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Abiotic stress resistance, plant growth promotion and antifungal potential of halotolerant bacteria from a Tunisian solar saltern

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ABSTRACT

The uses of halotolerant bacteria isolated from naturally saline habitats have the potential to be useful crop protection agents for plants in stressful conditions. These beneficial microbes generate several plant growth regulators and bioactive molecules, which enhance plant protection from adversities, such as plant pathogens, salts and metals stresses. In this study, 15 halotolerant bacterial strains endowed with important antimicrobial activities were isolated from Sfax solar saltern (Tunisia). All of these strains were characterized by biochemical and molecular tools aiming to investigate their *in-vitro* and *in-vivo* antifungal potentialities, plant growth promotion capabilities and metal tolerance abilities under saline stress condition. The 16S rRNA gene sequencing showed that the isolated strains were affiliated to different phylum and three species were described for the first time as plant growth promoting strains (*Idiomarina zobelli* FMH6v, *Nesterenkonia halotolerans* FMH10 and *Halomonas janggokensis* FMH54). The tested strains exhibited several potentialities: to tolerate high salt and heavy metal concentrations, to produce biosurfactants, exopolysaccharides and extracellular hydrolytic enzymes, to form biofilms and to liberate plant promoting substances. Eight strains were able to protect tomatoes fruits from the proliferation of the fungal disease caused by *Botrytis cinerea* and six strains improved plant vigor indexes. Principal component analysis showed an important correlation between *in-vitro* and *in-vivo* potentialities and two strains *Bacillus velezensis* FMH2 and *Bacillus subtilis subsp. spizizenii* FMH45 were statistically considered as the most effective strains in protecting plants from fungal pathogens attack and promoting the growth of tomatoes seedlings under saline and multi heavy-metals stress conditions.

1. Introduction

Soil pollution and degradation has become a serious problem with an impact on world food security. Increasing urbanization, industrial development and decline of the soil ecosystem negatively influence the growth, and crop yield of agriculture products (Akcil et al., 2015; Tirry et al., 2018). Moreover, soil contamination and chemical products increase the risk of antimicrobial resistance in pathogenic microorganisms and the potential for food spoilage (Heuer et al., 2009). Thus, the worldwide production is badly affected by biotic and abiotic stresses such as fungal diseases, salinity and heavy metals contamination (Jamil et al., 2014).

Currently, several efforts have been made to develop sustainable strategies useful to remediate agricultural soil, to restore soil fertility and to improve production. Microbial biotechnologies for improvement of plant growth and resistance to several biotic and abiotic stresses have attracted several researchers. Plant growth promoting bacteria (PGPB) have been the object of particular attention (Dimkpa et al., 2009; Majeed et al., 2018).

PGPB can influence plant growth and resistance directly or indirectly. Directly, when bacteria produced several kinds of bioactive molecules (antibiotics, siderophores, extracellular hydrolytic enzymes, *etc.*), able to reduce or avoid proliferation and harmful effects of phytopathogenic organism (biocontrol) (Glick, 1995; Mayak et al., 1999; Ahemad and Kibret, 2014). Indirectly, when PGPB synthesized compounds facilitating the uptake of essential nutrients from the soil and

Abbreviations: EPS, exopolysaccharides; PGPB, plant growth promoting bacteria; PGPR, Plant Growth Promoting Rhizobacteria

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act as elicitors in a wide spectrum of signaling pathways such as provision of nitrogen, sequestration of iron through siderophores, phosphate solubilization (Glick, 1995; Ahemad and Kibret, 2014).

Despite many reports that have described a number of effective bacterial strains (del Carmen Orozco-Mosqueda et al., 2018d; Radhakrishnan et al., 2017; Santoyo et al., 2016), soil contamination by different toxic substances (salt, heavy metal, *etc.*) could negatively influence the efficiency of these PGPB. Thus, bioprospecting novel microbial resources which can improve growth and resistance of plants in contaminated soils are required.

