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ORIGINAL ARTICLE

Antifungal activity of bioactive compounds produced by the endophyte *Bacillus velezensis* NC318 against the soil borne pathogen *Sclerotium rolfsii* Sacc

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Abstract

In a previous study, the endophytic *Bacillus velezensis* NC318 was isolated from the rhizosphere of date palm and showed strong antifungal activity against the soil-borne plant pathogenic fungus, *Sclerotium rolfsii* Sacc, the causal agent of Southern blight. The potential of the *Bacillus* genus in the inhibition of plant pathogens is mainly due to the production of certain bioactive compounds. In the present study, secondary metabolites extracted from the cell-free supernatant of strain NC318 showed strong antifungal activity on the mycelial growth and germination of *S. rolfsii* sclerotia *in vitro*. With 50 µl of bioactive compounds crude extracts, the mycelial growth inhibition rate was 97% and any germination of sclerotia was reported. Chemical analysis of the secondary metabolite crude extracts performed by high performance liquid chromatography coupled with mass spectrometry (HPLC/MS), revealed that the secreted bioactive compounds belonged to the family of lipopeptides (iturin, fengycin, surfactin), polyketides (bacillaene, macrolactin, difficidin and bacilysin) and siderophores (bacillibactin). These results provide a better understanding of the biocontrol mechanism of the bacteria strain *B. velezensis* NC318 against the soil fungal pathogens, especially *S. rolfsii* root rot.

Keywords: antifungal activity, *Bacillus velezensis,* biological control, sclerotia, secondary metabolites

Introduction

Biological control as a way to control or reduce the density of pathogens and suppress plant diseases has received significant attention in recent years. This approach remains an alternative to chemical pesticides with the advantage of being environmentally friendly and eliminating health and pollution risks (Sibponkrung *et al.* 2017; Jiang *et al.* 2018). Several species of the genus *Bacillus* spp. are recognized as promising biological control agents. These species possess particular properties, including the production of molecules with anti-microbial activity, the formation of endospores, and their involvement in the promotion of plant growth, which has earned them the name Plant Growth Promoting Rhizobacteria (PGPR) (Sansinenea and Ortiz 2011; Zouari *et al.* 2016; Jin *et al.* 2017). *Bacillus velezensis*, a gram-positive aerobic bacterium, is a bacteria exploited as a biological control agent and has been reported to suppress the growth of microbial pathogens, including bacteria, fungi, and nematodes (Rabbee *et al.* 2019). Control of these plant pathogens could result from the synthesis of a variety of bioactive components, including antibiotics, antifungals, and siderophores (Nifakos *et al.* 2021).

Molecular research has confirmed that many genetic regions of *B. velezensis* are linked to the synthesis of antagonistic compounds. It is estimated that approximately 10% (340 kb) of the *B. velezensis* genome is dedicated to the production of antimicrobial compounds (Rabbee *et al.* 2019). These molecules are primarily antimicrobial peptides with generally cyclic, hydrophobic structures and contain particular amino acid fragments (Caulier *et al.* 2019). Among the molecules secreted by *B. velezensis* are cyclic non-ribosomal lipopeptides (e.g., iturin, surfactin, fengycin and bacillomycin), polyketides (e.g., difficidin and macrolactin), bacteriocins (e.g., plantazolicin, amylocyclicin) which are post-translationally modified peptide antibiotics and siderophores (bacillibactin) (Fan *et al.* 2018). These potent molecules are biodegradable, non-toxic, and exhibit significant antimicrobial activity against a wide variety of pathogens (Ongena *et al.* 2005). Their mode of action has been reported in several studies on biocontrol against plant pathogens *viz Botrytis cinerea* (Nifakos *et al.* 2021), *Verticillium dahliae* (Azabou *et al.* 2020), *Sclerotinia sclerotiorum* (Teixeira *et al.* 2021), *Alternaria solani* (Zhang *et al.* 2021), *Fusarium graminearum* (Xu *et al.* 2020) and *Phytophtora infestans* (Kim *et al.* 2021).

