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Unravelling the secondary metabolome and biocontrol potential of the recently described species *Bacillus nakamurai*

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ABSTRACT

In the prospect of novel potential biocontrol agents, a new strain BDI-IS1 belonging to the recently described *Bacillus nakamurai* was selected for its strong *in vitro* antimicrobial activities against a range of bacterial and fungal phytopathogens. Genome mining coupled with metabolomics revealed that BDI-IS1 produces multiple non-ribosomal secondary metabolites including surfactin, iturin A, bacillaene, bacillibactin and bacilysin, together with some some ribosomally-synthesized and post-translationally modified peptides (RiPPs) such as plantazolicin, and potentially amylocyclicin, bacinapeptin and LCI. Reverse genetics further showed the specific involvement of some of these compounds in the antagonistic activity of the strain. Comparative genomics between the five already sequenced *B. nakamurai* strains showed that non-ribosomal products constitute the core metabolome of the species while RiPPs are more strain-specific. Although the secondary metabolome lacks some key bioactive metabolites found in *B. velezensis*, greenhouse experiments show that *B. nakamurai* BDI-IS1 is able to protect tomato and maize plants against early blight and northern leaf blight caused by *Alternaria solani* and *Exserohilum turcicum*, respectively, at levels similar to or better than *B. velezensis* QST713. The reduction of these foliar diseases, following root or leaf application of the bacterial suspension demonstrates that BDI-IS1 can act by direct antibiosis and by inducing plant defence mechanisms. These findings indicate that *B. nakamurai* BDI-IS1 can be considered as a good candidate for biocontrol of plant diseases prevailing in tropical regions, and encourage further research into its spectrum of activity, its requirements and the conditions needed to ensure its efficacy.

1. Introduction

The development of bacterial inoculants to control plant diseases is one of the most promising eco-friendly and efficient alternatives to the use of chemical pesticides in sustainable modern agriculture (Elnahal et al., 2022; Galli et al., 2024). This biocontrol strategy relies on the potential of selected beneficial species to reduce diseases caused by phytopathogens via two main often additive mechanisms which are direct inhibition of the infectious agent through antibiosis and stimulation of plant defences leading to the so-called induced systemic

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resistance (ISR) phenomenon (Benaissa, 2023; Serrão et al., 2024). ISR is particularly interesting since beneficial bacteria inoculated to the roots enhances the defensive capacity of the entire plant against various pathogens including those infecting the aerial parts (Salwan et al., 2023).

The biocontrol activity of these bacteria depends on the potential to produce bioactive secondary metabolites (BSMs) acting as antimicrobials and/or elicitors of host immunity (Abd-Elsalam and Mohamed, 2023; Lourenzi et al., 2022). However, other BSMs retain key ecological functions because they are involved in competition for nutrients (iron-chelating siderophores) or in root colonization (biofilm formation, motility) (Deb and Tatung, 2024; Liu et al., 2024). Several bacterial genera including *Pseudomonas, Streptomyces, Acetobacter, Azospirillum, Paenibacillus, Serratia, Burkholderia, Rhizobium and Bacillus* have been reported for their biocontrol activity (Backer et al., 2018) and in most cases these species are naturally living in close association with plants and were isolated from the rhizosphere. The potential of *Bacillus* to control plant diseases has been amply demonstrated from lab to field, especially species of the *Bacillus subtilis* group thanks to their ability of forming resistant endospores, colonizing quite efficiently the rhizosphere, secreting an arsenal of BSMs, and establishing a mutualistic relationship with plants (Dunlap, 2019; Miljaković et al., 2020; Wang et al., 2022). Bacilli dedicate up to 10–13 % of their genome to the biosynthesis of BSMs (Borriss, 2020; Fazle Rabbee and Baek, 2020) including volatile organic compounds (VOCs) mediating chemical signalling and antifungal activities (Kai, 2020) but also a wide range of soluble metabolites of different types such as non-ribosomal (NR) cyclic lipopeptides, polyketides (PKs), oligopeptides, ribosomally produced and post-translationally modified peptides (RiPPs) encompassing bacteriocins and lantibiotics, as well as various lytic enzymes (Iqbal et al., 2023). From an ecological perspective, evolving such diversity of BSMs can also be considered as an adaptive trait to improve rhizosphere fitness (Anckaert et al., 2021; Etesami et al., 2023a). Additionally, the importance of low molecular weight organic compounds in communication among microorganisms and between microorganisms and plants is increasingly appreciated, extending their ecological roles beyond microbial warfare, defence, and development (Mithöfer and Boland, 2016; Vaishnavi and Osborne, 2021; Xu et al., 2023).

Furthermore, as for other microbial species, the success of these *Bacillus*-based products as soil bioinoculants suffers from variable efficacy and inconsistency in real-world agricultural conditions (Marian and Shimizu, 2019). This is due to the high complexity of the ecological context and many factors may restrict proper invasion of the soil or rhizosphere niche by the introduced strain and hamper therefore its establishment and persistence within the crop-associated microbiome, at threshold populations necessary to provide the beneficial effects (Hossain et al., 2023; O'Callaghan et al., 2022). Successful invasion depends on the ability of bioinoculants to cope with specific abiotic soil factors including pH, temperature, water content, nutrient status but it is also driven by intricate interactions with the host plant and with the associated microbiome communities whose structure, functions and compositions are also dictated by the plant genotype and inherent soil physico-chemical conditions (Bonaterra et al., 2022; Hu et al., 2021; Santos and Olivares, 2021). This supports the importance of keeping mining soils to discover new bacterial isolates with biocontrol potential and well adapted to the specific agro-ecological conditions in which they will be used (Novello et al., 2023; Thakur et al., 2022).

In this work, we describe a new strain BDI-IS1, isolated in the highlands of tropical Africa (Burundi), belonging to the *Bacillus nakamurai* species and displaying a broad-spectrum antagonistic activity against bacterial and fungal phytopathogens. We combined genomics and metabolomics to provide a first characterization of the whole range of specialized metabolites formed by this understudied species. We identified some compounds that play a key role in the antimicrobial activity observed *in vitro*, but our data also suggest that this secondary metabolome may also contribute to the strong biocontrol potential of BDI-IS1 against diseases caused by *Alternaria solani* and *Exserohilum turcicum*, responsible for tomato early blight and northern corn leaf blight, respectively.

2. Materials and methods

2.1. Sample collection, isolation of beneficial bacteria and phytopathogenic fungi from Burundi

Arable soil samples containing bean and potato roots were collected in Murwi (North-west of Burundi) and Isare (West of Burundi) communes, respectively, for isolation of beneficial bacteria. Samples of tomato, bean, and maize leaves showing typical necrotic lesions of early blight, angular leaf spot and northern leaf blight, respectively, were collected in Mwakiro (North-East of Burundi), placed in paper bags, and taken to the laboratory for isolation of fungal pathogens.

For bacterial isolation, small roots were then selected from soil samples and mixed with peptone water + Tween 80 (2 %) and glass beads in a falcon (15 mL). The mixture was vortexed (5 min), serially diluted and plated (100 µL) on LBA (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L and agar 15 g/L). After 16 h of incubation (30°C), *Bacillus*-like colonies were selected based on their morphological traits and subcultured (three times) on LBA for purification. Pure isolates were cultured in LB broth at 30°C overnight and stored at −80°C in peptone water + 20 % glycerol until use.

For isolation of pathogenic fungi, diseased leaf samples were cut into small pieces (<1 cm^2), superficially disinfected with sodium hypochlorite (1 %) for 30 seconds, rinsed three times in sterile water and placed in Petri dishes containing water agar (10 g/L) with chloramphenicol (0.2 g/L) as antibiotic. After 24 h incubation at 22°C, pieces of freshly formed mycelia were taken from the edge of the colonies and transferred to potato dextrose agar (PDA) to obtain pure cultures of *A. solani*, *E. turcicum* and *Ascochyta rabiei,* which were then stored in a freezer at 4°C for further use. Identification of the fungal species was based on morphology (microscopic observation of fungal structures produced by *E. turcicum* and *A. rabiei* cultured on PDA, and *A. solani* cultured on V8) and molecular characterisation of the nuclear ribosomal internal transcribed spacer (ITS) region. For this purpose, fungal mycelia grown on PDA were gently scraped with a sterile blade and used to extract DNA using the cetyltrimethylammonium bromide (CTAB) protocol as described by Lee and Taylor (1990). The purity and integrity of the DNA was checked by electrophoresis on 0.8 % agarose gel for 45 minutes at 130 volts. ITS4 (TCC-TCC-GCT-TAT-TGA-TAT-GC) and ITS5 (GGA-AG-T-AAA-AGT-CGT-AAC-AAG-G) primers were used to amplify the ITS region for the isolated fungi as described by White et al. (1990). PCR products were run on 1.2 % agarose in 1X TAE buffer for 45 minutes at 130 volts and sequenced by Sanger method at Microsynth Seqlab GmbH (Germany). Both sequences of each isolated pathogen were analysed using Bioedit software and multiple alignment was performed using the Muscle algorithm based on MEGA X software. The consensus sequences were then blasted against the sequences available at NCBI platform.