Solar salterns are harsh environments with higher salt concentrations than seawater and present intraspecific or intrageneric biotic and abiotic interactions (Kastman et al., 2016). The genetic diversity of bacteria allow them to develop adaptive strategies to survive in harsh and changing environments (Oren, 2008, 2002; Koizumi et al., 2004). Moreover, fluctuation of nutrients, abiotic parameters (salinity, temperature, pH, moisture, and light) and biotic factors (inter, intraspecific interactions and competitor sensing) are involved in complex interactions which have the potential to significantly affect the production of bioactive secondary metabolites (Tyc et al., 2016).

Therefore, bacteria derived from saline environments may have a great potential in agriculture field (Oren, 2008; Chen et al., 2010). They could play an important role in the biological control of foodborne diseases and food spoilage microorganisms, considered among the most important challenging issues in the food industry (Essghaier et al., 2014; Haldar and Sengupta, 2016). The prevalence of salt-affected soils (major obstacle for agriculture in arid and semi-arid regions in Tunisia) opens up a possible important role of these microorganisms in promoting plant growth and tolerance under salt stress conditions for a better survival and performance in the field (Nabti et al., 2015). Moreover, numerous halotolerant and moderately halophilic bacteria possess metal tolerance and play a major role in remediation of heavy metals contaminated soil (Voica et al., 2016).

Despite many studies that have described the potential uses of halotolerant PGPB as efficient biocontrol and biostimulator agents (Sadfi-Zouaoui et al., 2008; Tirry et al., 2018), the majority of these agents are generally able to protect plants only against a single abiotic or biotic stress. However, plants and soil are generally exposed to multi-stress factors. Hence, the present work was conducted in an attempt to isolate halophilic bacteria exhibiting both direct and indirect effects. The plant growth direct effect was evaluated *via* the production of antifungal metabolites and Biosurfactants. The indirect influence on plants was assessed by the detection of PGP traits, Biofilm formation and EPS production. Such kind of effective bacterial strains could be able to protect plant against fungal plant pathogens and to support plant growth in presence of salt stress and heavy metal contamination. To support this goal, the antibacterial and antifungal capabilities of the cultivable halotolarant bacteria isolated from Sfax solar saltern were assessed. Active strains were identified using 16S rRNA sequence analysis. Their ability to promote plant growth was evaluated *in-vitro* and verified *in-vivo* in presence of different concentrations of salt and heavy metals.

2. Materials and methods

2.1. Isolation of antimicrobial bacteria

Sfax solar saltern is geographically located in the center of Eastern Tunisian coast (34°39' N, 10°43' E) (Fig. 1). From this solar saltern and during the four seasons, five water samples were collected, 5 cm from the surface, from five different ponds: Seawater at the entrance of the solar saltern (E1), primary pond manufacturing NaCl (E2), secondary pond manufacturing NaCl (E3), final pond manufacturing NaCl (E4) and pond manufacturing $MgCl_2$ (E5). Physicochemical parameters (pH, salinity, temperature) of the five samples were recorded.

Ten-fold serial dilutions of the collected samples were spread on Zobell Marine agar (Zobell, 1941). The salinity of the media was adjusted from 35 g L^{-1} until 300 g L^{-1} by adding NaCl to isolate the maximum number of halotolerant bacteria. The plates were incubated at 30 °C for 3 weeks (Berrada et al., 2012; Orhan and Gulluce, 2015). Based on morphological approach (colony color, size, shape microscopic observation), a total of 306 halophilic isolates were tested for their antimicrobial potentialities by observing growth inhibition of bacteria or fungi. Five bacterial species were used as indicator strains: *Salmonella enteritidis* (Food isolate), *Listeria monocytogenes* (Food isolate 2132), *Pseudomonas aeruginosa* (ATCC9027), *Bacillus cereus* (ATCC14579) and *Staphylococcus aureus* (ATCC6536). Four indicator strains of fungi were used, including *Altenaria alternata* (CTM10230), *Fusarium oxysporum* (CTM10402), *Botrytis cinerea* (LBPES15) and *Pythium aphanidermatum* (LPAP 32). The indicator microorganisms were obtained from international culture collections and local culture collections of the Centre of Biotechnology of Sfax (CBS), Tunisia. After incubation, all plates were examined daily for the determination of the inhibition zones. All measurements were carried out in triplicate and the experiments were repeated at least three times. Global activity indexes for each tested strain were calculated according to the following formula:

GABI = total of inhibited pathogenic bacteria / total of tested pathogenic bacteria.

