological characteristics of the LYSX1 strain were identified according to Logan and De Vos (2009), where chromosomal DNA of LYSX1 was extracted using a Bacterial Genomic DNA Extraction Kit (Axygen Scientific Inc. USA) following the manufacturer's protocol. Partial 16S rDNA and *gyrB* gene sequences were amplified using primer pairs (27F: 5′-AGAGTTTGATCMTGGCTCAG -3 and 1492R: 5′-GGYTACCTTGTTACGACTT-3′, and UP-2r: 5'-AGCAGGGTACGGATGTGCGAGCC-.

RTCNACRTCNGCRTCNGTCAT-3′ and UP-1: 5′-GAAG T C A T C A T G A C C G T T C T G C A Y G C N G G N G - GNAARTTYGA-3′, respectively). Amplicons were purified from gel, cloned into pMD18-T vector (Takara Biotechnology, Japan), and transformed into *E. coli* DH5α competent cells (Sambrook et al. 1989). Transformed cells were then cultured at 37 °C overnight on LB agar plates, supplemented with ampicillin (100 μg/mL). Individual colonies were selected and screened using PCR with vector sequencing primers (M13F: 5′-TGTAAAACGACGGCCAGT-3′ and M13R: 5′-CAGGAAACAGCTAT-GACC-3′). Nucleotide sequence data are included in the NCBI nucleotide sequence database with accession numbers MH359398 and MH359399. For phylogenetic analysis, 16S rDNA and *gyrB* gene sequences of most related bacteria strains were acquired separately from the GenBank database and aligned using ClustalX (Thompson et al. 1997), and a phylogenetic tree of combined 16S rDNA and *gyrB* gene in LYSX1 multiple sequence alignments was created in MEGA version 4.0 using neighbor-joining (NJ) trees, where genetic distances were computed using the maximum composite likelihood model, with bootstrapping analysis (1000 replicates) to evaluate tree topology confidence (Felsenstein 1985).

### Statistical analysis

Treatment effects were analyzed using analysis of variance, followed by Fisher's least-significant-difference test ($p \leq 0.01$) in SPSS software (SPSS Inc., Illinois, USA).

**Table 1** Effect of four strains of bacteria on in vitro mortality of *M. javanica* J2

| Strain | Mortality (%) | |
|---|---|---|
| | 12 h | 24 h |
| LYSX1 | 100.00 ± 0.00A | 100.00 ± 0.00A |
| LYSX2 | 86.67 ± 2.52B | 97.67 ± 0.58A |
| LYSX3 | 77.00 ± 2.00C | 87.67 ± 2.08B |
| LYSX4 | 75.33 ± 1.15C | 86.00 ± 2.65B |
| Control 1 | 0.00 ± 0.00D | 0.00 ± 0.00C |
| Control 2 | 0.00 ± 0.00D | 0.00 ± 0.00C |

Control 1: Landy medium without bacteria; control 2: sterile distilled water. Data are means ± SD from three repetitions. Different letters within a column indicate treatment difference at $p \leq 0.01$ by Fisher's least-significant-difference test

## Results

### Antagonistic bacteria

We isolated 15 bacteria from the tobacco rhizosphere soil, based on colonial morphology; of these, four, which were respectively named LYSY1, LYSY2, LYSY3, and LYSY4, showed antagonistic efficacy in vitro toward *M. javanica* (Table 1), causing 75.33 to 100% juvenile mortality after 12-h exposure. Strain LYXS1 exhibited greater levels of nematicidal activity than the other strains, so it was selected for subsequent assays.

