Bacillus mojavensis: biofilm formation and biochemical investigation of its bioactive metabolites

Ippolito Camele,[1] Hazem S. Elshafie,[1] Lucia Caputo,[2] Shimaa H. Sakr,[1] Vincenzo De Feo[2]

[1]School of Agricultural, Forestry, Food and Environmental Sciences, University of Basilicata, Potenza; [2]Department of Pharmacy, University of Salerno, Fisciano, Italy

Abstract

Bacillus mojavensis is an endophytic bacterium which has been reported to have fungicidal effect against some phytopathogens. Bioactive secondary metabolites produced by *B. mojavensis* could have promising applications in agricultural, food industry and clinical fields. The current research has been conducted to: i) evaluate the antagonistic effect of *B. mojavensis* isolate against some phytopathogens; ii) characterize chemically the principal bioactive substances produced by the studied isolate of *B. mojavensis* using Gas Chromatography-Mass Spectroscopy (GC-MS); iii) evaluate its ability to produce a biofilm using ELISA technique. Results showed that the studied isolate has an antagonistic activity against the majority of tested microorganisms. Results showed also that the studied isolated produced a biofilm in Supplemented Luria-Bertani Casamino acid (SLB) and Minimal Mineral (MM) medium. The substantial attached growth in SLB was significantly higher than MM media. GC-MS analysis revealed the presence of 9 compounds accounting 87.8% of the total extract, where oxygenated monoterpenes are the main constituents.

Correspondence: Vincenzo De Feo, Department of Pharmacy, University of Salerno, I-84084 Fisciano, Italy.
Tel.: +39.0899.69751 - Fax: +39.0899.69602.
E-mail: defeo@unisa.it

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Introduction

Bacillus mojavensis, that Roberts *et al.*[1] previously classified as *Bacillus subtilis*, was firstly isolated from Mojave desert (California, USA).[2] Several studies reported that all strains of this species have antagonistic activity against different fungi[2] and it is considered one of the most important endophytic biocontrol agents.[2-4] Several endophytes have been used in biological control and as plant growth promoters.[5,6] The growth promotion mechanisms of several endophytic bacteria include the production of volatile, diffusible bioactive substances[7-10] and plant hormones such as auxin, cytokinin and gibberellin.[11,12]

Recently, a lot of researchers all over the world have been interested to reduce the dependency on the synthetic chemicals as antimicrobial agro-pharmaceutical agents.[13] Worldwide, there is a great interest for the human health, animal welfare and also for environmental risks correlated to the excess consumption of commercial pesticides.[13,14] The use of natural pestiticides extracted from plant essential oils and obtained from microbial secondary metabolites is considered one of the most important eco-friendly strategy for reducing the environmental pollution and preserve the human health.[13,15]

On this regard, the current research is trying to biochemically characterize the secondary metabolites produced by *B. mojavensis* for eventually use them in natural products pharmaceutical industry. Bacon and Hinton[16] have reported that *B. mojavensis* demonstrated inhibitory effects against *Fusarium verticillioides* (Sacc.) by decreasing its toxin accumulation and disease incidence and also promoted the seedling growth of maize.

Biofilm is an important chemically complex structure, formed by various bacterial and fungal pathogens for regulating several biological activities (antibiotic resistance, metabolic processes, *etc.*).[17-19] The biofilm formation is regulated by quorum sensing phenomena by different bacterial species.[20] In addition, the biofilm plays an essential role for better adaptation of bacterial cells in different conditions.[21] Bacterial biofilms are associated with serious medical microbial infections that are resistant to antibiotics such *Pseudomonas aeruginosa*.[20,22] Most phytopathogenic bacteria, such as *Xylella fastidiosa*, form biofilm in order to

protect the bacterial aggregation, thus increasing the damages effects to the host plants.[23,24]

Many Authors have reported that lipopeptides and other related biosurfactants can induce the biofilm formation.[25,26] The investigation of bacterial biofilms for plant and human diseases give a clear sight about the bacterial behavior and infection and the possible way for its control. For this reason one of the main motivations of the current research is to study the ability of the studied isolate to form a biofilm and to evaluate the effects of some nutrient media and other growth conditions on its intensity.