GAFI = total of active inhibited phytopathogenic fungi / total of tested phytopathogenic fungi.

With: **GABI**: Global antibacterial activity index.

GAFI: Global antifungal activity index.

2.2. Molecular taxonomy of the active isolates

Based on the biological tests, 16S rRNA genes of the hyperactive isolates were amplified using PCR and two specific primers: Forward primer 16F27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR reactions were performed in minicycler (Applied Biosystems) with 3 min at 95 °C for initial denaturation, 35 cycles including 30 s at 95 °C, 30 s at 53 °C and 1 min 30 s at 72 °C, and a final extension at 72 °C for 4 min (Salaün et al., 2010). Amplified PCR products were purified using bacterial DNA purification kit (FAVORGEN BIOTECH CORP ®), sequenced and identified using the EzBioCloud 16S database (Yoon et al., 2017). The 16S rDNA sequences were deposited in the Genbank database and accession numbers were obtained.

2.3. Effect of salt concentration on bacteria growth

The effect of salt concentration on the growth of active halophilic strains was determined as described by Joshi et al. (2008). Each bacterial strain was inoculated into 250 mL Erlenmeyer flask containing 50 mL Zobell marine medium with increasing salt concentrations (0, 10, 50, 100, 170, 220 and 300 g L^{-1}). All cultures, adjusted to 0.5 McFarland standards (10^8 CFU mL^{-1}) by Densimat spectrophotometer (BioMérieux, Italy), were kept for incubation on a rotary shaker (INNOVA ® 44) at 30 °C and 200 rpm for 24 h. Then, the optical density was measured at 600 nm to check the relative level of bacterial growth at each salinity concentration. All measurements were carried out in triplicate and the experiments were repeated at least three times.

2.4. Effect of heavy metals concentration on bacteria growth

The selected bacterial strains were tested for their resistance against four different heavy metals Cr (VI) ($K_2Cr_2O_7$), Ni (II) ($NiCl_2$), Cu (II) ($CuSO_4$), and Co (II) ($CoSO_4$) in presence of 15 g L^{-1} NaCl. Minimum inhibitory concentrations (MIC) were determined for each selected isolate using a two-fold serial dilution concentrations ranging from 160 mM to 0.75 mM. MIC corresponds to as the lowest concentration at which no viable colony-forming units (CFU) were grown after 48 h of

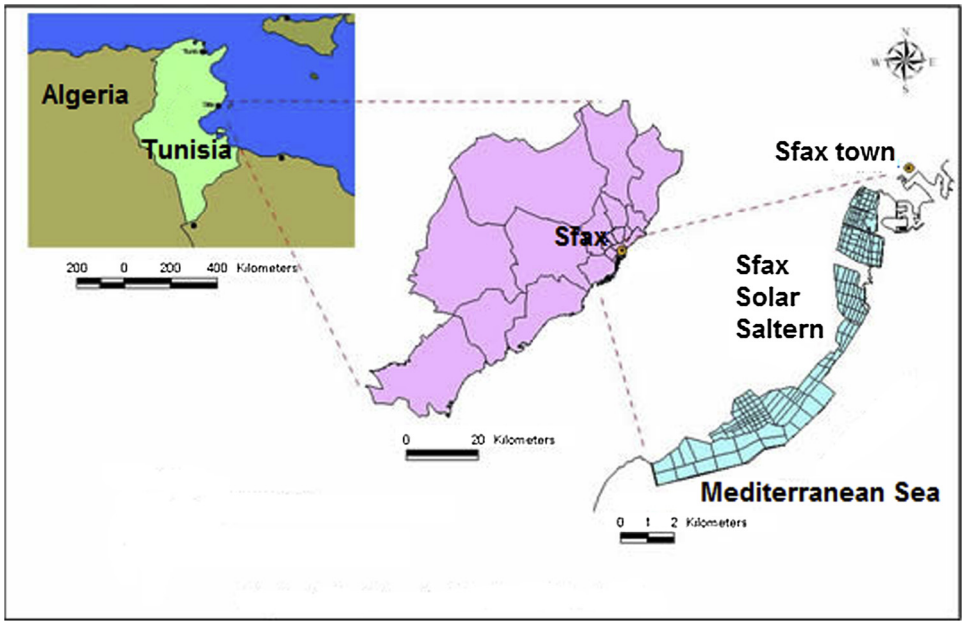

Fig. 1. Localization of Sfax Solar Saltern.

incubation at 30 °C (Mallick et al., 2018).