2.2. Direct antagonism assays

2.2.1. Biological materials

Six plant pathogenic bacteria i.e. *Clavibacter michiganensis* subsp. *michiganensis*, *Rhodococcus fascians*, *Pectobacterium carotovorum*, *Xanthomonas campestris* subsp. *campestris*, *Pseudomonas cichorii*, *Pseudomonas fuscovaginae* and six phytopathogenic fungi i.e. *Fusarium oxysporum*, *Botrytis cinerea*, *Rhizoctonia solani*, *Aspergillus niger*, *Pyricularia oryzae* and *Colletotricum* sp. available in the collection of the Microbial Processes and Interactions (MiPI) Laboratory, Gembloux Agro-Bio Tech, University of Liège, were used in the antagonism assays with the bacterial isolates from Burundi. In addition, fungi isolated from diseased leaves collected in Burundi, i.e. *A. solani*, *E. turcicum* and *A. rabiei* were tested. *Bacillus velezensis* QST713, also from the MiPI

collection, was used as a reference standard antagonist bacterium.

2.2.2. Culture preparation

Bacillus-like isolates and plant pathogenic bacteria initially stored at −80℃ were plated on LBA and incubated at 30℃ for 24 h. Individual colonies were then pre-cultured the day before the experiment. On the day of the experiment, all bacterial cultures were centrifuged, and the pellet was washed twice with LB broth, resuspended in the same medium, and adjusted to OD_{600} 0.1 and OD_{600} 2 per mL for pathogenic and isolated bacteria, respectively. *B. velezensis* QST713 (hereafter referred to as QST713) was prepared under the same conditions. Five days' old fungal mycelia (cultured on PDA at 25°C) were used in dual confrontation assay with the most promising isolate based on antibacterial activity.

2.2.3. Antagonistic activity assessment

A dual culture assay was performed to evaluate the antibacterial and antifungal activity of the bacterial isolates. Following the historical background of the sampling site where potatoes were previously grown, solidified Solanaceae root exudates mimicked medium (REM) (glucose 1 g/L, fructose 1.7 g/L, maltose 0.2 g/L, ribose 0.3 g/L, citrate 2 g/L, oxalate 2 g/L, succinate 1.5 g/L, malate 0.5 g/L, fumarate 0.5 g/L, casamino acids 0.5 g/L, $(NH_4)_2SO_4$ 1 g/L, KH_2PO_4 0.3425 g/L, MOPS 11.5 g/L, $MgSO_4 \times 7\ H_2O$ 0.25 g/L, KCl 0.25 g/L, yeast extract 0.5 g/L, 50 µL of $Fe_2(SO_4)_3$ 1.2 %, 50 µL of $MnSO_4$ 0.4 %, 50 µL of $CuSO_4$ 1.6 %, 50 µL of Na_2MoO_4 4 %, Agar 15 g/L) (Nihorimbere et al., 2012) was selected for antibacterial activity assessment. Suspension of pathogenic bacteria ($OD_{600\,nm}$ 0.1) was spread homogeneously on REM agar medium in square plates (12 cm×12 cm) and were incubated at room temperature for approximately one hour to dry. Bacterial isolates and QST713 suspensions ($OD_{600\,nm}$ 2) were spotted (5 µL) on the plate coated with the pathogenic bacteria. Incubation was performed at 30℃ for 48 h. Each bacterial suspension was tested in triplicate against each plant pathogen, with two independent experiments (n = 6). Activity was expressed as mean values (± δε) of the inhibition diameter (mm) around the bacterial spot. The antibacterial potential of the cell-free REM culture supernatant was tested in a 96-well microtitre plate (200 µL per well including a mixture of LB culture of phytopathogenic bacteria adjusted at $OD_{600\,nm}$ 0.1 and 15 % of filtered (0.22 µm pore size) cell-free REM culture supernatants of the tested bacteria grown for 48 h at 30°C, 160 rpm) and incubated at 30°C, 250 rpm in microplate incubator. Optical density readings (at 600 nm) were performed on a microplate reader (TECAN, SPARK) and the experiment was performed two times independently with three replicates per experiment (n = 6). For monitoring the kinetic growth of pathogens treated with BDI-IS1 cell-free extracts, incubation of microplates was performed however inside the TECAN machine with continuing shaking (30°C) and readings (at 600 nm) recorded automatically over the time (each 30 min). The antibacterial activity of various constructed mutants (see Section 2.5) of the most prominent isolate was evaluated using the dual culture assay protocol as described above.

For antifungal activity evaluation, a mycelial plug (5 mm of diameter) of each fungus grown on PDA was placed at the centre of a plate on PDA medium and 5 µL of the best bacterial isolate (based on antibacterial potential) suspension ($OD_{600\ nm}$ 2) was spotted 25 mm away from the centre. The inhibition radius was recorded when control growth reached a radius of 25 mm and the percentage of inhibition was calculated using the following formula: Inhibition (%) = ((A-B)/A) X 100), where A and B are the growth radius of the control and treated fungi, respectively. Each confrontation was tested in triplicate and two independent experiments were performed (n=6). The antifungal activity of different constructed mutants of BDI-IS1 (see Section 2.5) was assessed following the same protocol as detailed above.

2.3. Bacterial isolates molecular characterization

Genomic DNA from bacterial isolate was extracted using the Thermo Fischer Scientific Gram-positive bacteria genomic DNA Extraction Kit according to the manufacturer's standard procedure and was quantified using a Nanodrop ND-1000 spectrophotometer. The 16 S rRNA gene was amplified by PCR using the primers 8 F (5′ - AGAGTTTGATCHTGGCTCAG - 3′) and 1492 R (5′ - GGHTACCTTGTTACGACTT - 3′) and Q5® High-Fidelity DNA Polymerase (New England BioLabs). After electrophoresis (1 % agarose gel), the amplicons were further purified using the Thermo Fischer Scientific GeneJET Genomic DNA Purification Kit according to the manufacturer's standard procedure. The 16 S rRNA amplified fragments were sequenced by Eurofins Genomics (Anzinger, Germany) and were blasted for homology searching to the NCBI Genebank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Whole genome sequencing (wgs) was also performed for the most promising isolate. Libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina), and the sequencing was performed on the Illumina MiSeq v3 platform using 600 bp paired-end chemistries. Illumina read adapters and low-quality bases were trimmed with Trimmomatic0.39. Draft genomes were assembled using SPAdes v3.13.0.

Comparative genome mining for important biosynthetic gene clusters (BGCs) was carried out using the web tools AntiSMASH 7.0.1 (Blin et al., 2023) and BAGEL4 (Van Heel et al., 2018) in the available public genome sequences of strains belonging to the same species as the one sequenced in this study.

2.4. Bioactive secondary metabolites identification of the isolate BDI-IS1

Metabolome characterization of the BDI-IS1 cell-free supernatant was performed by LC-ESI-qTOF-MS (Agilent 1290 Infinity II coupled with mass detector (Jet Stream ESI-Q-TOF 6530)) in positive mode with the following source parameters: capillary voltage of 3.5 kV, nebuliser pressure of 35 psi, drying gas of 8 $L.min^{-1}$, gas temperature of 300°C, sheath gas flow rate of 11 $L.min^{-1}$, sheath gas temperature of 350°C, fragmentor voltage of 175 V, skimmer voltage of 65 V, and octopole radiofrequency of 750 V. Accurate mass spectra were recorded in the *m/z* range of 100–1700 (acquisition rate 2 spectra/s). For an optimal separation, a C18 Acquity UPLC BEH column (2.1 mm; 50 mm; 1.7 µm; Waters) was used at a flow rate of 0.6 mL min^{-1} and a temperature of 40°C (injection volume: 10 µL). A gradient of acidified water (0.1 % formic acid) (solvent A) and acidified acetonitrile (0.1 % formic acid) (solvent B) was chosen as the mobile phase, starting at 10 % B, and rising to 100 % B in 20 min. Solvent B was kept at 100 % for 4 min before returning to the initial ratio. Where possible, metabolites were identified based on retention time and accurate mass, by comparison with *in-house* database constructed with *B. velezensis* mutants impaired in the production of the different metabolites (Andrić et al., 2023). In addition, all the structures were confirmed by MS/MS fragmentation.

2.5. BDI-IS1 mutants' construction

Knockout mutant strains of the promising isolate BDI-IS1 depleted in the biosynthesis of each of the bioactive secondary metabolites and in all non-ribosomally produced peptides (sfp mutant) were constructed by gene replacement and homologous recombination. The primers used for this purpose are listed in the supplementary material (Table A.1). A cassette containing a chloramphenicol resistance gene flanked by about 1 kb of the upstream and downstream regions of each target gene was constructed by three fragments joining PCR. BDI-IS1 transformation was carried out according to the protocol of Hoff et al. (2021) with minor modifications. Shortly, one colony of BDI-IS1 was grown in LB medium (37°C, 150 rpm) for 4 h, and washed twice with MMG liquid medium (19 $g.L^{-1}$ K_2HPO_4 anhydrous, 6 $g.L^{-1}$ KH_2PO_4, 1 $g.L^{-1}$ $Na_3C_6H_5O_7$ anhydrous, 0.2 $g.L^{-1}$ $MgSO_4\ 7\ H_2O$, 2 $g.L^{-1}$ Na_2SO_4, 50 mM $FeCl_3$, 2 mM $MnSO_4$, 8 $g.L^{-1}$ glucose, and 2 $g.L^{-1}$ L-glutamic acid; pH 7.0) and the

OD_{600} was adjusted to 0.01. The recombinant cassette (0.5 – 1 μg) was added to 1 mL of the latter prepared suspension and incubated at 37°C for 24 h. Transformed colonies that have integrated the recombinant cassette, were further selected on LBA medium supplemented with chloramphenicol (5 μg/mL).