Moyne *et al*.[27] studied the bioactivity of various species of *Bacillus* and results showed that they are able to produce remarkable distribution of three cyclic lipopeptides: surfactin, iturin and fengycin. Bacon and Hinton[2] reported that *Bacillus* spp., in general, are able to produce lipopeptides biosurfactants such as surfactin, pumilacidin, esperin, fengycin and iturin. The lipopeptides have a potential antifungal activity in managing infections caused by multidrug resistant and biofilm forming pathogens.[4] Surfactin is considered a characteristic feature of *Bacillus* spp. including *B. mojavensis*[28] and plays an essential role in interspecies competition, biofilm formation, cell motility, root colonization and plants systemic protection.[29]

In this research, antagonistic bacterial activity of *B. mojavensis* was investigated against *B. megaterium* de Bary, *Clavibacter michiganensis* Smith (G+ve), *Xanthomonas vesicatoria* Doidge, *Pseudomonas fluorescens* Flügge, *Pseudomonas savastanoi* (Janse) Gardan and *Pseudomonas syringae* pv. *phaseolicola* Van Hall (G-ve). Furthermore, antifungal activity has been also evaluated against *Monilinia laxa* (Aderh. & Ruhland) Honey, *Monilinia fructicola* (G. Winter) Honey, *Monilinia fructigena* Honey, *Fusarium oxysporum* Snyder and Hansen, *Rhizoctonia solani* J.G. Kühn, *Botrytis cinerea* Pers., *Aspergillus ochraceus* Wilhelm, *Penicillium digitatum* Sacc., *Sclerotinia sclerotiorum* (Lib.) de Bary., *Colletotrichum acutatum* J.H. Simmonds and *Cryphonectria parasitica* (Murrill) Barr. Biofilm formation was screened by measuring the absorbance at λ 540 nm of bacterial cells adherent to Microplate-*96* wells in two different nutrient media: supplemented Luria-Bertani Casamino acid (SLB) and Minimal Mineral (MM). Finally, the chemical characterization of the purified filtrate was carried out using Gas Chromatography-Mass Spectroscopy (GC-MS).

Materials and Methods

Tested bacteria

The tested bacteria were: *B. megaterium*, *C. michiganensis* (G+ve), *X. vesicatoria*, *P. fluorescens*, *P. savastano*i and *P. syringae* pv. *phaseolicola* (G-ve). All tested bacteria were cultured on agar King B (KB) media and incubated at 30±2°C for 72 h and stored as pure freeze-dried cultures (-20°C) in the collection of School of Agricultural, Forestry, Food and Environmental Sciences (SAFE), Basilicata University, Potenza, Italy.

Tested fungi

The tested fungi were: *M. laxa*, *M. fructicola*, *M. fructigena*, *F. oxysporum*, *R. solani* J.G. Kühn., *B. cinerea*, *A. ochraceus, P. digitatum*, *Sclerotinia sclerotiorum*, *C. acutatum* and *C. parasitica*. The above-mentioned studied fungi were maintained as pure cultures in the mycotheca of SAFE and re-cultured on Potato Dextrose Agar (PDA) at 24±2°C.

Antagonistic bacterial assay

B. mojavensis was evaluated for its antagonistic activity against target bacteria.[30] In particular, single small mass from fresh culture (24 h) of *B. mojavensis* were deposited in the centre of Petri dish containing KB as nutrient media. Successively, suspension of each tested bacteria (10^8 CFU·mL$^-$) was applied and, then, all plates were incubated at 30°C for 24 h. As negative control, for each treatment, three KB plates without *B. mojavensis* have been used. The diameter of inhibition zone was measured and the bacterial inhibition percentage was calculated (Equation 1):

$$BIP\ (\%) = 100 - [\frac{GC\text{-}GT}{GC} \times 100] \quad \text{Equation 1}$$

Where BIP: bacterial inhibition percentage; GC: average diameter of bacterial grown in cm (control); GT: average diameter of inhibition zone in cm (treatments).

Antagonistic fungal assay

Antifungal activity of *B. mojavensis* was evaluated following contact-phase method.[31] Ten µL of the bacterial suspension was deposited on a PDA-Petri dish previously inoculated with a fungal disc (0.5 cm^2). All plates were incubated at 22±2°C for 96 h. The diameter of fungal mycelium growth was measured in mm. Each treatment was carried out in triplicate. As negative control, for each treatment, three PDA plates without *B. mojavensis* have been used. The growth inhibition percentage (GIP) was calculated according to Zygadlo *et al*.[32] formula (Equation 2):

$$GIP\ (\%) = 100 \times \frac{GC\text{-}GT}{GC} \quad \text{Equation 2}$$

where GC: average diameter of fungus colony grown on PDA alone (control); GT: average diameter of fungus colony grown on PDA containing each treatment.