2.5. Biofilm formation

Biofilm formation was assessed using a previously described 96-well cell culture plate method (O'Toole et al., 2000). An optical density of 0.01 of each strain culture determined at 595 nm was inoculated in culture medium with 1.5% (w/v) NaCl (without shaking) for 48 h at 30 °C. One hundred microlitter of crystal violet solution (0.1%) was added to each well and kept for 20 min at room temperature in order to stain adhered cells. Excess of the crystal violet solution was removed, and all the wells were rinsed three times with distilled water. After that, according to the method of Frikha-Gargouri et al. (2017), 100 µL of dimethyl sulfoxide (DMSO) was added to each dry well and the plate was incubated for 20 min, then their optical densities were measured at 620 nm using a plate reader. All measurements were carried out in triplicate and the experiments were repeated at least three times.

2.6. Biosurfactant production

Biosurfactant production was performed according to the method previously described by Cooper and Goldenberg (1987). After centrifugation at 4500 rpm for 20 min, obtained supernatant from the culture medium containing 1.5% (w/v) NaCl were used for the surface tension measurement with a modified de Nouy apparatus. Emulsifier activity was measured in mm after addition of 2 mL of mineral oil to 2 mL of supernatant and incubation of samples for 24 h at ambient temperature. The commercial chemical surfactants "Triton X100" (1 g L^{-1}) and "TrisHcl" (50 mM) were used as positive control. The emulsion index was calculated after the division of the emulsion layer by the total height, and its multiplication by 100.

2.7. Exopolysaccharides (EPS) production

The 15 isolated bacteria were checked for the production of EPS in presence of 1.5% **(w/v)** NaCl. Cell-free supernatant of each strain was recovered by centrifugation for 20 min at 10,000 rpm and two volumes of ice-cold isopropanol were added. All mixtures were stored overnight at 4 °C, the precipitated material was collected by centrifugation for 20 min at 10,000 rpm and obtained pellets were dried at 100 °C (Mallick et al., 2018). Microbial extracellular polymeric substances (EPS) are composed of polysaccharides, proteins, humic substances and nucleic acids (Zeng et al., 2016). Therefore, each EPS fraction has been quantified for the proteins concentration using standard Bradford protein assay (Bradford, 1976) and for the total carbohydrate content by the phenol-sulphuric acid method (Dubois et al., 1956). This method consists of mixing 1 mL of EPS solution with 1 mL phenol 5% (w/v) and 5 mL of sulphuric acid 96% (v/v). OD was determined at 490 nm after incubation for 5 min in 100 °C and 30 min in the obscurity. The amount of total carbohydrate was calculated using glucose as standard.

2.8. Ability of active strains to produce plant growth promoting substances and hydrolytic enzymes in-vitro

Different agar media with 15 g L^{-1} of NaCl were prepared to detect the ability of the selected strains to produce plant growth promoting substances. Pikovskaya's agar media was used to test phosphate solubilization (Pikovskaya, 1948). Chrome azural-S (CAS) agar medium was adopted to test siderophore production (Alexander and Zuberer, 1991). Proteolytic activity was evaluated using Skim milk agar (Smibert and Krieg, 1994) and lipolytic potentiality was detected by mixing LB with 1% tween (Sierrea, 1957). A colony of each potent strain was inoculated on the different media. After incubation at 30 °C, positive results were indicated when clear zones appeared around colonies. All measurements were carried out in triplicate.