We reported in a previous study, that *B. velezensis* NC318 isolated from date palm rhizosphere showed very high antifungal activity against *Sclerotium rolfsii* Sacc *in vitro* and *in vivo* (Bidima *et al.* 2021). In the present work, we sought to better understand the biocontrol potential of the endophytic bacterial strain NC318 by: 1 – evaluating the antifungal potential of the secondary metabolites secreted by the strain NC318 against *S. rolfsii in vitro* and by 2 – identifying the secreted antifungal active compounds using high-performance liquid chromatography coupled with mass spectrometry (HPLC/MS).

Materials and Methods

Microorganisms

The bacterial strain used in this study, *Bacillus velezensis* NC318, was isolated from the rhizosphere of a date palm crop. The fungal pathogen used was a strain of *S. rolfsii* Sacc isolated from an infected sugar beet root. These strains were from the collection of the phytopathology laboratory of the Hassan II Agronomic and Veterinary Institute (Rabat, Morocco). Before any experimental use, strain NC318 was grown at 27°C and the fungal pathogen at 30°C on potato dextrose agar (PDA) medium.

Culture conditions for secondary metabolites production

A pure culture of 7-day-old bacterial strain NC318 grown on PDA medium was taken to seed sterile flasks containing 100 ml of lysogeny broth (LB) liquid medium. The flasks were incubated at 28°C in the dark with continuous agitation at 150 rpm for 72 h. The culture broths of strain NC318 were filtered through Whatman n°1 filter paper, and then the supernatant was centrifuged at 6,000 rpm for 20 min and sterilized with 0.45 µm Millipore filters to remove bacterial cells.

Extraction and purification of secondary metabolites

The cell-free supernatant was acidified to pH 2 with concentrated HCl to precipitate secondary metabolites and then stored for 16 h at 4°C and centrifuged at 10,000 rpm for 20 min. The precipitate was recovered and extracted three times with 10 ml of 100% methanol (MeOH) (Zouari *et al.* 2016). The methanol crude extracts were evaporated by a rotary evaporator and were redissolved in 1 ml of pure methanol and then filtered through a 0.2 µm filter and stored at –80°C until used for antifungal assay and chemical analysis.

In vitro antagonistic activity of secondary metabolites secreted by strain NC318

Effect of crude extracts on mycelial growth of *Sclerotium rolfsii*

The crude extracts of the bioactive compounds were evaluated for their antifungal activity by a double culture assay on PDA according to the method of Sarwar *et al.* (2018). Briefly 10, 20 and 50 µl of crude extracts (2 µg · µl^{-1}) were spread on Petri dishes containing PDA medium and then a 5 mm mycelial plug, taken from 7-day-old *S. rolfsii* culture, was placed in the center of the petri dishes. The diameter of the mycelial growth was measured after 5 days of incubation at 30°C. Sterile methanol was used to replace the crude extracts in the control dishes. The experiment was repeated three times.

The antifungal activity of the crude extracts was calculated using the following equation:

$$\text{Mycelial growth inhibition [\%]} = \frac{D-d}{D} \times 100,$$

where: D – mycelial growth in control Petri dishes; d – mycelial growth in treated Petri dishes.

To detect the inhibition of crude extract on mycelial growth of *S. rolfsii* the well diffusion method of Jiang *et al.* (2018) was used. A Petri dish was inoculated in the center with 5 mm of a 7-day-old culture of *S. rolfsii*. Then 50 µl of crude extract (2 µg · µl^{-1}) was added to wells opposite each other on the culture dish

by using an oxford cup and 50 µl of methanol was used to serve as a control in the other two wells. The inhibition zones were recorded 5 days after incubation at 30°C. Antifungal activity was determined by observing the zone of inhibition of fungal growth around the wells.