Biofilm formation assay

Biofilm formation assay has been carried out to reveal the ability of *B. mojavensis* to adhere the wells of Microplate-*96* (MaxiSorp™ Surface, Denmark) as explained by Conway *et al*.[20] The tested bacteria was cultured on Luria-Bertani (LB) media and 100 µL of bacterial suspension were injected in each well. Two types of media were employed: i) SLB composed of LB with 0.5% (w/v) and Casamino Acids, and ii) MM [(K_2HPO_4 , 10.5 g; KH_2PO_4% , 4.5 g; $(NH_4)2SO_4$, 1 g; Na_3C_6 $H_5O_7(2H_2O$, 0.5 g; $MgSO_4$ $7H_2O$, 0.2 g; dextrose, 5 g; agar 16 g l^{-1})]. Microplate-*96* were covered and incubated at 37°C for three different incubation period (24, 48 and 72 h). The plates were washed after that and stained with 125 µL of 1% (w/v) crystal violet for 15 min. it was added after that 200 µL ethanol (95%) to each well to release the stain. Absorbance of the remaining stain dye was measured at λ 540 nm using the *Elisa Microplate reader* instrument (DAS s.r.l., Rome, Italy). This assay was repeated twice with three replicate per each ±SDs.

Gas chromatography and gas chromatography/mass spectroscopy analyses

Preparations of bacterial cell-free culture filtrate

The studied bacteria were cultured at 30°C in Erlenmeyer flask-500 mL of MM broth media (150 mL). About 1.5 mL of *B. mojavensis* suspension (10^8 UFC·mL^{-}1) was transferred into the MM flask and then was putted in a shacked-incubator (180 rpm, 5 days, 24°C).

Extraction of diffusible secondary metabolites

The bacterial broth was centrifuged at 21.000 *g* (15 min). The supernatant was filtered by sterile Millipore (USA) filter 0.22 mm and subsequently lyophilized using CHRIST ALPHA 1-4 (B. Braun Biotech International, Germany) and stored at –20°C. Lyophilized aliquots of 300 mg were suspended in 10 mL sterile distilled water (SDW) and injected after that into a cartridge syringe (Strata C18-T) washed with 2 mL of methanol and 2 mL of SDW. Each cartridge was washed after that with 1 mL SDW and the purified bioactive substances were recovered by 1 mL of methanol. The purified filtrate was analyzed by GC and GC-MS for components identification.

Gas chromatography-flame ionization detector analysis

GC analyses were performed using Perkin-Elmer Sigma-115 gas-chromatograph (PerkinElmer, Waltham, MA, USA) with a data handling system and a flame ionization detector. The separation was achieved using a HP-5 MS fused-silica capillary column (30 m×0.25 mm, 0.25 µm film thickness). The operative conditions were as follows: Column temperature: 40°C, with 5 min initial hold, and then to 270°C at 2°C/min, 270°C (20 min); injection mode splitless (1 µL of a 1:1000 n-hexane solution). Injector and detector temperatures were 250 and 290°C, respectively. Analysis was also run by using a fused silica HP Innowax polyethylene glycol capillary column (50 m×0.20 mm, 0.25 µm film thickness). In both cases, helium was used as carrier gas (1.0 mL/min).

GC-MS analyses were performed on an Agilent 6850 Ser. II apparatus (Agilent, Roma, Italy), fitted with a fused silica DB-5 capillary column (30 m×0.25 mm, 0.33 µm film thickness), coupled to an Agilent Mass Selective Detector MSD 5973 (Agilent); ionization energy voltage 70 eV; electron multiplier voltage energy 2000 V. Mass spectra were scanned in the range 40–500 amu, scan time 5 scans/s.

The identity of oil components was established from their GC retention indices, by comparison of their MS spectra with those reported in literature[33-35] or those of authentic compounds purchased from Sigma Aldrich, Co., Milan, Italy; available in our laboratories by means of NIST 02 and Wiley 275 libraries.[36] Component relative concentrations were calculated based on GC area peaks normalization.

Statistical analysis

The obtained results from biofilm and antagonistic assays were statistically processed using Statistical Package for the Social Sciences (SPSS) version 13.0 (Prentice Hall: Chicago, USA, 2004). The analysis of variance one-way ANOVA and *Tukey* B Post Hoc multiple comparison tests have been performed with a probability of $P<0.05$.

Results and Discussion

Antagonistic bacterial activity

The results of antibacterial assay showed high antagonistic effect of *B. mojavensis* strain against *X. vesicatoria*, *P. savastanoi*, *P. fluorescens* and *P. syringae* pv. *phaseolicola* and a moderate effect against *B. megaterium* (Figure 1). *X. vesicatoria* and *P. savastanoi* were the highest significant inhibited bacteria and slightly higher than *P. fluorescens* and *P. syringae* pv. *phaseolicola* (Figure 1). On the other hand, *B. mojavensis* did not show any antagonistic activity against *C. michiganensis* (Figure 1).