### Antagonistic properties of LYSX1 against M. javanica

Figure 1 shows that all concentrations of LYSX1 resulted in mortality of *M. javanica* J2s, and mortality tended to increase

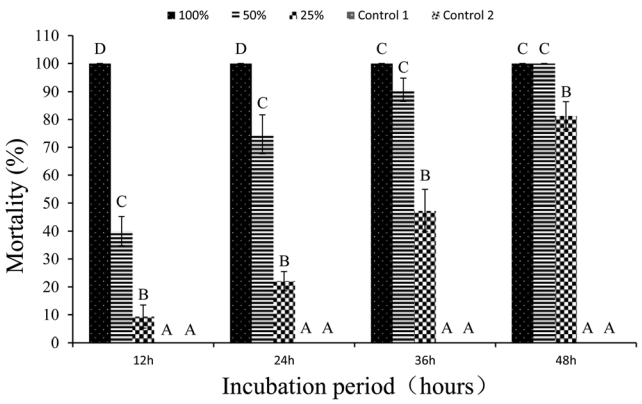


**Fig. 1** Effect of LYSX1 concentration in vitro *M. javanica* mortality over time. Control 1: Landy medium without bacteria; control 2: sterile distilled water. Data are means ± SE and different letters within a time period indicate treatment differences at $p \leq 0.01$ by Fisher's least-significant-difference test. Three independent biological experiments were performed and yielded similar results, so representative results from one experiment are presented

**Table 2** LYSX1 treatment effects on cumulative in vitro hatch rate of *M. javanica* egg masses after exposure for 14 days

| Treatment | No. of juveniles hatched | Hatching inhibition rate (%) |
|---|---|---|
| 100% | 26.33 ± 8.74 D | 98.81 |
| 50% | 240.00 ± 47.47 C | 89.17 |
| 25% | 535.33 ± 53.48 B | 75.85 |
| Control | 2216.67 ± 103.71 A | |

Control: sterile distilled water. Data are means ± SD from three repetitions. In each column, data followed by the same letter are not significantly different at $p \leq 0.01$ by Fisher's least-significant-difference test

in a time- and dose-dependent manner. The mortality rate of J2s treated with high-concentration culture supernatants was remarkably higher than for those treated with low concentrations and controls at 12 h and 24 h ($p \leq 0.01$). Levels of mortality in the 50% and 100% concentration treatments were similar after 36 and 48 h; 25% supernatants caused 47.33% and 81.33% J2s mortality at 36 h and 48 h, respectively.

In vitro, LYSX1 treatments resulted in significant inhibition of egg hatching compared with the control ($p \leq 0.01$) (Table 2). One hundred percent concentration treatment brought about 98.81% inhibition of egg hatching after 2 weeks of incubation. The effect of hatching inhibition was significantly reduced with decreased concentration.

## Effect of LYSX1 on host plant growth and M. javanica

To determine the biocontrol efficacy of LYSX1 in greenhouse conditions, tomato plants were cultivated in the presence or absence of nematodes and LYSX1. Following inoculation with 100% and 50% concentrations of strain LYSX1, the numbers of egg masses and root knots per plant 50 days after inoculation displayed reductions of 39.72% and 42.00% (100% treatment), or 31.36% and 30.83% (50% treatment) compared to the control, respectively (Table 3). The fresh weights of root and shoot showed an increment of 53.30% and 28.70% (100% treatment), or 43.91% and 21.59% (50% treatment), respectively. However, the 25% concentration treatment produced no significant difference in biomass or disease density in tomato plants compared with the control ($p \leq 0.01$).

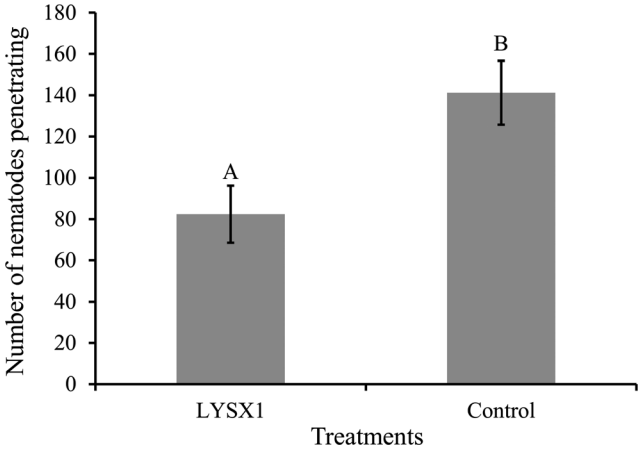


**Fig. 2** Effect of 100% concentration of LYSX1 on penetration of *M. javanica* J2s in split roots of tomato seedlings 15 days after inoculation. Control: sterile distilled water. Data are means ± SD and different letters indicate treatment differences at $p \leq 0.01$ by Fisher's least-significant-difference test. Three independent biological experiments were performed and yielded similar results, so representative results from one experiment are presented