Effect of crude extracts on germination of *Sclerotium rolfsii* sclerotia

The same scheme as described in section 4.1 was used to study the effect of crude extracts on the germination of *S. rolfsii* sclerotia. A total of 12 mature sclerotia disinfected with sodium hypochlorite were used. The germination of the sclerotia was evaluated after 10 days of incubation at 30°C. The experiment was repeated three times.

$$\text{Sclerotia germination inhibition [\%]} = \frac{N-n}{N} \times 100,$$

where: N – number of germinated sclerotia in control Petri dishes; n – number of germinated sclerotia in the treated Petri dishes.

Analysis of compounds secreted by strain NC318 by HPLC/MS

Analysis of the chemical profiling of crude extracts was performed by a high-performance liquid chromatography (HPLC) system (Thermo Scientific™ Dionex Ultimate 3000 HPLC, Germany) coupled with a mass spectrometer (MS) (Thermo Scientific™ Q Exactive™ Orbitrap MS, Germany). An aliquot of 10 µl was injected into a Hypersil C18 column (150 × 4.6 mm, 5 µm) for chromatographic separation. Eluent A (water + + 0.1% formic acid) and eluent B (acetonitrile + 0.1% formic acid) were used in an elution gradient mode as follows: 0 to 21 min (95% A and 5% B); 21 to 24 min (5% A and 95% B); 24 to 30 min (95% A and 5% B) with a flow rate of 0.5 ml · min^{-1}. Full MS scan was performed in positive (ESI+) and negative (ESI-) ion mode, high resolution (70 000), data acquisition was performed over a mass range of 100–1500 m/z with a sheath gas flow rate of 60, an auxiliary gas flow rate of 20 arbitrary units, and 3.5 kV spray voltage.

Statistical analysis

Data on the evaluation of crude extracts of bioactive compounds on mycelial growth and sclerotia germination of *S. rolfsii* were analyzed using SPSS software (version 20). Tukey's test was performed after an analysis of variance (ANOVA). Values of $p < 0.05$ were considered statistically significant.

Results

In vitro antagonistic activity of metabolites secreted by *Bacillus velezensis* NC318 against *Sclerotium rolfsii*

Most biocontrol agents affect fungal plant pathogens by inhibiting mycelial growth and sporulation. The antifungal activity of strain NC318 crude extracts was evaluated by the mycelial growth and sclerotia germination inhibition method and was represented in Figure 1. Strong inhibitory activity was observed against *S. rolfsii*. As shown in the results of Figure 2A,

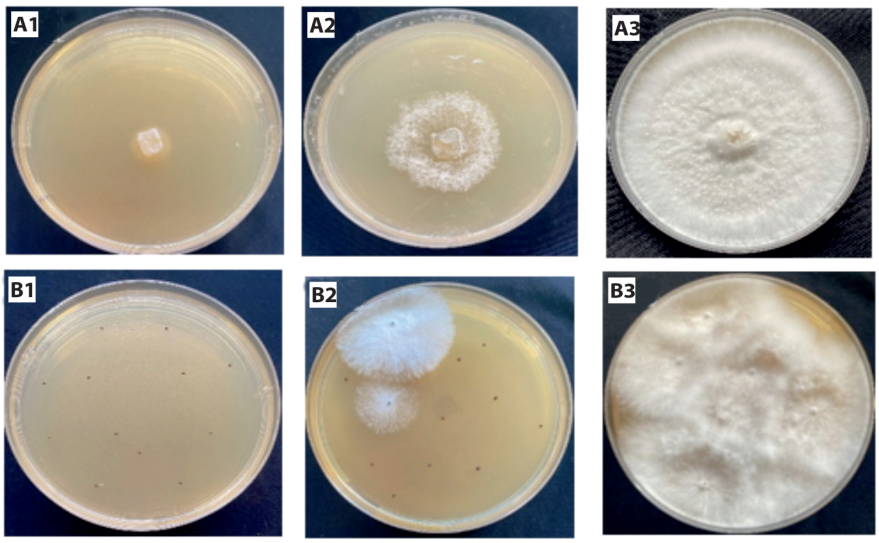

Fig. 1. Antifungal activity of *Bacillus velezensis* NC318 crude extracts on *Sclerotium rolfsii* mycelial growth (A1 – 50 µl crude extracts, A2 – 10 µl crude extracts and A3 – 50 µl methanol – control) after 5 days of incubation at 30°C and sclerotia germination (B1 – 50 µl crude extracts, B2 – 10 µl crude extracts and B3 – 50 µl methanol – control) after 10 days of incubation at 30°C