Results demonstrated that tested G-ve bacteria have been inhibited significantly higher than G+ve ones in contrast to the general statement indicating that the systemic resistance of G-ve is more than G+ve ones. The high systemic resistance of G-ve is due to the structure of the outer cell membrane that composed of two layers of peptidoglycan and lipopolysaccharides whereas G+ve has only one peptidoglycan layer.[37,38] The obtained results are interesting in this regard for controlling some G-ve phytopathogens. The enhancement of antagonistic activity of the studied bacterium is explained by different mechanisms such as the increase of the cell membrane permeabilization and also the loss of cell ions which probably lead to the bacterial growth inhibition and hence the complete cell death.

Antagonistic fungal activity

Results demonstrated that *B. mojavensis* has antagonistic activity against most tested fungi (Figure 2). In particular, high significant antagonistic activity was observed for *P. digitatum*, *M. laxa*, *M. fructigena*, *C. acutatum*, *C. parasitica* and *S. sclerotio-*

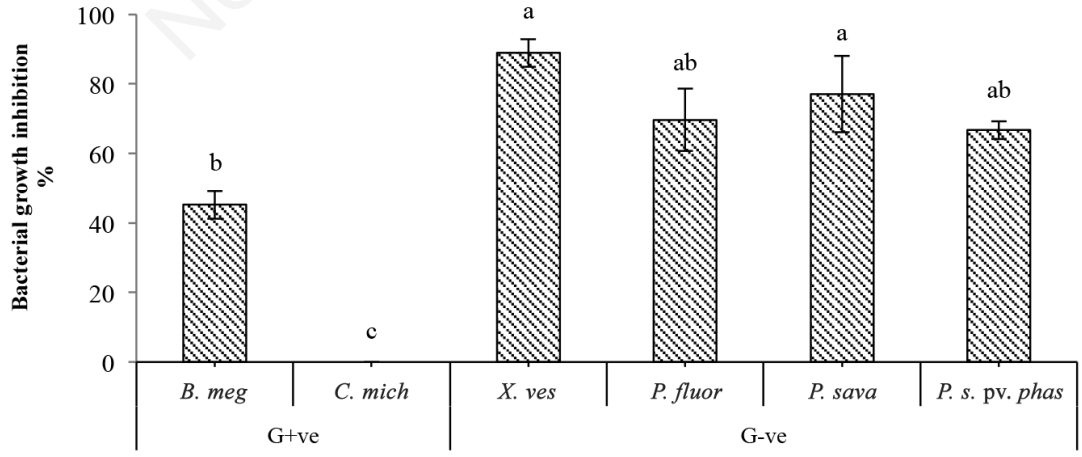

Figure 1. Bactericidal activity of *B. mojavensis* against some pathogenic bacteria. Bars with different letters indicate means values significantly different at $P<0.05$ according to Tukey post hoc test. Data are expressed as the mean of three replicates ± SDs. *B. meg*, *Bacillus megaterium*; *C. mich*, *Clavibacter michiganensis*; *X. ves*, *Xanthomonas vesicatoria*; *P. fluor*, *Pseudomonas fluorescens*; *P. sava*, *Pseudomonas savastanoi* and *P. s.* pv. *phas*, *Pseudomonas syringae* pv. *phaseolicola*.

rum. Moderate antagonistic fungal activity was observed for *M. fructicola*, *B. cinerea* and *F. oxysporum.* There is no notable inhibition activity against *R. solani* and *A. ochraceus.* The moderate activity against *B. cinerea* is presumptively due to its resistance especially in Agar-nutrient media.[14]

The potential antagonistic efficacy of the tested bacterium may be correlated to indirect antagonism of the produced bioactive substances which stimulate the host resistance response.[39] The biological effects of surfactin have been reported in several studies having an inhibition effect against some pathogenic fungi, bacteria, mycoplasmas and viruses.[40-42] On the other hand, the synergetic effect between two or more of the main chemical constituents of the studied isolate could have a distinctive role in its antagonistic activity.

The obtained results are promising especially in controlling *P. digitatum* which is considered one of the most aggressive post-harvest pathogen especially on *Citrus* species. Post-harvest diseases are the main reason of the fruit loss following harvesting. *P. digitatum* is responsible for 90% of citrus fruits lost in post-harvest causing green rot or mold in citrus fruits leading to severe economic losses worldwide.[43,44]

Biofilm formation

Results revealed that the tested isolate was able to form a biofilm in two different nutrient media SLB and MM (Figure 3). In addition, the biofilm formation ability was also assessed after 24, 48, and 72 h of the incubation at 37°C.