## Induced systemic resistance to M. javanica

The ability of strain LYSX1 to induce systemic resistance was assessed in this study. Fifteen days after nematode inoculation, the 100% concentration of LYSX1 significantly reduced nematode penetration from one half of the split-root system to the other, by 41.64% when compared with the control (Fig. 2).

## Identification of the LYSX1 strain

The detailed phenotypic characteristics of LYSX1 (Table 4) indicate that it belongs to the genus *Bacillus* (Logan and De Vos 2009). The sequences of the genes 16S rDNA (1437 bp) and *gyrB* (1257 bp) were concatenated to form a single sequence of 2694 bp (16S rDNA-*gyrB*) and compared with a concatenate of orthologous genes from *Bacillus*. Sequences were aligned, and a phylogenetic tree was constructed using the maximum likelihood model with 1000 bootstrap replicates (Fig. 3). Most branches were supported by high bootstrap values in this tree. Based on Fig. 3, strain LYSX1 belongs to the cluster of *B. halotolerans*.

**Table 3** LYSX1 treatment effects on tomato plant biomass, and *M. javanica* root-knots, and egg masses densities

| Treatment | Root fresh weight per plant (g) | Shoot fresh weight per plant (g) | No. of egg masses per plant | No. of root knots per plant |
|---|---|---|---|---|
| 100% | 6.04 ± 0.47B | 11.03 ± 0.84C | 34.60 ± 5.64C | 60.20 ± 4.09B |
| 50% | 5.67 ± 0.22B | 10.42 ± 0.64BC | 39.40 ± 4.82BC | 71.80 ± 7.40B |
| 25% | 4.48 ± 0.40A | 9.62 ± 0.49AB | 47.60 ± 4.04AB | 94.80 ± 4.97A |
| Control | 3.94 ± 0.36A | 8.57 ± 0.81A | 57.40 ± 7.02A | 103.80 ± 8.11A |

Control: sterile distilled water. Values are the means ± SD from five repetitions. Means in one column followed by the same letter are not significantly different at $p \leq 0.01$ by Fisher's least-significant-difference test

**Table 4** Phenotypic characteristics of strain LYSX1

| Characteristic | Result | Characteristic | Result |
|---|---|---|---|
| Shape | rod | Acid from: | |
| Endospore | + | D-Glucose | + |
| Gram stain | + | D-Mannitol | + |
| Citrate utilization | + | Glycerine | + |
| Nitrate reduction | + | Sucrose | + |
| MR reaction | – | Lactose | – |
| Indole production | – | Gas from: | |
| Starch hydrolysis | + | D-Glucose | – |
| Casein hydrolysis | + | D-Mannitol | – |
| Anaerobic growth | – | Glycerine | – |
| V-P test | + | Sucrose | + |
| Catalase test | + | Lactose | – |
| Litmus milk test: | | Growth at: | |
| Acid reaction | – | 5 °C | – |
| Alkaline reaction | – | 10 °C | + |
| Curd formation | – | 20 °C | + |
| Peptonization | + | 45 °C | + |
| Growth in 10% NaCl | + | 50 °C | – |