2.6. Biocontrol assays against early blight and northern leaf blight

The biocontrol potential of the best antimicrobial bacterial isolate BDI-IS1 against two important plant diseases prevalent in Burundi, tomato early blight and northern corn leaf blight, was assessed under greenhouse conditions. The bacterium *B. velezensis* QST713 (ingredient of Serenade®) was tested under the same conditions for comparison purposes.

2.6.1. Plant material and culture conditions

The varieties Ruganda for tomato and ZM605 for maize were selected for their relative susceptibility to early blight of tomato and northern leaf blight of maize, respectively. Maize and tomato seeds were disinfected in sodium hypochlorite (0.05 %), washed 3 times in distilled water, pregerminated and then sown in pots (2 L) containing potting soil, then placed in greenhouse at a temperature of 22°C ± 2°C and 18 °C ± 2°C day and night respectively, a relative humidity of 80 % ± 5 % and a photoperiod of 16 h.

2.6.2. Treatments and experimental design

The activity of the two tested bacteria (BDI-IS1 and QST713), after leaf or root application, against tomato early blight and northern corn leaf blight was evaluated in separate bioassays (see subsection 2.6.3) on tomato or maize, including twelve treatments: T_1= plant + BDI-IS1 leaf + fungus, T_2= plant + BDI-IS1 root + fungus, T_3= plant + QST713 leaf + fungus, T_4= plant + QST713 root + fungus, T_5= plant + BDI-IS1 leaf + water tween, T_6= plant + BDI-IS1 root +water tween, T_7= plant + QST713 leaf + water tween, T_8= plant + QST713 root + water tween, T_9= plant + $MgCl_2$ buffer on leaf + water tween, T_{10}= plant + $MgCl_2$ buffer on root + water tween, T_{11}= plant + $MgCl_2$ buffer on leaf + fungus,T_{12}= plant + $MgCl_2$ buffer on leaf. The treatments were tested in a completely randomised block design (CRBD) in two independent experiments, with 16 (4 replicates of 4 plants: N=16) and 12 (3 replicates of 4 plants: N=12) plants per treatment in trial one and two, respectively.

2.6.3. Culture preparation, biocontrol agents' application and pathogen inoculation

A. solani was cultured on V8 media (V8 20 % (v/v), $CaCO_3$ 1 g/L, Agar 15 g/L) under neon lamp light, alternating light, and darkness (12 h/12 h) for 10 days. *E. turcicum* was cultured on PDA (potato dextrose agar 39 g/L, agar 2 g/L) and incubated at 27°C for 21 days. To collect the conidia, 3 mL of sterilised water and Tween 20 (0.05 %) were added to Petri dishes and the mycelia and conidia were repeatedly scraped with a sterilised blade and collected in Falcon tube. Conidia were obtained by filtration through a double layer of cheesecloth and the concentration of the conidial suspension was counted using Fuchs Rosenthal cell and light microscopy. Adjusted concentrations of 2×10^4 conidia/mL for *A. solani* and 4.5×10^4 conidia/mL for *E. turcicum* were used for plant inoculation. The bacterial isolate BDI-IS1 and QST713 were revived from cryotubes (-80°C) on LBA, 16 h at 30°C. An individual bacterial colony was used to carry out a pre-culture incubated for 24 h at 30°C, 120 rpm, then used to perform a culture (initial $OD_{600\ nm} = 0.001$, 100 mL in 250 mL flasks) for 24 h, at 30°C, 120 rpm. The cultures were centrifuged, the pellets washed twice with $MgCl_2$ (2.5 g/L) and resuspended in 10 L of $MgCl_2$ (equivalent to10^6 CFU/mL) before application on plants.

Two application methods were tested, i.e. on roots (root treatment) or on leaves (leaf treatment). For the root treatment, 100 mL of the bacterial suspension was poured onto the plant substrate (about 2 L potting soil/plant), twice, at seven days and one day before pathogen (or control) inoculation, i.e. on 24 and 28 days-old tomato plantlets and 11 and 16 days-old maize plantlets, respectively, for trial one and two. For foliar treatment, the bacterial suspension was sprayed on all leaves of treated plants until run-off, the day before inoculation. The control plants were treated with $MgCl_2$ solution only.

The fungal inocula of *A. solani* or *E. turcicum*, as prepared above, were sprayed until run off on the leaf surface of plants of 25 and 29 days-old tomato plantlets and 12 and 17 days-old maize plantlets, respectively for trial one and two.

2.7. Data collection and analysis

Leaf necrotic area (LNA) was used to assess the severity of early blight and northern leaf blight on three (low, middle and top) and two leaves (middle and top) of each plant for tomato and maize, respectively. The LNA of tomato early blight was estimated daily using a scale from 1 to 9, where 1 = 0–5 % of leaf area affected by the disease, 3 = 5–10 %, 5 = 10–20 %, 7 = 20–50 % and 9 = 50–100 %. Northern leaf blight severity was scored at two-day intervals on a scale from 1 to 9 (Fig. A.6), where 1 = 0 % of leaf area affected by the disease, 2 = 10 %, 3 =20 %, 4 = 30 %, 5 = 40 %, 6 = 50 %, 7 = 60 %, 8 = 70 %, 9 = 80–100 %. The disease severity (DS) for NLB was obtained from the mean of the disease severity of the related scores recorded on two leaves, while DS for TEB represent the mean of disease scores recorded on three leaves obtained using the following formula as described by Willocquet et al. (2023):

$DS = \sum_{i=1}^{k}(SR_i \times NP_i)/TNP \times HS) \times 100$, where i is the disease score, k the number of classes in disease scale (k = 5), while SR_i and NP_i represent the score rating and number of plants with i score, respectively. TNP and HS stand for the total number of assessed plants and high score (HS = 9), respectively.

The area under disease progress curve (AUDPC) for tomato early blight and northern corn leaf blight was calculated using the following formula:

$AUDPC = \sum_{i=1}^{n-1}\left(\frac{y_i+y_{i+1)}}{2}\right)X(t_{i+1} - t_i)$, Where y_i: disease severity of i^{th} day,y_{i+1}: disease severity of i^{th+1} day, t_i:i^{th} day, t_{i+1}: i^{th+1} day and n = total number of observations.

The Protection Index (PI) conferred by a treatment was calculated as described by Caulier et al., (2018):

$$PI = \left[\left(1 - \frac{\text{AUDPC treatment}}{\text{AUDPC Control}}\right) * 100\right]$$

The data on disease severity collected in the greenhouse for tomato early blight and northern corn leaf blight were analysed using Generalized Linear Mixed Model (GLMM) with R 4.0.5 software, where the bacteria, treatment method and trial were fixed factors, and block was a random factor. Degrees of freedom were calculated using the Kenward-Roger method, while the Estimated Marginal Means (emmeans) package was used to compare the different means. The differences between PI were assessed using t-test within R 4.0.5.

For the *in vitro* antagonist assays, data analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test (p value = 0.05). All the graphs were constructed using Prism GraphPad 9.4.1 software.

3. Results

3.1. B. nakamurai strain BDI-IS1 as strong antagonist of plant pathogens

This work was initiated by screening bacteria in rhizosphere soil samples collected from crop fields at two different locations in Burundi. Our objective was to isolate endemic bacilli and we selected a subset of sixteen isolates characterized by a rapid growth ($\leq$ 20 h) and typical white *Bacillus*-like colonies on solid LB medium, with most of the isolates from Murwi (thirteen) than Isare sites (three). These isolates were first

tested for their antagonistic potential against a range of phytopathogenic bacteria including species known as important plant pathogens with high economic impact notably in tropical and subtropical regions (Ndayihanzamaso et al., 2016; Nihorimbere et al., 2024). Antibacterial activity was chosen as prime criterion for screening because this trait is not only critical to the overall efficacy of broad-spectrum biocontrol agents (Wang et al., 2022) but also important for the ecological fitness and persistence in the competitive rhizosphere niche (Afridi et al., 2022; Andrić et al., 2023; Weiland-Bräuer, 2021). Moreover, the outcome of bacterial interactions in general and of antagonistic competition in particular may vary according to various conditions including the medium (Garrido-Sanz et al., 2023; Sun et al., 2022). It is why, instead of common laboratory media, we performed these confrontation assays on an agar-solidified Root Exudate-Mimicking medium (REM) designed to reflect the nutritional context of the rhizosphere in Solanaceae plants (Hoff et al., 2021).