SLB and MM media have been used for evaluating the biofilm formation based on the bibliographic research which reported that a rich medium could accelerate the substantial attached growth.[20]

In particular, results showed a high significant variation of the biofilm formation between the tested media. The substantial attached growth of the studied bacterium in case of SLB media was significantly higher than MM media where the growth in SLB was ranged between 0.792 to 1.081 nm compared to 0.195 to 0.287 nm in case of MM (Figure 3). Further investigation showed that there are low significant differences within SLB media regarding the

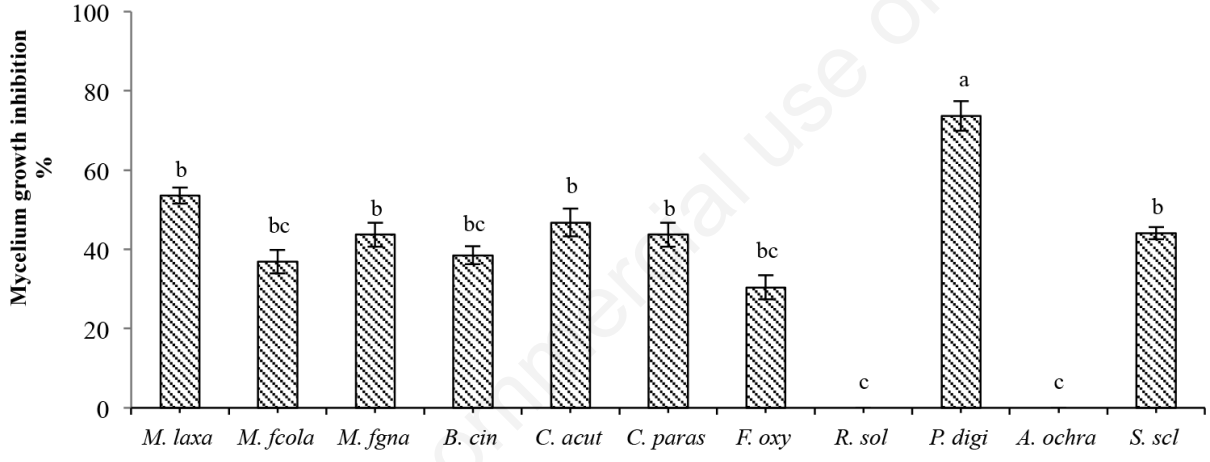

Figure 2. Fungicidal activity of *Bacillus mojavensis* against some phytopathogenic fungi. Bars with different letters indicate means values significantly different at P<0.05 according to Tukey post hoc test. Data are expressed as the mean of three replicates ± SDs. *M. laxa*, *Monilinia laxa*; *M. fcola*, *Monilinia fructicola*; *M. fgna*, *Monilinia fructigena*; *B. cin*, *Botrytis cinerea*; *C. acut*, *Colletotrichum acutatum*; *C. paras*, *Cryphonectria parasitica*; *F. oxy*, *Fusarium oxysporum*; *R. sol*, *Rhizoctonia solani*; *P. digi*, *Penicillium digitatum*; *A. ochra*, *Aspergillus ochraceus* and *S. scl*, *Sclerotinia sclerotiorum*.

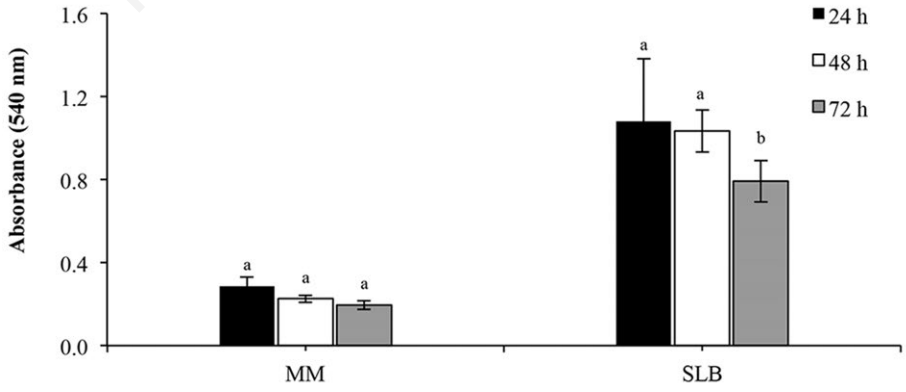

Figure 3. Biofilm formation by *Bacillus mojavensis* at different incubation times. Tested strain was grown in *96*-well polypropylene microtiter dishes in Supplemented Luria-Bertani Casamino acid (SLB) and Minimal Mineral (MM) media. Biofilm formation was assessed at 24 h (black bars), 48 h (white bars), and 72 h (grey bars). Each bar represents the average for three replicates. Bars with different letters for each nutrient media indicate means values significantly different at P<0.05 according to Tukey post hoc test. Data are expressed as the mean of three replicates ± SDs.