"+" and "–" indicate positive and negative reactions, respectively

## Discussion

We evaluated nematicidal efficacy on *M. javanica* of bacterial strains isolated from a tobacco rhizosphere, and selected strain LYSX1 for further analysis due to its greater in vitro nematicidal activity. Phenotypic characteristics and nucleotide sequences of 16S rDNA and *gyrB* genes of strain LYSX1 indicated that it belongs to the *Bacillus halotolerans* group. Previous studies have demonstrated biocontrol activity of *Bacillus* spp. against plant-parasitic nematodes (Siddiqui and Mahmood 1999; Engelbrecht et al. 2018); however, previously recorded biocontrol activity of *B. halotolerans* was for fusarium wilts and root rots caused by soil-borne fungi, such as *Fusarium oxysporum*, *Phytophthora capsici*, and *Rhizoctonia solani* (Sagredo-Beltrán et al. 2018; Slama et al. 2019). Thus, this study is the first to demonstrate suppression of plant-parasitic nematodes by a strain of *B. halotolerans*. Unpublished data show that culture filtrates of strain LYSX1 were also antagonistic to *M. incognita*, *M. arenaria*, and *M. hapla*, similar to the nematicidal activity against *M. javanica* reported in this study, and also inhibited mycelial radial growth in plant pathogenic fungi, such as *F. graminearum*, *R. cerealis*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Gloeosporium fructigenum*, indicating that LYSX1 may have a broad spectrum of biocontrol activity against multiple plant diseases. Similar findings have been reported by Siddiqui et al. (2001), who demonstrated antagonistic activities of bacterial culture supernatants to the phytopathogenic nematode *M. javanica* and fungi *Macrophomina phaseolina* and *R. solani*. Some strains of *Bacillus* obtained from agricultural soils have been shown to suppress the growth of soil-borne fungal pathogens (*V. dahlia*, *R. solani*, and *F. culmorum*) and a bacterial pathogen (*Ralstonia solanacearum*) and cause in vitro mortality in *M. incognita*

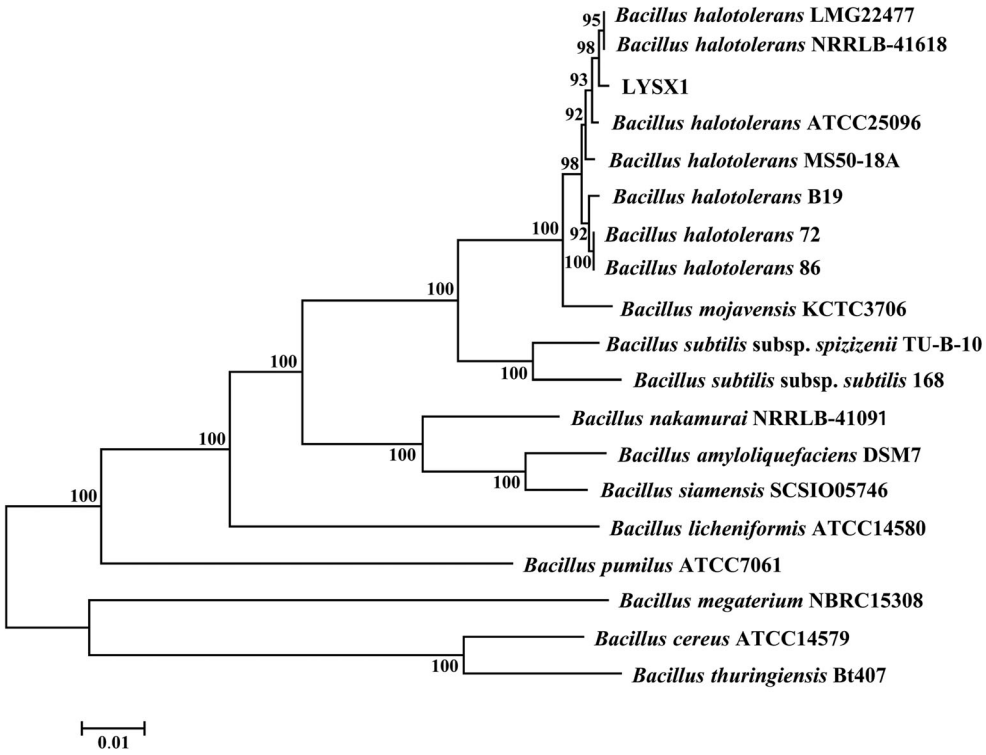


**Fig. 3** Phylogenetic tree based on concatenation of sequences of the genes 16S rDNA and *gyrB* of strain LYSX1. Numbers at the nodes represent the percent bootstrap replicates from 1000 replicates. Scale bar estimate sequence divergence and accession numbers to the NCBI GenBank database are shown in parentheses