Based on the size of the inhibition zone developed around the colony (Fig. 1A&B), the isolate BDI-IS1 displayed the highest and most consistent antibacterial activity against all the tested bacterial pathogens and at a level similar to the one observed for the commercial strain *B. velezensis* QST713, a well-known biocontrol agent in agriculture (Anastassiadou et al., 2021). A very high inhibition was observed against *C. michiganensis*, *R. fascians* and *X. campestris* for both BDI-IS1 and QST713, while *Pectobacterium carotovorum*, *Pseudomonas cichorii* and *Pseudomonas fuscovaginae* were moderately inhibited (Fig. 1A). We next used microplate-based assays to test the inhibitory effect of cell-free extracts obtained from BDI-IS1 liquid cultures in REM. Growth of *C. michiganensis* and *R. fascians* cells were fully repressed upon addition of BDI-IS1 extract (15 % v/v); while *X. campestris*, *P. carotovorum* and *P. cichorii* growth inhibitions were partial, ranging from 90 % to 40 % depending on the pathogen (Fig. 1C & 1D). This correlates well with results obtained in plate confrontation assays and evidences the crucial role of secreted soluble BSMs in mediating antibacterial activities. The isolate BDI-IS1 was also evaluated for its antifungal activity against a range of phytopathogenic fungi reported as threats to agriculture in tropical regions including species that we isolated from diseased plants in Burundi (Fig. 1E). Data revealed the broad-spectrum activity of BDI-IS1, at levels comparable to QST713, with very high antifungal potential against *P. oryzae* and *Colletotrichum* sp., good inhibitory potential against *A. rabiei*, *A. solani* and *E. turcicum*, and moderate inhibition towards *A. niger*, *B. cinerea* and *F. oxysporum* (Fig. 1E & F).

Genomic DNA was further extracted and the 16 S rRNA gene was sequenced (NCBI accession number OP546292) for identification purpose. The sequence alignment with NCBI database showed the highest homology to *Bacillus nakamurai* NRRL B-41091 with 97.7 % of identity. This was supported by the black-pigment production by the strain when cultured on tryptic soy agar (TSA) (Fig. A.1), a phenotypic feature typical of newly described species "*Bacillus nakamurai*" (Dunlap et al., 2016). The black, melanin-like pigment observed with *B. nakamurai* strains is also reported in other members of *Bacillus subtilis* group including among others *B. atrophaeus* (Nakamura, 1989), and *B. subtilis* 4NP-BL (Ghadge et al., 2020). This melanic pigment is associated to environmental stresses resistance such as UV rays oxidative stress (Ghadge et al., 2020; Saxena et al., 2002) and was found to have antibacterial activity against some plant pathogens including *X. campestris* (Ghadge et al., 2020). *B. nakamurai* is a less-studied species that has been first described in 2016 and only five strains including BDI-IS1, were sequenced up to date: *B. nakamurai* NRRL B-41091 (NZ_LSA Z00000000.1) and *B. nakamurai* NRRL B-41092 (NZ_LSBA00000000.1), *B. nakamurai* MZ03–67 (NZ_JANBMN000000000.1) and *B. nakamurai* B-41093 (NZ_JARLZY000000000.1). Other isolates that have not been fully sequenced are also described as putative strains of *B. nakamurai* based on 16 S rRNA gene sequencing (Chaouachi et al., 2021; Shaikh et al., 2023).

3.2. Characterization of the B. nakamurai secondary metabolome

The strong antagonistic potential of BDI-IS1 reflects the ability of the strain to efficiently secrete antimicrobial metabolites and therefore, we inspected its genomic content for biosynthetic gene clusters (BGCs) responsible for the synthesis of BSMs. The whole genome (3,811,863 bp of size) of BDI-IS1 was sequenced via Illumina MiSeq (NCBI acc. # NZ_JAJJBV000000000.1) and genome mining was performed using the softwares antiSMASH 7.0.1 (Blin et al., 2023) and BAGEL 4 (Van Heel et al., 2018). It first revealed the presence of BGCs responsible for the biosynthesis of a range of non-ribosomal products such as the two families of cyclic lipopeptides surfactin and iturin A (Wang et al., 2023), the polyketide bacillaene (and its variant dihydrobacillaene) (Miao et al., 2023), the siderophore bacillibactin (Dertz et al., 2006), and the dipeptide bacilysin (Islam et al., 2022). Genes encoding several ribosomally produced and post-translationally modified peptides (RiPPs) including amylocyclicin (Scholz et al., 2014), plantazolicin (Molohon et al., 2016), bacinapeptin (Xue et al., 2022) and LCI (Gong et al., 2011) were also identified (Fig. 2). Plantazolicin is a thiazole and oxazole heterocycle-containing molecule with a demethylated bioactive form (plantazolicin A) and its inactive non-methylated precursor peptide (plantazolicin B) (Kalyon et al., 2011).

Further chemical characterization of BSMs produced by BDI-IS1 in REM was performed via UPLC-q-TOF-MS^2 analysis of cell-free extracts. All the predicted non-ribosomal compounds and plantazolicin were detected and identified based on their exact mass and fragmentation patterns (Fig. 3A and Fig. A.2). With this method we also elucidated the panoply of co-produced structural variants for the lipopeptides surfactin and iturin families, which differ in the length of the fatty acid chain ranging from C_{12} to C_{16} for surfactin and C_{14} to C_{17} for iturin A.

We next inspected the genomic content in BGCs responsible for BSMs synthesis in the other strains of *B. nakamurai* sequenced so far in order to evaluate to what extent this secondary metabolome is conserved at the species level. It reveals that synthesis of the non-ribosomal compounds surfactin, iturin A, bacillaene, bacilysin and bacillibactin is conserved across the strains. Together with plantazolicin predicted in four out of five strains, these molecules thus constitute the core metabolome typical of the *B. nakamurai* species. By contrast, the content in BGCs predicted to encode for RiPPs (bacinapeptin, thermoactinoamide, ericin_S, plantazolicin, amylocyclicin, LCI, pumilarin and mersacidin) is more variable and strain-specific (Fig. 3B).

3.3. Specific involvement of BSMs in the antimicrobial activity of BDI-IS1

We next wanted to determine the relative contribution of each BSM in the broad-spectrum antimicrobial activity displayed by *B. nakamurai*. For that purpose, we generated a library of mutants specifically repressed in the synthesis of one compound and a *Δsfp* mutant lacking the 4′-phosphopantetheinyl transferase (sfp) gene essential for proper NRPS functioning (Wu et al., 2023) and thus impaired in the synthesis of all non-ribosomal metabolites (Table A.1).

All these mutants were tested for their antagonistic activity against a set of bacterial and fungal pathogens described above and data revealed different situations (Fig. 4). In some cases, the *Δsfp* mutant completely lost its inhibitory potential but the antimicrobial activity clearly relies on one single compound such as bacillaene (and/or its −2 H form) against *P. carotovorum* and *R. fascians* or iturin A against *A. niger* and *R. solani*. In other cases, the antagonistic activity of *Δsfp* is drastically reduced and we could identify bacilysin (against *P. fuscovaginae*) and iturin A (against *E. turcicum*) as main active ingredients, but other unidentified compounds also contribute to the inhibition. As a third situation, some sfp-independent metabolites are mainly involved in the inhibition of pathogens like plantazolicin which plays some role in the antagonistic effect against *X. campestris* (partial loss of activity of *ΔpznA*). However, the main metabolite(s) interplaying in the antagonism of the latter pathogen together with *C. michiganensis*, *P. cichorii*,