Table 1. Gas chromatography-mass spectroscopy analysis of metabolites extract from *Bacillus mojavensis*.

N.	Compound Name	%	KI*	KI°	Identification#
1	2,5-Diethenyl-2-methyl-tetrahydrofuran	6.0	759	916	1, 2
2	α-Pinene	6.8	927	939	1, 2, 3
3	Verbenene	1.1	942	967	1, 2
4	*trans*-Isolimonene	1.1	945	984	1, 2
5	δ-2-Carene	1.0	997	1002	1, 2
6	*m*-Cymenene	4.3	1000	1085	1, 2
7	*cis*-Thujone	4.1	1049	1102	1, 2, 3
8	*p*-Cymen-8-ol	27.1	1095	1182	1, 2
9	3-Thujanol	36.3	1180	1168	1, 2
	Total	**87.8**	-	-	-
	Monoterpenes hydrocarbons	14.3	-	-	-
	Oxygenated monoterpenes	67.5	-	-	-
	Other compounds	6.0	-	-	-

KI, Kovats retention index. *Linear retention index on a HP-5MS column; °Linear retention index on a HP Innowax column; #Identification method: 1=linear retention index; 2=identification based on the comparison of mass spectra; 3=co-injection with standard compounds.

incubation time, whereas there is no significant difference within MM media in relation to the incubation time. Surfactin might play an important role in regulation of the biofilm formation and other biological activities.[29]

On the other hand, the obtained results showed that the biofilm formation can be induced by modifying the nutrient media components and especially in case of the rich media SLB which has accelerated the growth rate of the studied bacterium and hence a strong formation of biofilm could be a probable result. As conclusion, the biofilm formation might help *B. mojavensis* against many microbial agents, furnish resistance to antibiotics and hence increasing its antagonizing effect.[20]

Gas chromatography/mass spectroscopy analysis

Table 1 shows the chemical composition of *B. mojavensis* extract in percent; compounds are listed according to their elution order on a HP-5MS column. Altogether, 9 compounds were identified accounting for 87.8% of the total extract. Oxygenated monoterpenes are the main constituents with *p*-cymen-8-ol (27.1%) and 3-thujanol (36.3%). Monoterpene hydrocarbons are present in lesser amounts, with α-pinene (6.8%) and 2,5-diethenyl-2-methyl-tetrahydrofuran (6.0%).

In our study the studied isolate produced 3-thujanol (36.3%) as main constituent by using KB medium which contain some other nutrient minerals such as dipotassium hydrogen phosphate and magnesium sulphate heptahydrate. Instead, Youcef-Ali *et al.*[10] reported that surfactin and iturin were the principal substances in methanolic extract obtained from the culture supernatant of *B. subtilis* and *B. mojavensis.*[10]

Monoterpenes, in general, are considered as plant secondary metabolites, but isoprenoids, chemical unit of terpenes, are synthetized also by prokaryotes and are essential for cells principal functions and to assure their growth and survival.[45] Many of compounds (monoterpenes and oxygenated monoterpenes) of the *B. mojavensis* extract are present also in plant essential oils and determine their antimicromicrobial activity against different microorganism.[46]

Our results are in agreement with previous studies reported that terpenoids can be isolated from endophyte cultures.[47,48] Moreover it has been reported that *B. subtilis*, a close relative of *B. mojavensis,* produces volatile compounds that play an important role in activation of plant defence.[49] Previous chemical investigations on *B. mojavensis* have highlighted the antibacterial effects of some furan derivatives such as furan, 2- furaldehyde, 2-furfuryl alcohol, 2-furoic acid and nitrofuran compounds against both G+ve and G-ve bacteria and less activity against *P. aeruginosa*.[50]

Conclusions

The genus *Bacillus* in recent years has gained a great attention for its antagonistic activity and production of several diffusible bioactive metabolites. The potential biological activity of the studied *B. mojavensis* might correlate with its ability to produce some specific lipopeptides biosurfactants and ooxygenated monoterpenes. The above-mentioned metabolites may play an essential role in several biological properties of *Bacillus* species such as biofilm formation, cell motility, root colonization and plants systemic protection. These metabolites could have also promising applications in agricultural, food industry and clinical fields. Further biological characterization assays seem necessary for the best application of the studied active metabolites on the large scale in pharmaceutical industry.