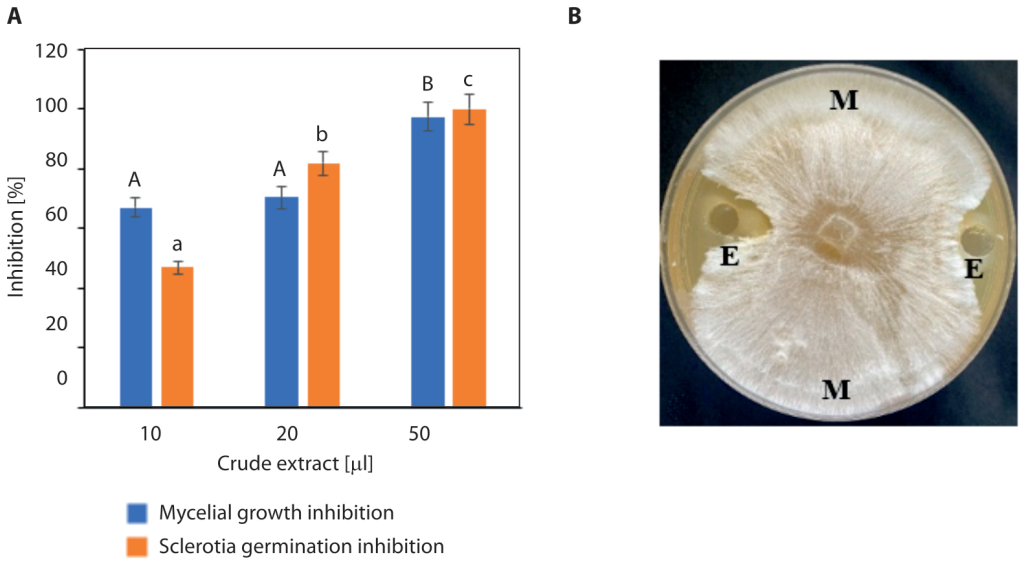

Fig. 2. A – effect of crude extracts of *Bacillus velezensis* NC318 on mycelial growth and germination of *Sclerotium rolfsii* sclerotia. Means with the same letters are not significantly different with Tukey's test at $p < 0.05$. B – inhibition of *S. rolfsii* growth by crude extracts produced by *B. velezensis* NC318 in the agar well diffusion assay: E – 50 µl aliquot of the crude extracts preparation; M – 50 µl of pure methanol (control)

B. velezensis NC318 significantly ($p < 0.05$) inhibited the mycelial growth of *S. rolfsii*. An inhibition rate of 97% was detected with 50 µl of crude extracts. The results of antifungal activity of crude extracts of strain NC318 using the well diffusion method are shown in Figure 2B. A line of inhibition was formed around the wells containing the crude extracts, with an absence of mycelium development, in contrast to the mycelium around the wells containing methanol. In addition, the germination of sclerotia also plays a crucial role in the infection process of plant diseases. Therefore, the activity of suppression of sclerotia germination by crude extracts was also evaluated. *B. velezensis* NC318 was able to significantly ($p < 0.05$) inhibit the germination of *S. rolfsii* sclerotia compared to the control treatment (Fig. 2A). With 10 µl of crude extracts, a germination inhibition rate of 46.78% was observed whereas with 50 µl of crude extracts no germination was observed (Fig. 1). These results suggest that *B. velezensis* NC318 can inhibit mycelial growth and germination of *S. rolfsii* sclerotia by secreting antifungal compounds.

HPLC/MS analysis of metabolites secreted by strain NC318

To identify the secondary metabolites produced by strain NC318, the methanolic crude extracts were analyzed by high performance liquid chromatography-mass spectrometry (HPLC/MS). HPLC/MS chemical analysis revealed the presence of lipopeptides, polyketides and, siderophore (Table 1).