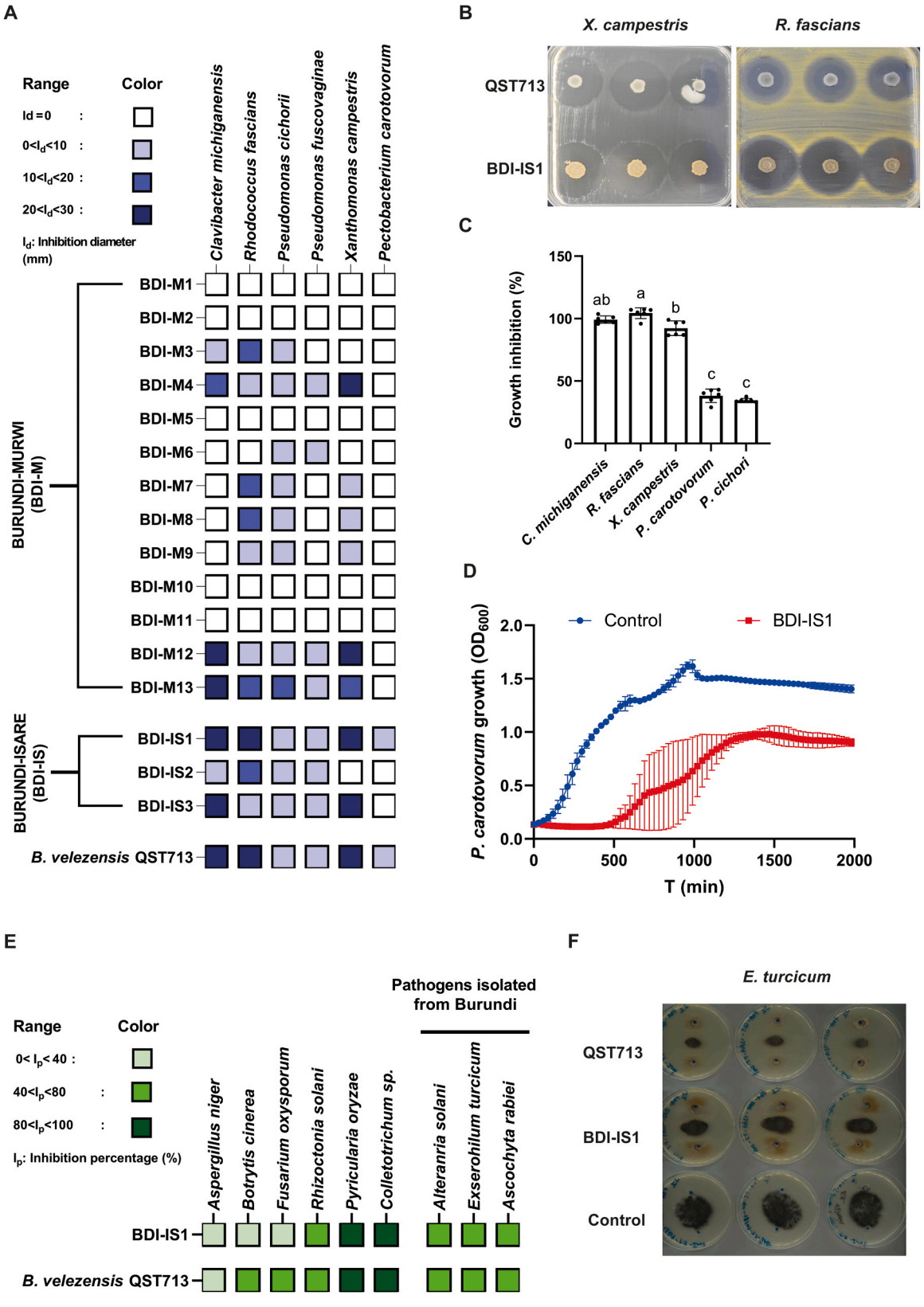

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Fig. 1. The Burundi isolated strain BDI-IS1 retains a great antagonistic potential against phytopathogens. A. Antibacterial activity of bacterial isolates (BDI-M1 to BDI-M13 and BDI-IS1 to BDI-IS3) against a set of bacterial phytopathogens in comparison to *Bacillus velezensis* QST 713 (Serenade®). This was assessed through a dual culture assay carried out on gelified REM (30 mL, 30°C, 48 h) and the inhibition potential is illustrated in blue boxes with a gradual increase of color font in line with the increasing range of inhibition diameter (Id); data are means of inhibition diameter (± SE, mm) and evolve from two independent experiments conducted each in triplicate (n=6) (Table A.2). B. Illustration of inhibition growth of *X. campestris* and *R. fascians* exhibited by the isolate BDI-IS1 and *B. velezensis* QST713. C. Growth reduction of bacterial phytopathogens by cell-free REM culture supernatants (CFS) of BDI-IS1; overall growth reduction (%) after 48 h of bacterial (pathogenic) culture supplemented with 15 % (V/V) of CFS. Bar graphs (with error bars) represent mean (± SD) values calculated from three repetitions with two independent assays (n=6), letters a to c indicate significant statistical differences of means following the one-way analysis of variance (ANOVA) and Tukey's HSD test (Honestly significant different, $\alpha = 0.05$). D. Growth curve of *P. carotovorum* cultured with or without 15 % (v/v) BDI-IS1 CFS (red and blue curves, respectively); data points are means (± SD) values calculated from three repetitions with two independent assays (n=6). E. Antifungal activity of the isolate BDI-IS1 and *B. velezensis* QST713 against fungal phytopathogens. Dual confrontation assay was used and inhibition percentages (partitioned into different ranges) are represented in green boxes with color intensity increasing with inhibition percentage range, data are means (± SE) of triplicates repeated independently twice (n=6) (Table A.3). F. Illustration of antagonistic activity of BDI-IS1 and QST713 against *Exerohilum turcicum* isolated from diseased maize leaves from Burundi, in a dual culture assay set-up.

B. cinerea and *A. solani* still remain to be identified (Fig. 4).

3.4. Biocontrol potential of BDI-IS1

Greenhouse experiments were next conducted to evaluate the potential of BDI-IS1 to control two major diseases prevalent in Burundian agro-ecological zones and caused by the foliar pathogens *A. solani* on tomato and *E. turcicum* on maize. Prior to infection, plants were treated with BDI-IS1 either by aerial spraying (as a foliar treatment) or by soil drenching (as a root treatment) to evaluate the biocontrol activity mediated via direct antagonism in the first case or via the induction of systemic resistance in the second case. The leaves of tomato or maize plants were then inoculated with *A. solani* or *E. turcicum*, respectively. Tomato early blight and northern leaf blight diseases were assessed by determining the necrotic leaf area according to a specific scale of symptoms (Fig. A.3). Comparison of the dynamics of disease severity revealed that symptoms occurrence was delayed by 1–3 days in plants pretreated with BDI-IS1, depending on the pathosystem and application method (Fig. 5). We also observed a slower progression of disease severity compared to untreated plants, particularly on tomato plants upon leaf treatment with the bacteria, and on maize plants upon root and leaf treatments (Fig. 5).

The reduction of disease severity was significant from the 13th day post inoculation until the time when symptoms no longer evolved on control plants (Table A.4 & A.5). BDI-IS1 delayed disease development by acting efficiently at early stage, as shown by the disease progression curves (Fig. 5). Root-treated plants did not show symptoms until 4 days post inoculation with *A. solani*, while a 10 % disease severity (DS) was observed on control plants and for those treated on leaves. NLB symptoms appeared on control and treated plants from the 7th and 10th day post inoculation, respectively. Overall protection indexes of approximately 25 % and 35 % for TEB and 65 % and 50 % for NLB (Fig. 5) were obtained after root and leaf treatments in two independent greenhouse experiments under severe infection pressure, and no significant differences were observed between leaf and root treatments. These results highlight the biocontrol potential of BDI-IS1 acting locally as antagonist (leaf treatment) or systemically by eliciting plant resistance (root treatment). Furthermore, the levels of protection provided by BDI-IS1 against TEB or NLB are in the same range as the ones observed for *B. velezensis* QST713 tested under the same conditions, except for leaf treatment on tomato, where no disease reduction was observed with that strain (Fig. A.4 & A.5 and Table A.6). Despite the same global trend in protection levels for each treatment method adopted, the data collected from the two independent trials show a huge variation due to the different weather conditions in which the trials were conducted (Table A.7).

4. Discussion

In this work, we provide a first comprehensive genomic and chemical characterization of the secondary metabolome produced by an isolate belonging to the rare species *B. nakamurai*. Comparative genome mining for cognate BGCs revealed that a subset of this metabolome with compounds produced via the non-ribosomal pathway is well conserved in all strains sequenced so far. Due to the absence of the lipopeptide fengycin and the polyketides difficidin and macrolactin, this core metabolome of *B. nakamurai* appears less diversified compared to other closely related species such as *B. velezensis* or *B. amyloliquefaciens* in the *B. subtilis* clade but still can be used as a chemical fingerprint typical for the species and can be exploited in taxonomic identification (Yin et al., 2023). By contrast with the well-conserved non-ribosomal products and plantazolicin, the type and number of RiPPs are more strain-specific. This diversification of RiPPs at the strain level may be explained by the acquisition of BGCs from close relatives or other bacterial genera sharing the same agro-ecological niche via horizontal gene transfer (Grubbs et al., 2017; Malit et al., 2021) which may be facilitated by the *ComS* gene located inside the *srfA* operon, involved in natural competence and quorum-sensing (Rahman et al., 2021), and present in all *B. nakamurai* strains with 82.98 % of similarity, while aligned using NCBI Blast against the one of *B. subtilis* 168 (Pedreira et al., 2022).

Furthermore, we show for the first time possible genetic modification in this undervalued species and data generated from testing the different mutants allow to further exemplify the key role played by some of these specialized metabolites in the broad-spectrum antimicrobial activity displayed by BDI-IS1 (Ajuna et al., 2024; Saiyam et al., 2024). Some bacterial phytopathogens were antagonized by BDI-IS1 via the production of bacillaene and/or its 2 H-form or bacilysin or a synergy between the two compounds. The polyketide bacillaene (and its 2 H-form) is an antibacterial compound against various pathogenic bacteria by interfering with protein synthesis (Erega et al., 2021; Miao et al., 2023), and it is reported to sustain other ecological roles like protecting the producing strain from predation by soil competitors such as *Mycoplasma xanthus* (Andrić et al., 2023). Although not evidenced in our study, this compound was also shown to exhibit antifungal activity (Miao et al., 2023). Bacilysin retains strong antibacterial activity against a range of plant and food-borne pathogenic bacteria, presumably by inhibiting the synthesis of glucosamine 6-phosphate and hence the formation of peptidoglycan (Chen et al., 2009; Islam et al., 2022). Plantazolicin, a thiazole/oxazole containing-molecule was found to be involved in the inhibition of the brassica's black rot-causing pathogen *X. campestris*. Beyond exhibiting nematicidal activity against *Caenorhabditis elegans* (Liu et al., 2013), this metabolite was already reported to have antibacterial activity, but only against Gram positive bacteria including the obligate pathogen *B. anthracis* (Molohon et al., 2016; Scholz et al., 2011).