References

1. Roberts MS, Nakamura LK, Cohan FM. *Bacillus mojavensis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. Int J Syst Bacteriol 1994;44:256-64.
2. Bacon CW, Hinton DM. Bacteria in agrobiology: plant growth responses. In: Maheshwari DK, ed. Structure. Berlin-Heidelberg: Springer-Verlag; 2011. pp 21-40.
3. Gold SE, Blacutt AA, Meinersmann RJ, Bacon CW. Whole-genome shotgun sequence of *Bacillus mojavensis* strain

RRC101, an endophytic bacterium antagonistic to the mycotoxigenic endophytic fungus *Fusarium verticillioides*. Genome Announ 2014;2:e01090-14.
4. Jasim B, Sreelakshmi S, Mathew J, Radhakrishnan EK. Identification of endophytic *Bacillus mojavensis* with highly specialized broad spectrum antibacterial activity. Biotech 2016;6:1-20.
5. Silva HS, Tozzi JP, Terrasan CR, Bettiol W. Endophytic microorganisms from coffee tissues as plant growth promoters and biocontrol agents of coffee leaf rust. Biol Control 2012;63:62-7.
6. Herrera SD, Grossi C, Zawoznik M, Groppa MD. Wheat seeds harbour bacterial endophytes with potential as plant growth promoters and biocontrol agents of *Fusarium graminearum*. Microbiol Res 2016;186:37-43.
7. Compant S, Duffy B, Nowak J, et al. Use of plant growth promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol 2005;71:4951-9.
8. Glick BR. Plant growth promoting bacteria: mechanisms and applications. Scientifica 2012;1-15.
9. Santoyo G, Moreno-Hagelsieb G, del Carmen Orozco-Mosqueda M, Glick BR. Plant growth-promoting bacterial endophytes. Microbiol Res 2016;183:92-9.
10. Youcef-Ali M, Chaouche NK, Dehimat L, et al. Antifungal activity and bioactive compounds produced by *Bacillus mojavensis* and *Bacillus subtilis*. Afr J Microbiol Res 2014;8:476-84.
11. García de Salamone IE, Hynes RK, Nelson LM. Cytokinin production by plant growth promoting rhizobacteria and selected mutants. Can J Microbiol 2001;47:404-11.
12. Gutiérrez-Mañero FJ, Ramos-Solano B, Probanza A, et al. The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. Physiol Plant 2001;111:206-11.
13. Elshafie HS, Amato M, De Feo V, Camele I. Chemical composition and antimicrobial activity of Chia (*Salvia hispanica* l.) essential oil. Europ Food Res Technol 2018;244:1675-82.
14. Elshafie HS, Gruľová D, Baranová B, et al. Antimicrobial activity and chemical composition of essential oil extracted from *Solidago canadensis* L. growing wild in Slovakia. Molecules 2019;24:1-13.
15. Elshafie HS, Armentano MF, Carmosino M, et al. Cytotoxic activity of *Origanum vulgare* L. on hepatocellular carcinoma cell line HepG2 and evaluation of its biological activity. Molecules 2017;22:1-16.
16. Bacon CW, Hinton DM. Endophytic and biological control potential of *Bacillus mojavensis* and related species. Biol Control 2002;23:274-84.
17. Branda SS, Vik S, Friedman L, Kolter R. Biofilms: the matrix revisited. Trends Microbiol 2005;13:20-6.
18. Houry A, Briandet R, Aymerich S, Gohar M. Involvement of motility ad flagella in *Bacillus cereus* biofilm formation. Microbiol 2010;156:1009-18.
19. Abee T, Kovacs AT, Kuipers OP, van der Venn S. Biofilm formation and dispersal in Gram-positive bacteria. Curr Opin Biotech 2011;22:172-9.
20. Conway BD, Venu V, Speert DP. Biofilm formation and acyl homoserine lactone production in the *Burkholderia cepacia* complex. J Bacteriol 2002;184:5678-85.
21. Van Loosdrecht MCM, Lyklema J, Norde W, Zehnder AJB. Influence of interfaces on microbial activity. Microbiol Rev 1990;54:75-87.
22. Anwar H, Strap JL, Costerton JW. Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy. Antimicrob Agents Chemother 1992;36:1347-51.
23. Killiny N, Martinez RH, Dumenyo CK, et al. The exopolysaccharide of *Xylella fastidiosa* is essential for biofilm formation, plant virulence, and vector transmission. Mol Plant Microbe Interact 2013;26:1044-53.
24. Padmavathi AR, Bakkiyaraj D, Pandian SK. Biochemical and molecular mechanisms in biofilm formation of plant associated bacteria. In: Ahmad I, Husain FM, eds. Biofilms in plant and soil health. Hoboken, NJ: John Wiley & Sons Ltd; 2017. pp 195-214.
25. Davey ME, Caiazza NC, O'Toole GA. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PA01. J Bacteriol 2003;185:1027-36.
26. Kalia VC. Quorum sensing inhibitors: an overview. Biotech Adv 2013;31:224-45.
27. Moyne AL, Cleveland TE, Tuzun S. Molecular characterization and analysis of the operon encoding the antifungal lipopeptide bacillomycin D. FEMS Microbiol Lett 2004;234:43-9.
28. Snook ME, Mitchell T, Hinton DM, Bacon CW. Isolation and characterization of leu,7-surfactin from the endophytic bacterium *Bacillus mojavensis* RRC101, a biocontrol agent for *Fusarium verticillioides*. Agric Food Chem 2009;57: 4287-92.
29. Raaijmakers JM, De Bruijn I, Nybroe O, Ongena M. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiol Rev 2010;34:1037-62.
30. Lavermicocca P, Iacobellis NS, Simmaco M, Graniti A. Biological properties and spectrum of activity of *Pseudomonas syringae* pv. *syringae* toxins. Physiol Mol Plant Pathol 1997;50:129-40.
31. Elshafie HS, Camele I, Racioppi R, et al. *In vitro* antifungal activity of *Burkholderia gladioli* pv. *agaricicola* against some phytopathogenic fungi. Int J Mol Sci 2012;13:16291-302.
32. Zygadlo JA, Guzman CA, Grosso NR. Antifungal properties of the leaf oils of *Tagetes minuta* L., *T. filifolia* Lag. J Essent Oil Res 1994;6:617-21.
33. Davies NW. Gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicone and Carbowax 20M phases. J Chromatogr A 1990;503:1-24.
34. Adams RP. Identification of essential oil components by gas chromatography/mass spectroscopy. 4th ed. Carol Stream, IL, USA: Allured Publishing Corporation; 2007.
35. Goodner KL. Practical retention index models of OV-101, DB-1, DB-5, and DB-Wax for flavor and fragrance compounds. LWT-Food Sci Technol 2008;41:951-8.
36. McLafferty FW. Wiley registry of mass spectral data, with NIST spectral data CD Rom. 7th ed. New York, NY, USA: John Wiley & Sons; 1998.
37. Sakr SH, Elshafie HS, Camele I, Sadeek SA. Synthesis, spectroscopic, and biological studies of mixed ligand complexes of gemifloxacin and glycine with Zn(II), Sn(II), and Ce(III). Molecules 2018;23:1-17.
38. Elshafie HS, Sakr SH, Sadeek SA, Camele I. Biological investigations and spectroscopic studies of new Moxifloxacin/Glycine-Metal complexes. Chem Biodivers 2019;16:1-13.
39. Blacutt A, Mitchell TR, Bacon CW, Gold SE. *Bacillus mojavensis* RRC101 lipopeptides provoke physiological and metabolic changes during antagonism against *Fusarium verticillioides*. Mol Plant Microbe Interact 2016;29:713-23.