2.9. In vivo-tests

2.9.1. Phytotoxicity, phytostimulation and germination potentiality of treated tomato seeds exposed to saline and heavy metals conditions

Tomato seeds (*Solanum lycopersicum var.Rio grandi*) were sterilized using 0.1% (v/v) $HgCl_2$ for 5 min, then 70% (v/v) ethanol for 10 min followed with washes using distilled water. Sterilized seeds were treated with 10^8 spores mL^{-1} of the selected isolates for 30 min. For the phytotoxicity/ phytostimulation test, each ten imbibed seeds were placed in plates with filter paper and irrigated with 5 mL distilled water. Tomato seeds exposed to salt stress were irrigated with 5 mL of a salt solution containing 0.1 M NaCl. For the tomato seeds exposed to heavy metal stress, ten imbibed seeds were irrigated with a mixture solution containing 0.5 mM of four different heavy metals (Cr (VI), Cu (II), Co (II) and Ni (II)). Seeds used as control without bacterial treatment were treated with sterile distilled water, salt solution or a aqueous

mixture of the four heavy metals. Each treatment was repeated for 3 times. All plates were putted in dark at 24 °C (Ramadoss et al., 2013). After 5 days, the number of germinated seeds as well as their length was determined. Vigor indexes (VI) were calculated according to the formula of Abdul-Baki and Anderson (1973):

Vigor index = % Sg* Sl

With: **% Sg:Seedling** germination rate (%)
Sl: **Seedling** length **(cm)**

2.9.2. Tomatoe fruit bioassay

Antifungal efficiency of the 15 strains was tested against the grey mould disease caused by the fungus *B. cinerea*. Tomato fruits (*Solanum lycopersicum* var. *cerasiforme*) were sterilized with bleach (0.3%) for 5 min and rinsed with sterile distilled water. On each tomato fruit, a wound of 0.5 cm × 0.5 cm was created then each 5 fruits of uniform size were putted together. As a negative contol, five fruits were kept without any treatment. As a positive control, five fruits were inoculated with 10^5 spores mL^{-1} of *B. cinerea* and 10^8 CFU mL^{-1} spore suspension of each tested strain or the commercial biofungicide "Prevam" and "Biobac" (Kilani-Feki et al., 2016). Treated fruits were stored for 5 days at 25 °C then the necrosis width (W) and the necrosis depth (D) were measured. The penetration (P) of tissues was calculated according to the formula described by Lapwood et al. (1984):

P = (W/2 + (D-5))/2.

2.10. Statistical analysis

Analyses of variance (ANOVA) and of the principal component were done using the Statistical Package for the Social Sciences (SPSS V.11; SPSS Inc., Chicago, IL, USA). The one way analysis was carried out using the Duncan's multiple range test to compare mean values among treatments at the 5% level of significance ($p = 0.05$). The principal component analysis was performed for the different studied traits *in-vitro* and *in-vivo* as well as for the studied strains to assess the relationships between them.

3. Results

3.1. Physico-chemical parameters of the different ponds of the solar saltern

During the sampling campaigns, the physicochemical parameters of each pond were recorded (Table 1). Obtained results showed, during each season, temperature stability for all ponds and pH average of 7.5 for the first four ponds and 5.9 for the pond manufacturing $MgCl_2$ (E5). We noted also an increasing salinity from 40 g L^{-1} to 150 g L^{-1} along the salt ponds circuit of salt production. The minimal was noted in seawater entry pond of the solar saltern corresponding to the initial evaporation pond (E1). The maximal salinity was detected in the secondary pond manufacturing NaCl (E3) and the final pond manufacturing NaCl (E4).

3.2. Isolation of antimicrobial halobacteria

Isolation of halotolerant bacteria exhibiting antibacterial and/or antifungal activities was carried out at different salt concentration ranging from 35 g L^{-1} to 300 g L^{-1}. A total of 306 halophilic strains were selected based on the morphological characteristics of the colony and microscopic examinations (110 were isolated from winter samples, 52 from spring samples, 81 from summer samples and 63 from autumn samples). All these strains were screened for their antimicrobial activities. Based on the antimicrobial test and the activity indexes (GABI and GAFI) values, 15 different strains, showing a GABI and/or a GAFI equal or superior to 3, were selected and classified into three groups (Table 2). The first class harbors strains (FME1, FMP5 and FME7) able to inhibit only fungi proliferation (GAFI > 3 and GABI ≤ 1). The second one groups strains (FMH3z, FMH6 and FMH6v) with high antibacterial activity (GABI ≥ 3) and weak or absent antifungal one (GAFI ≤ 1). The last group includes nine strains (FMH2, FMH9, FMH10, FMH11, FMH12, FMH28, FMH45, FMH54 and FMH77) with broad antibacterial and antifungal spectrum.