juveniles (Köberl et al. 2013; Adam et al. 2014). This study demonstrated that LYSX1 elicits concentration-independent antagonistic activity against *M. javanica* J2s and egg masses, suggesting extracellular nematicidal substances may be present in the culture supernatant. Indeed, Slama et al. (2019) reported that plipastatin A1, Inthomycin-A, cyclo (L-Val-L-Phe), and 5-deoxybutirosamine generated by *B. halotolerans* BFOA1–BFOA4 had antimicrobial properties. Analysis of the LYSX1 supernatant showed that the nematicidal active ingredients were non-protein, heat and cold resistant, and were highly polar (data no shown), so further studies are required to couple with gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) analysis to identify specific nematicidal active substances.

Numerous *Bacillus* strains have been proposed as efficient alternatives to synthetic pesticides for the sustainable management of phytonematodes in agricultural systems, due to promotion of plant growth and nematicidal potential. These biocontrol bacteria mainly comprise *B. subtilis*, *B. amyloliquefaciens*, *B. firmus*, *B. pumilus*, *B. megaterium*, *B. cereus*, *B. methylotrophicus*, *B. velezensis*, *B. mojavensis*, *B. tequilensis*, *B. flexus*, and *B. altitudinis* (Burkett-Cadena et al. 2008; Xia et al. 2011; Engelbrecht et al. 2018; Xiang et al. 2018); however, there are no reports of *B. halotolerans* as a biocontrol agent, to our knowledge. Our evaluation of the strain LYSX1 for its nematode management potential under glasshouse conditions showed its potential for the promotion of plant growth in tomato seedlings (greater fresh root and shoot weight) and the reduction of disease severity caused by *M. javanica* (lower number of egg masses and root knots) when applied at 50 or 100% concentrations; while these concentrations elicited similar effects, treatment with 25% concentration of LYSX1 had no effect on plant growth or disease density of tomato plants. Thus, our results indicate an application rate of 50% concentration is optimal for the suppression of *M. javanica* under glasshouse conditions; however, additional studies are required to evaluate the efficacy of LYSX1 against RKN under field conditions.

ISR in nematode host plants was first reported by Hasky-Günther et al. (1998), who indicated that culture filtrate of *B. sphaericus* B43 induced systemic resistance against *Globodera pallida* in potato. Our results from a split-root system clearly demonstrated that *B. halotolerans* LYSX1 reduced nematode penetration rates by inducing systemic resistance in tomato plants to *M. javanica*; this indicates that *B. halotolerans* LYSX1 secondary metabolites acted as signals to induce systemic resistance against the RKNs. Similarly, Siddiqui and Shaukat (2003) found that the antibiotic secondary metabolite, 2, 4-diacetylphloroglucinol (2, 4-DAPG), produced by *Pseudomonas fluorescens* strain CHA0 may function as an inducing agent of systemic resistance in tomato roots against *M. javanica*. The lipopolysaccharide of *Rhizobium etli* strain G12 is also essential for eliciting systemic resistance in potato against the potato cyst nematode *G. pallida* infestation (Reitz et al. 2000). However, the compound in *B. halotolerans* LYSX1 involved in inducing systemic resistance remains unclear, so further research is required to identify inducement factors and confirm mediation of ISR against RKN in tomato.

## Conclusion

Our results demonstrated that *B. halotolerans* strain LYSX1 antagonized the RKN *M. javanica* through induced systemic resistance in tomato plants.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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