These metabolites were identified based on their observed peak m/z with those already known in the literature (Table 1). The mass peak profiles are shown in Figure 3, the ions with m/z values of 1058.66861 $[M\text{-}Na]^+$ and 1036.68632 $[M\text{-}H]^+$ were attributed to surfactin A C15 and surfactin A C16, respectively. In addition, chemical analysis revealed the presence of ions with m/z values of 1043.54892 $[M\text{-}H]^+$, 1079.54658 $[M\text{-}Na]^+$, and 1071.57994 $[M\text{-}H]^+$ which were assigned to iturin C14-16. Ions with m/z values of 1449.78229 $[M\text{-}H]^+$, 1463.79775 $[M\text{-}H]^+$, 1477.81313 $[M\text{-}H]^+$ were assigned to fengycin B C13, fengycin B C14, and fengycin B C16, respectively. The compounds with m/z values of 881.245 $[M\text{-}H]^-$ and, 581.35672 $[M\text{-}H]^+$, 271.31805 $[M\text{-}H]^+$, 511.25382 $[M\text{-}Na]^+$, 525.24476 $[M\text{-}Na]^+$, and 559.8010 $[M\text{-}H]^-$ were attributed to the siderophore bacillibactin and the polyketides bacillaen A, bacilysin, 7-O-malonyl macrolactin, 7-O-succinyl macrolactin A, and oxydifficidin, respectively. These results suggest that strain NC318 co-produces several families of secondary metabolites and these bioactive compounds play a key role in the antagonistic activity.

Discussion

Antagonistic bacteria of the *Bacillus* genus have received much attention in recent years as biocontrol agents against plant diseases. These endophytic microorganisms are potential components of a strategy for

Table 1. HPLC/MS analysis of secondary metabolites secreted by strain NC318

Families	Compounds	Observed peak [m/z]	Adduct	RT [min]	References
Lipopeptides	surfactin A C15	1058.66861	$[M\text{-}Na]^+$	28.06	(Mácha *et al.* 2021)
	surfactin A C16	1036.68632	$[M\text{-}H]^+$	28.6	(Chen *et al.* 2018)
	iturin A C14	1043.54892	$[M\text{-}H]^+$	17.3	(Chen *et al.* 2018)
	iturin A C15	1079.54658	$[M\text{-}Na]^+$	18.5	(Wang *et al.* 2021)
	iturin A C16	1071.57994	$[M\text{-}H]^+$	20.02	(Chen *et al.* 2018)
	fengycin B C13	1449.78229	$[M\text{-}H]^+$	19.37	(Wang *et al.* 2021)
	fengycin B C14	1463.79775	$[M\text{-}H]^+$	20.1	(Wang *et al.* 2021)
	fengycin B C16	1477.81313	$[M\text{-}H]^+$	20.72	(Chen *et al.* 2020)
Polyketides	bacillaen A	581.35672	$[M\text{-}H]^+$	23.04	(Chen *et al.* 2018)
	bacilysin	271.31805	$[M\text{-}H]^+$	23.82	(Chen *et al.* 2018)
	7-O-malonyl macrolactin A	511.25382	$[M\text{-}Na]^+$	15.56	(Li *et al.* 2021)
	7-O-succinyl macrolactin A	525.24476	$[M\text{-}Na]^+$	22.48	(Chen *et al.* 2018)
	oxydifficidin	559.28010	$[M\text{-}H]^-$	29.25	(Nifakos *et al.* 2021)
Siderophores	bacillibactin	881.245	$[M\text{-}H]^-$	14.43	(Nifakos *et al.* 2021)