In some cases, we could not identify the metabolites involved in the antimicrobial activity suggesting that other products may interplay solely or in synergy with known BSMs in these antagonisms. The predicted antimicrobial peptide LCI was shown to have a strong inhibitory potential against *X. campestris* pv *oryzae* and *Pseudomonas solanacearum* PE1 (Gong et al., 2011), whereas the LCI-like APC_2 is reported to antagonize *Fusarium solani*, the causal agent of ginseng root rot (Wang

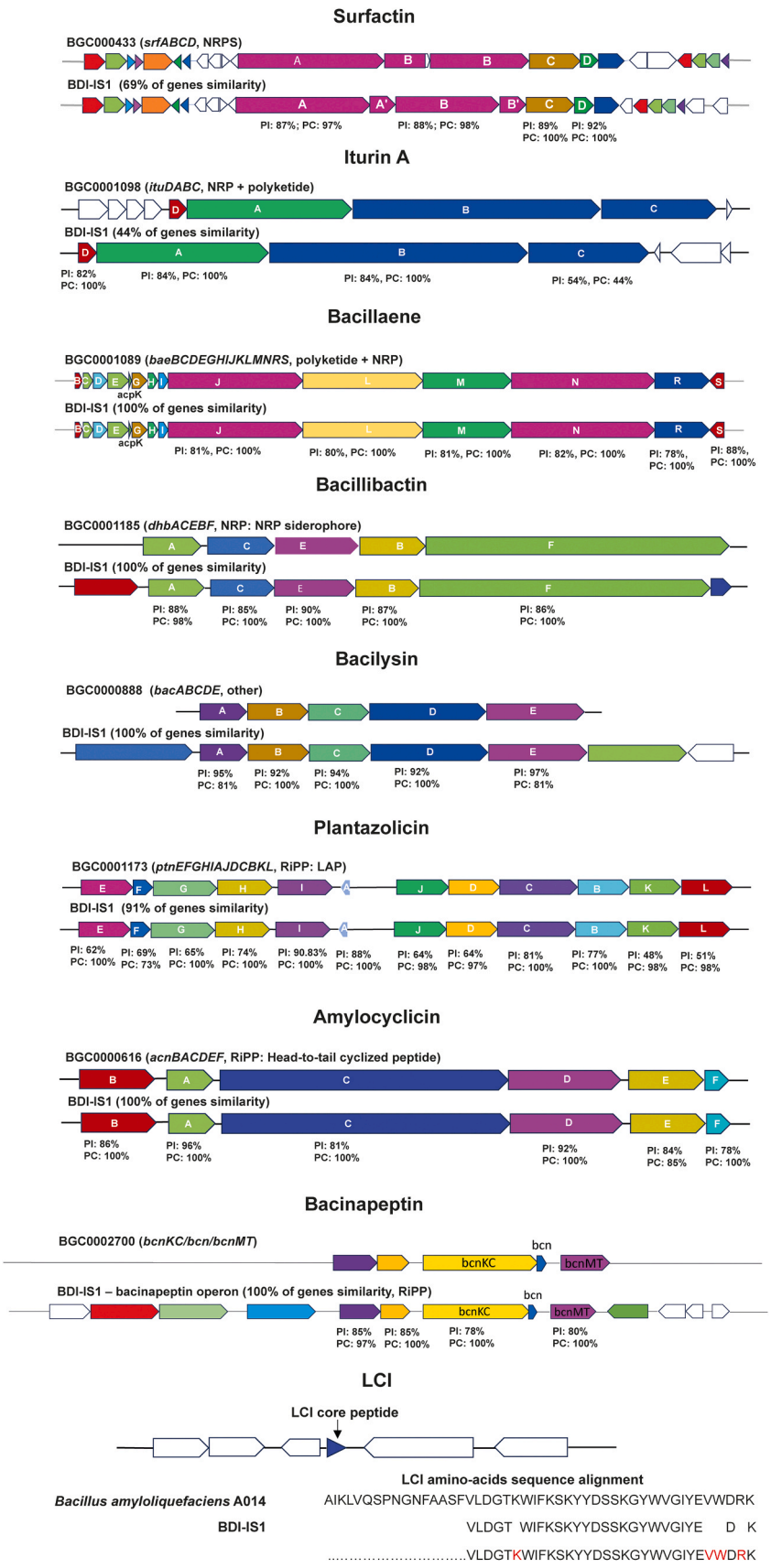
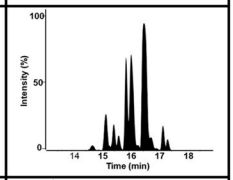
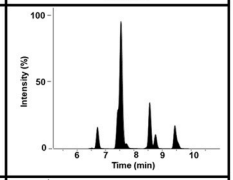
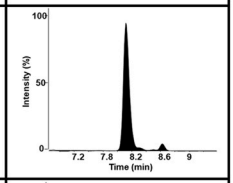
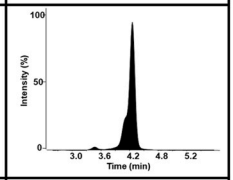
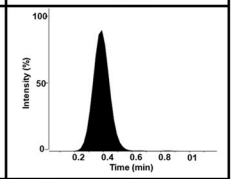
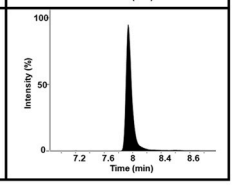
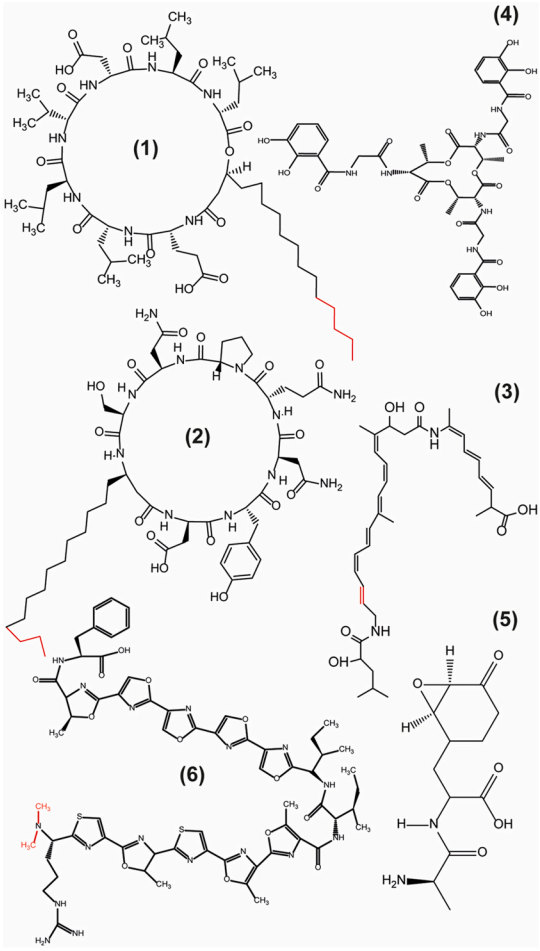


Fig. 2. Predicted biosynthetic gene clusters (BGCs) in BDI-IS1 genome sequence. Prediction was performed using AntiSMASH 7.0 (relaxed detection strictness) and Bagel 4 softwares. The percentage of identity and coverage below each gene of BDI-IS1 were generated from alignment to corresponding gene sequence of the reference strain (reference strain above and query strain, BDI-IS1 below). The architecture of the genes was adapted in line with the prediction tools information.

A

BSM	Detected m/z (mass error (ppm))	EIC
Surfactin (1)	C_{12} : 994.6585 (15.2) C_{13} : 1008.6762 (16.94) C_{14} : 1022.6935 (18.32) C_{15} : 1036.7088 (17.74) C_{16} : 1050.7231 (16.22)	
Iturin A (2)	C_{14} : 1043.5620 (9.56) C_{15} : 1057.5800 (11.66) C_{16} : 1071.5953 (11.18) C_{17} : 1085.6118 (11.82)	
Bacillaene (3)	Bacillaene : 581.3601 (2.33) Dihydro : 583.3585 (-25.85)	
Bacillibactin (4)	883.2717 (10.04)	
Bacilysin (5)	271.1355 (24.53)	
Plantazolicin (6)	A: 1336.4905 (9.75) B: 1308.4748 (21.86)	



B

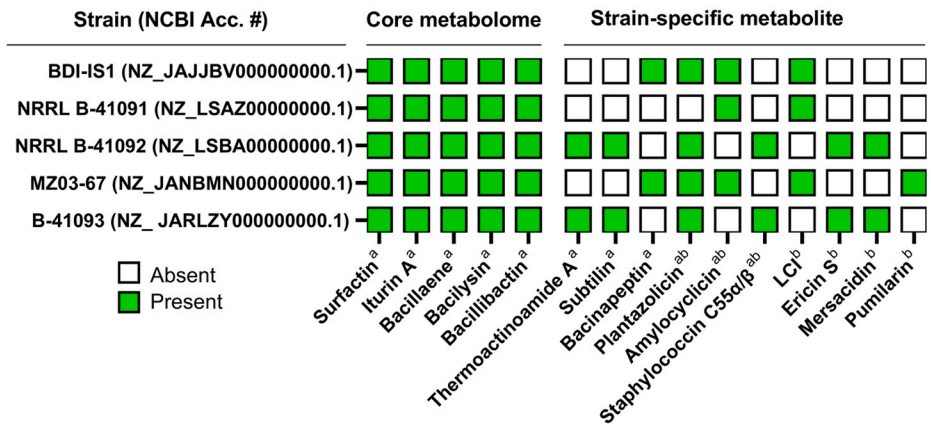


Fig. 3. Secondary metabolome of BDI-IS1 and comparative genome mining profile of all the *B. nakamurai* strains. A. Detected bioactive secondary metabolites with their *m/z* ratio (error in ppm) and extracted ion chromatogram (EIC); cell-free supernatant from a 48 h old culture of BDI-IS1 (REM) was analysed with the hyphenated method ultra-perfomance liquid choromatography (UPLC)- mass spectrometry (MS) with quadrupole time of flight (qtof) as method of detection in a positive mode. Chemical structures of different detected compounds were drawn with ChemDraw softaware. C. Comparative genome mining of BGCs in all available genome sequences of the different *B. nakamurai* strains. The letters a, b, ab imply that the typical compound was predicted either by AntiSMASH or by Bagel 4 or by the two webtools respectively.