40. Peypoux E, Monmatin JM, Wallach J. Recent trends in the biochemistry of surfactin. Appl Microbiol Biotechnol 1999;51: 553-63.
41. Bonmatin J, Laprevote O, Peypoux F. Diversity among microbial cyclic lipopeptides: iturins and surfactins. Activity-structure relationships to design new bioactive agents. Comb Chem High Throughput Screen 2003;6:541-56.
42. Seydlova G, Svobodova J. Review of surfactin chemical properties and the potential biomedical applications. Cent Eur J Med 2008;312:123-33.
43. Macarisin D, Cohen L, Eick A, et al. *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. Phytopathology 2007;97:491-500.
44. Chen PS, Peng YH, Chung WC, et al. Inhibition of *Penicillium digitatum* and citrus green mold by volatile compounds produced by *Enterobacter cloacae*. J Plant Pathol Microbiol 2016;7:1-8.
45. Pérez-Gil J, Rodríguez-Concepción M. Metabolic plasticity for isoprenoid biosynthesis in bacteria. Biochem J 2013;452:19-25.
46. Chouhan S, Sharma K, Guleria S. Antimicrobial activity of some essential oils – present status and future perspectives. Medicines 2017;4:1-21.
47. Firáková S, Šturdíková, M, Múčková M. Bioactive secondary metabolites produced by microorganisms associated with plants. Biologia 2007;62:251-7.
48. Tan RX, Zou WX. Endophytes: arich source of functional metabolites. Nat Prod Rep 2001;18:448-59.
49. Shafi J, Tian H, Ji M. *Bacillus* species as versatile weapons for plant pathogens: a review. Biotechnol Biotechnol Equip 2017;31:446-59.
50. Dodd M, Stillman W, Roys M, Crosby C. The *in vitro* bacteriostatic action of some simple furan derivatives. J Pharmacol Exp Therap 1944;82:11-8.