managing plant pathogens (Ab Rahman *et al.* 2018). The antifungal or antibiotic compounds they produce are generally considered to be responsible for biocontrol activities. In this study, we showed that the metabolites produced by *B. velezensis* strain NC318 reduced the development, growth and germination of the fungal pathogen *S. rolfsii*. The crude metabolites extracted from the cell-free supernatant of strain NC318 showed a broad spectrum of antifungal activity on mycelial growth and germination of *S. rolfsii* sclerotia. This suggests that strain NC318 is capable of secreting bioactive antifungal compounds against the pathogen. Recent studies with other *B. velezensis* strains also describe this potential. For example, culture filtrates of *B. velezensis* strain LHSB1 resulted in inhibition of hyphal growth, sclerotia formation and germination of *S. rolfsii*, accompanied by the presence of hyphal abnormalities and damage to membrane integrity (Chen *et al.* 2020). According to Darma *et al.* (2016), crude extracts of *B. velezensis* strain BMB26 showed inhibition of *S. rolfsii* in *in vitro* and *in vivo* assays.

Furthermore, the results of our chemical analysis of strain NC318 crude extracts revealed the presence of different families of bioactive compounds that are known to exert antifungal or antibacterial activity against plant pathogens. Among the metabolites detected in our analysis, we found products synthesized by non-ribosomal pathways, namely lipopeptides (iturin, fengycin and surfactin), polyketides compounds (bacillaen, macrolactin, difficidin and bacilysin) and siderophore (bacillibactin). Similar results have been obtained in recent studies on other strains of *B. velezensis* (Teixeira *et al.* 2021; Wang *et al.* 2021). These are potent molecules that present specific antifungal activities. From these molecules produced by strain NC318, lipopeptides are known to exert great antimicrobial activity (Rabbee *et al.* 2019). For example, fengycin and iturin exert strong fungitoxic activity on fungi and this activity is induced by the modification of cell membrane permeability by inhibition of fungal sterol synthesis, morphological change and destruction of the fungal cell wall (Zouari *et al.* 2016). Fengycin and iturin were the major metabolites of *B. velezensis* BA-26 and both had strong antagonistic effects on *B. cinerea* (Wang *et al.* 2021). Surfactin, on the other hand, stimulates plant root tissue colonization, nutrient acquisition, biofilm formation and acts as an activator of plant defense mechanisms against several microbial pathogens (Yamamoto *et al.* 2015; Rabbee *et al.* 2019). They also function as wetting agents, reducing surface tension. Polyketides such as difficidin, bacilysin, macrolactin and bacillaene exhibit high antimicrobial activity against a broad spectrum of bacteria and filamentous fungi (Wu *et al.* 2015; Ortiz and Sansinenea 2020). The bacillibactin produced by strain NC318 are siderophores capable of suppressing fungal growth. These are iron chelating molecules and when produced by bacteria they appropriate ferric ions making them unavailable to phytopathogenic fungi, resulting in an iron depletion of the medium which inhibits the development of pathogens (Carmona-Hernandez *et al.* 2019). Iron is an essential component of various proteins and for many biochemical reactions. It plays a very important role in DNA