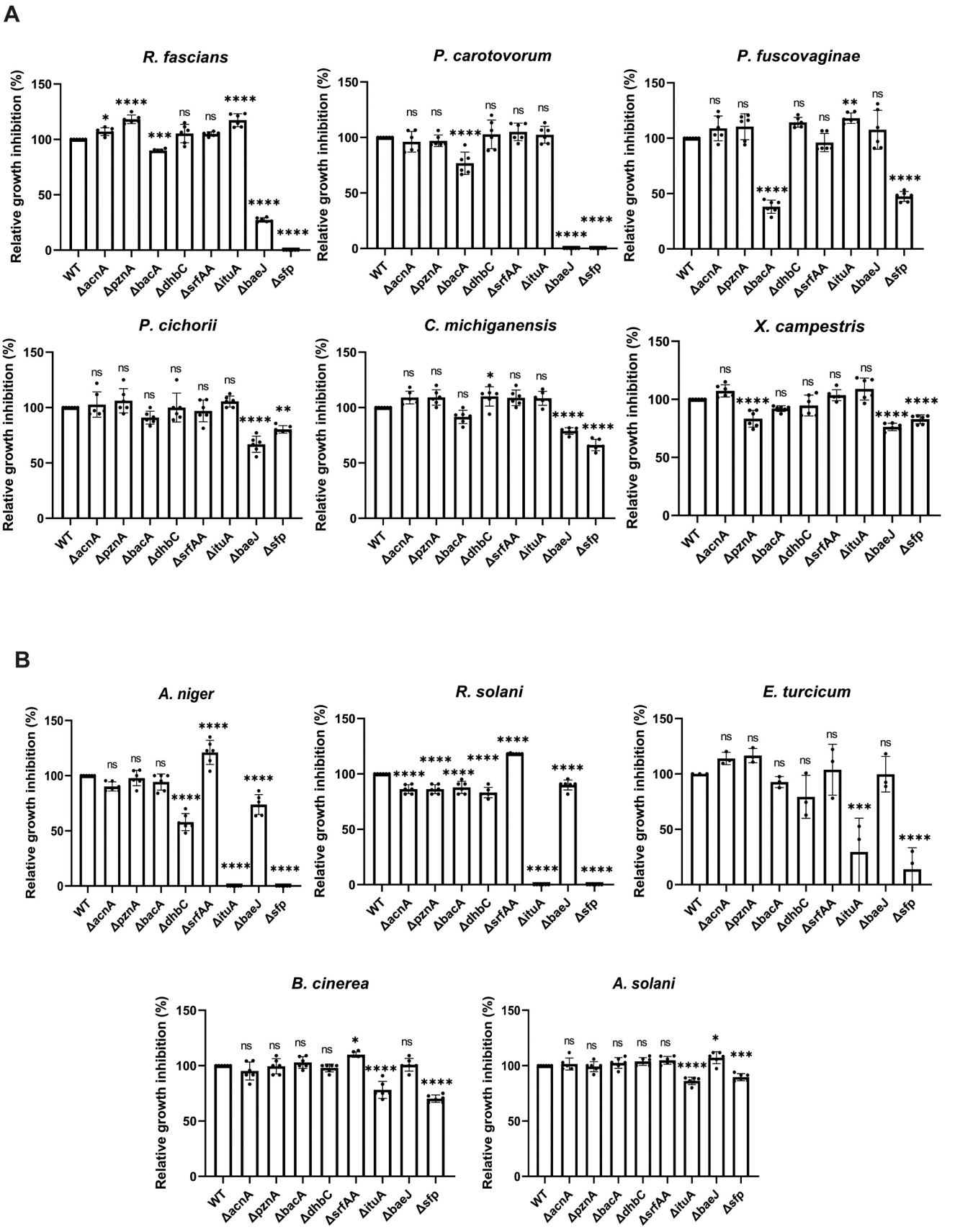


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Fig. 4. Antagonistic activities of BDI-IS1 mutants against bacterial and fungal phytopathogens. A. Antibacterial activity (relative growth inhibition) of different BDI-IS1 mutants against some bacterial phytopathogens, in comparison with the wild type. The confrontation assay was set on gelified REM (30 mL, 30°C, 48 h) and inhibition diameter (mm) was recorded and expressed in percentage (compared to the wild type). B. Antifungal activity of BDI-IS1 mutants against some important fungal phytopathogens. Relative growth inhibition of pathogens in confrontation with the different mutants was observed after seven days (20 mL of PDA, 23–25°C), except for *A. solani* where incubation was for 21 days and grown on REM Agar. The plotted data (with error bars) for A&B are means (±SD) of three technical replicates, conducted twice in independent experiments (n=6). Statistical comparison of means was performed with one-way ANOVA coupled with Tukey's test ($\alpha = 0.05$) and ns represent non-significant difference, while asterisks *, **, ***, **** imply significant differences at $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$ respectively. *WT* represent the *B. nakamurai* BDI-IS1 wild type, while *ΔacnA, ΔpznA, ΔbacA, ΔdhbC, ΔsrfAA, ΔituA, ΔbaeJ* and *Δsfp* stand for BDI-IS1 mutant strains depleted in the biosynthesis of amylocyclicin, plantazolicin, bacilysin, bacillibactin, surfactin, iturin A, bacillaene and all non-ribosomal produced peptides respectively.

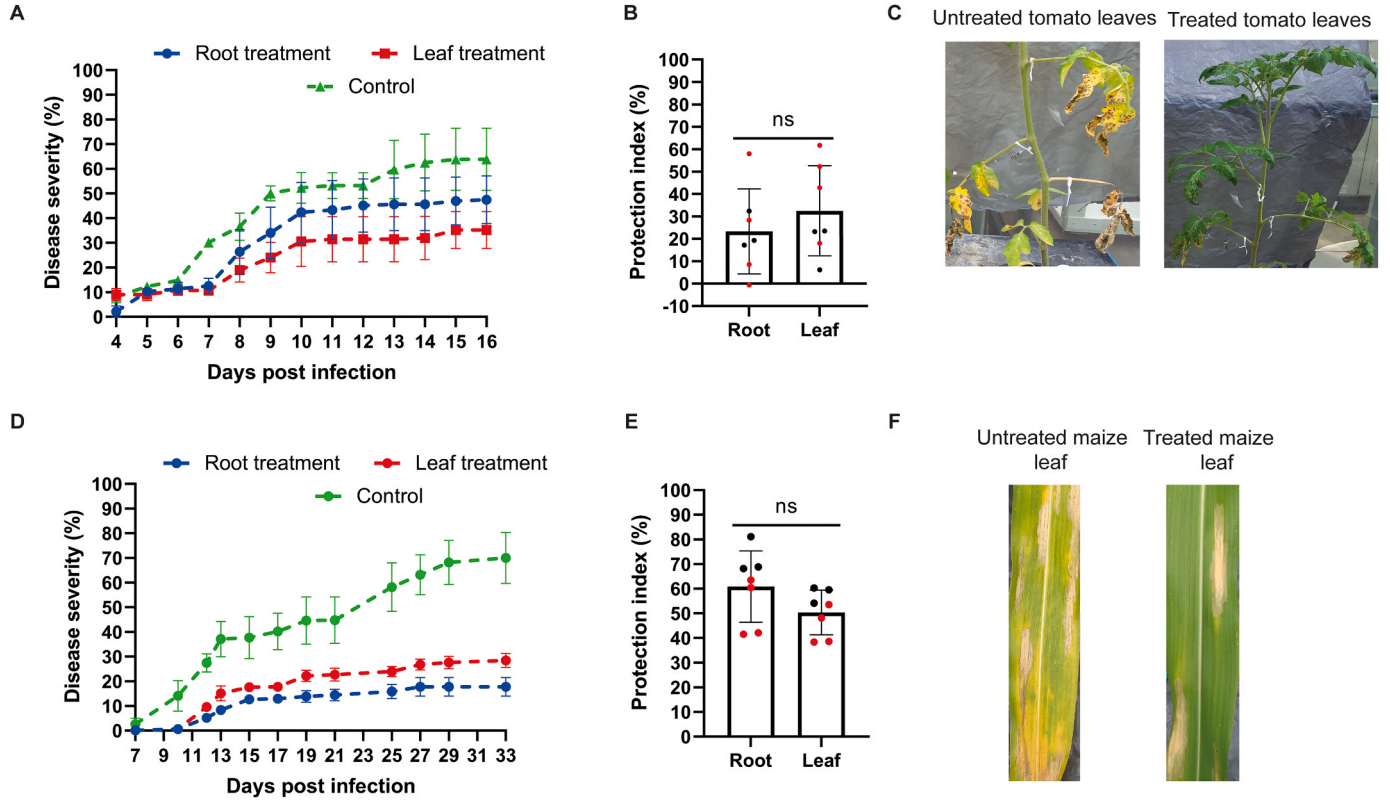

Fig. 5. Biocontrol efficacy of BDI-IS1 against tomato early blight (TEB) and nothern corn leaf blight (NLB). A&D represent the reduction in disease severity of TEB and NLB on plants treated in the roots (blue) or leaves (red) with BDI-IS1 followed by inoculation with *Alternaria solani* or *Exserohilum turcicum*, respectively, and control plants (in green) inoculated with the respective fungus, under greenhouse conditions. Each point in graphs A and D represents the mean (±SE) of disease severity from four replicates (n=4) and three replicates (n=3) for experiments one and two, respectively. B&E represent the protection index (PI) of BDI-IS1 when applied to roots or leaves of tomato and maize plants inoculated with *A. solani* and *E. turcicum*, respectively. PIs represent means (± SE) of two independent experiments (n=7) with four (red) and three (black) replicates per treatment for experiment one (n = 4) and two (n = 3), respectively. Means of PI are calculated from area under progress curve (AUDPC) data, which in turn depend on disease severity scores and the time interval between successive recording points (see formula in Section 2.7). The differences between PI of BDI-IS1 obtained from root and leaf treatment were analyzed using t-test method and there was no significant difference (ns) at p-value ≤5 %. C&F show the TEB and NLB necrotic lesions on tomato and maize plants treated on leaves with BDI-IS1 compared to untreated but inoculated plants.

et al., 2021). Other lanthipeptides predicted by AntiSMASH such as bacinapeptin (Xue et al., 2022) have been described for their antibiotic activity against various Gram-positive bacteria.

Beyond these already predicted and elucidated compounds, we suspect that other unknown metabolites derived from the T3PKS (type III polyketide synthase), terpene and RRE-containing protein BGCs (Fig. A.6) could putatively be among the mediators of the antagonistic activity of BDI-IS1. Indeed, although not yet identified in *Bacillus*, some compounds synthesized via the T3PKS like diacetylphloroglucinol in *Pseudomonas* sp., are described to play important biological functions via the inhibition of fungal phytopathogens (Katsuyama and Ohnishi, 2012). Most terpenes produced by strains of the *B. subtilis* group are volatile compounds (Caulier et al., 2019; Iqbal et al., 2023; Shafi et al., 2017), except the C-35 terpenes reported in broth culture of *B. subtilis* KSM 6–10 (Takigawa et al., 2010) and the oxidative stress alleviator sporulenes, tetracyclic terpenoids found in *B. subtilis* spores (Bosak et al., 2008). The roles of these terpenes in the antimicrobial potential of bacilli against phytopathogens is not well described, but it was shown that isoprene and monoterpene α-terpineol from *Bacillus subtilis* exhibit antagonistic activity against cyanobacteria and nematodes (Caulier et al., 2019). The RiPP Recognition Element (RRE) domain is represented in almost half of RiPPs-producing prokaryotes (Ren et al., 2023) and this RRE-containing protein BGC in BDI-IS1 would code for a novel RiPP with putative antagonistic properties (Hudson and Mitchell, 2018). Other BSMs like surfactin and bacillibactin are not involved in direct antagonistic activity but still these compounds can play key functions for the ecological fitness of *B. nakamurai* considering their well-recognized role in space colonization through cell motility and biofilm formation for

surfactin (Crouzet et al., 2020; Hoff et al., 2021) and iron acquisition for bacillibactin (Yu et al., 2011).

Globally, our data illustrate the huge chemical diversity of BSMs in *B. nakamurai* and this probably explains the broad-spectrum antimicrobial activity of strains like BDI-IS1, which extends much beyond the antagonistic activity of *B. nakamurai* occasionally reported against *Erwinia amylovora* (Leathers et al., 2020), *Fusarium poae* (Zanon et al., 2024) and *B. cinerea* (Chaouachi et al., 2021). As *B. nakamurai* targets bacterial and fungal pathogens affecting different crops of worldwide economic importance, this antagonistic potential is an important trait for any biocontrol candidate.

Basing our prospect on the new strain *B. nakamurai* BDI-IS1 isolated from arable soils collected from highlands of tropical region (1880 m of altitude, Burundi), we unravel the great biocontrol potential of this endemic *Bacillus* strain against two important tropical plant diseases, tomato early blight and northern corn leaf blight. The biocontrol efficacy of BDI-IS1 against important fungal diseases such as northern corn leaf blight and tomato early blight is interesting as it reaches a level similar or higher than the commercialized strain QST713 in greenhouse experiments. The bacteria were applied to the roots or leaves of the plants, followed by a fungal inoculation on the leaves. When applied preventively, BDI-IS1 delayed the severity of TEB and NLB by one to three days and thus reduced disease pressure on young plants, allowing faster growth and higher resistance to pathogen invasion or to abiotic stresses. This observation was also reported by Galiano-Carneiro and Miedaner (2017), where high yield losses were observed after early infection by *E. turcicum*, before silking.

Our results showed an average protection of 25 % and 35 % for TEB after root or leaf treatment with BDI-IS1. Previously, other *Bacillus* species such as *B. amyloliquefaciens* strain bact-03 have been reported to control TEB caused by *A. solani* strain As-9003, with a significant disease reduction (50 %) after leaf treatment under greenhouse conditions (Imran et al., 2022). Although BDI-IS1 protects tomato plants by either root or leaf treatment, the fact that the leaf treatment guaranteed relative high protection for BDI-IS1 strongly suggest that the strain utilizes mostly the direct route in controlling TEB. This may involve to some extent the antifungal iturin A active against several pathogens such as *Phytophthora infestans* (Wang et al., 2020), *B. cinerea* (Ambrico and Trupo, 2017) and *Fusarium* spp. (Liu et al., 2020). Noteworthy, the latter pathogens are also prevalent in the tropics, threatening various economic crops and could be controlled by BDI-IS1 alone or in combination with other plant disease control strategies.

The efficacy of *Bacillus* spp. in controlling NLB was previously reported, with a reduction in disease severity ranging from 40 % to 56 % following leaf treatment in a greenhouse experiment (Sartori et al., 2017). Here, we demonstrate for the first time the high potential of *B. nakamurai* to protect maize against NLB upon the two modes of application, suggesting a dual mode of action relying on direct antagonism and/or induction of plant systemic resistance. Based on *in vitro* assays, production of iturin A may be tightly involved in local restriction of *E. turcicum* development on leaves. Regarding ISR, the cyclic lipopeptide surfactin produced by all species of the *B. subtilis* clade, including *B. nakamurai*, has been widely reported as elicitor of plant defense in several pathosystems (Caulier et al., 2019; Crouzet et al., 2020; Khan et al., 2021; Pršić and Ongena, 2020). However, iturin A has also been reported as trigger of ISR (Han et al., 2015; Lam et al., 2021; Yamamoto et al., 2015) and further experiments are needed to identify the molecule(s) from *B. nakamurai* that is (are) active on tomato and maize in our study.

Although not equipped like *B. velezensis* QST713 in terms of secondary metabolites reported to be involved in the biocontrol of fungal diseases (i.e. absence of fengycin), BDI-IS1 demonstrated significant protection against TEB and NLB in a tropical mimicked-greenhouse conditions either upon leaf or root treatment at levels similar to QST713; except in the tomato/*A. solani* pathosystem upon leaf treatment where QST713 did not reduce tomato early blight severity (Fig. A.4, A.5 and A.7). This suggests a specific adaptation of BDI-IS1 to tropical conditions to provide similar or better protection against local plant diseases, as it has been shown that abiotic factors affect the *in planta* efficacy of biocontrol agents (Ayaz et al., 2023).

5. Conclusions

Isolated from Burundian soil, *B. nakamurai* BDI-IS1 exhibited strong *in vitro* antimicrobial activities against a range of important bacterial and fungal phytopathogens. We characterized the core metabolome of the species and identified some non-ribosomal specialized metabolites for their involvement in pathogen inhibition. However, the weaponry of *B. nakamurai* is quite diverse and several RiPPs may also contribute to the global antimicrobial activity. A lot remains to be discovered in the diversity of bioactive natural products potentially formed by this understudied species. The biocontrol potential assessment against tomato early blight and northern maize leaf blight revealed that BDI-IS1 provides its protective effect upon leaf or root treatment and to a level similar or higher compared to *B. velezensis* QST713 (Serenade®) under greenhouse conditions, suggesting two main modes of action including direct and indirect antagonism. Further studies should explore the mechanisms underlying these biocontrol properties and investigate other potential benefits in terms of plant growth promotion or resistance to abiotic stresses (Enebe and Babalola, 2018; Etesami et al., 2023b).

The high potential of this locally isolated strain to control major plant diseases prevalent in the tropical region highlights the importance of seeking adapted solutions to local problems by exploiting indigenous microbial resources (Liu et al., 2023; Novello et al., 2023).

Author contributions

FN, GN, AL and MO conceived the project. AAA contributed to chemical analysis and corrected the manuscript. SS participated in BDI-IS1 mutants' construction. CL contributed to fungal inocula production and greenhouse experiments. FN and GN carried out the experiments, analyzed the data, and wrote the initial manuscript. VN, AN, AL and MO corrected the manuscript. All authors checked the final version of the manuscript and approved it.

CRediT authorship contribution statement

Marc Ongena: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **François Nimbeshaho:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Anaclet Nibasumba:** Writing – review & editing. **Venant Nihorimbere:** Writing – review & editing, Funding acquisition. **Anne Legrève:** Writing – review & editing, Supervision, Conceptualization. **Gaspard Nihorimbere:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Anthony Argüelles Arias:** Writing – review & editing, Investigation. **Charlotte Liénard:** Investigation. **Sébastien Steels:** Investigation.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Data availability

All data supporting the findings of this work are available within the paper and in supplementary materials

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2024.127841.

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