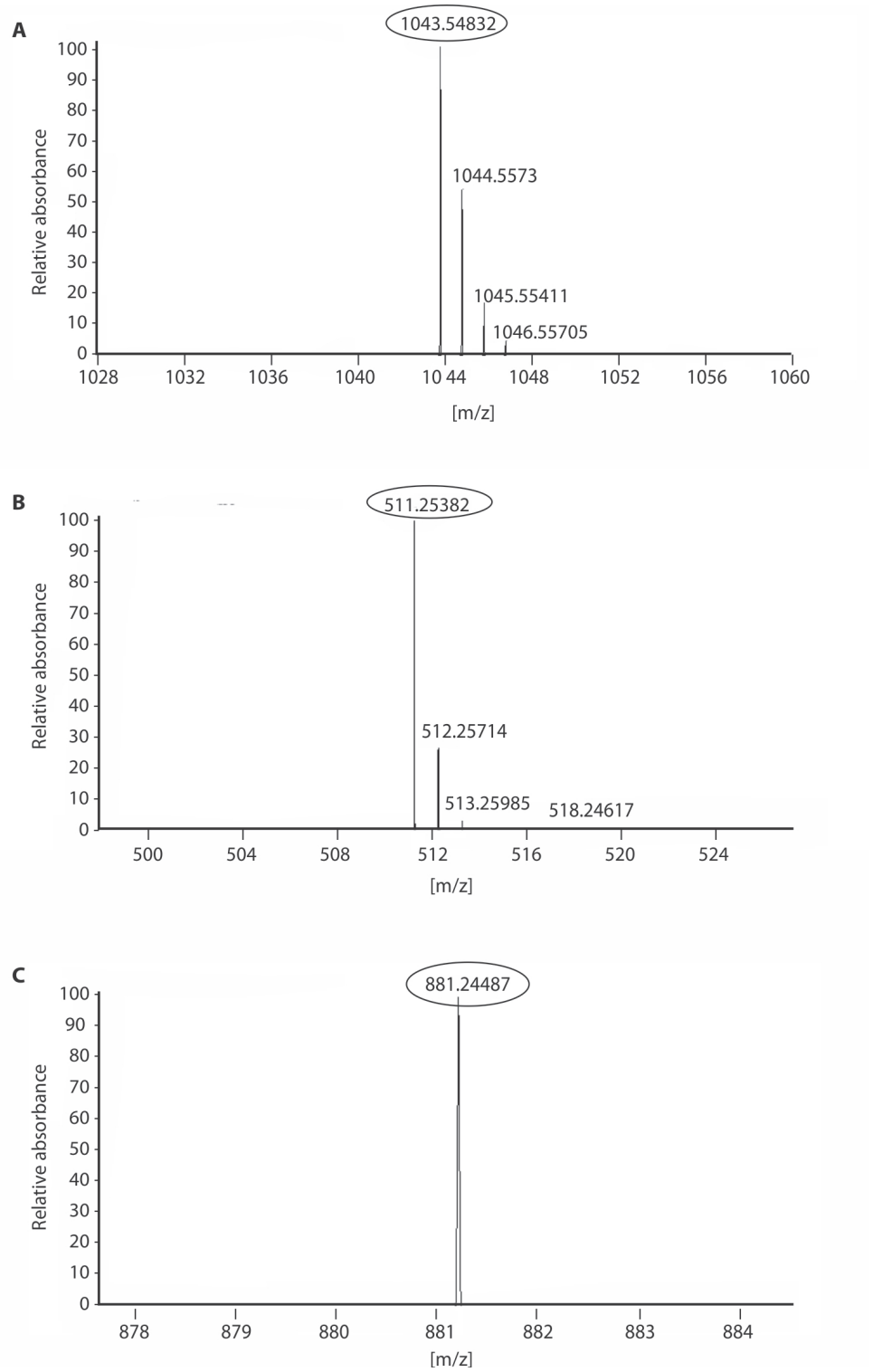

Fig. 3. Mass spectrometry (HPLC/MS) analysis spectra of metabolites secreted by NC318. A – iturin A C14; B – 7-O-malonyl macrolactin A; C – bacillibactin

replication and repair, oxygen transport, carbon metabolism, regulation of gene expression and oxidative phosphorylation where iron reduction/oxidation facilitates electron transfer in the respiratory chain (Caza and Kronstad 2013). Caulier *et al.* (2018) observed enhanced inhibition of *Phytophthora infestans* by siderophores produced by *Pseudomonas brenneri* and *B. amyloliquefaciens* under competitive conditions for iron acquisition. The results of this study revealed that the *B. velezensis* NC318, bacteria strain isolated from the soil rhizosphere, inhibits the growth and sclerotia germination of *S. rolfsii*. This is due, for example to the extracellular antifungal compounds produced by the bacterium such as fengycin, macrolactin and

bacillibactin. Thus, these results suggest that strain NC318 could be a useful biocontrol agent for the control of *Sclerotium* rot. However, further work is needed on the complete genome sequencing of *B. velezensis* NC318 to identify the gene clusters of the secondary metabolites, to understand the potential synergistic activities of the metabolites produced by *B. velezensis* NC318 and how they are able to influence plant defense mechanisms.

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