3.3. Taxonomical identification of the antimicrobial strains

The 15 effective strains were identified using a molecular approach. Analysis of the 16S rRNA gene sequences indicated that isolates belong to three different phylums: *Firmicutes* (12 strains), *Actinobacteria* (1 strain) and *Proteobacteria* (2 strains) (Table 3). Among the 12 strains of *Firmicutes*, *Bacillus* was the dominant genus followed by the *Oceanobacillus* and *Halobacillus* ones. The other strains belonged to *Halomonas*, *Idiomarina* and *Nesterenkonia* genera.

3.4. Effect of salinity variation on bacteria growth

In order to study salt requirement of the selected strains, they were cultured at different salt concentrations (0; 10; 50; 100; 170; 220 and 300 g L^{-1} NaCl) (Fig. 2). The obtained results showed that they could

Table 1
Physicochemical parameters recorded in the different ponds of Sfax solar saltern during the four season. **T:** temperature; **C:** conductivity; **S:** salinity; **E1:** Seawater at the entrance of the solar saltern; **E2:** primary pond manufacturing NaCl; **E3:** secondary pond manufacturing NaCl; **E4:** final pond manufacturing NaCl; **E5**: pond manufacturing $MgCl_2$.

	Winter sampling				Spring sampling			
	T(°C)	C(mS/Cm)	S(g/l)	pH	T(°C)	C(mS/Cm)	S(g/l)	pH
E1	10	45.2 ± 0.2	42.5 ± 0.3	7.4 ± 0.2	18	43.3 ± 2.0	32.8 ± 0.6	7.3 ± 0.1
E2	11	74. ± 0.7	72.7 ± 1.6	7.2 ± 0.1	18	70.9 ± 3.4	57.4 ± 0.8	7.4 ± 0.1
E3	10	129.5 ± 1.3	150 ± 2.1	7.5 ± 0.2	19	136.6 ± 4.3	123.7 ± 1.2	7.3 ± 0.0
E4	10	131.9 ± 1.4	153.7 ± 2.7	7.4 ± 0.1	19	134.9 ± 1.0	121.8 ± 1.0	7.5 ± 0.2
E5	12	84.6 ± 0.5	82.9 ± 2.4	5.9 ± 0.0	19	49.1 ± 0.7	36.8 ± 0.3	5.6 ± 0.1
	Summer sampling				**Autumn sampling**			
	T(°C)	C(mS/Cm)	S(g/l)	pH	T(°C)	C(mS/Cm)	S(g/l)	pH
E1	26	65.4 ± 0.9	43.4 ± 1.2	7.6 ± 0.3	24	53.6 ± 1.1	36.2 ± 0.1	7.6 ± 0.0
E2	25	92.8 ± 1	66.5 ± 1.7	7.3 ± 0.1	23	82.4 ± 1.3	60.6 ± 0.3	7.4 ± 0.1
E3	27	186.1 ± 1.9	150.8 ± 2.6	7.3 ± 0.1	23	159.6 ± 3.1	136.3 ± 1.1	7.3 ± 0.1
E4	27	193.4 ± 1.9	158.8 ± 3.9	7.4 ± 0.2	24	164.7 ± 1.6	138.5 ± 1.2	7.5 ± 0.2
E5	25	117.9 ± 1.2	88.6 ± 1.7	6.1 ± 0.1	22	91.2 ± 0.8	69.9 ± 0.6	5.8 ± 0.1

Table 2
Capability of selected isolates to secret antimicrobial metabolites and PGP features.

Strains	Pond	Season	Antibacterial activity						Antifungal activity					PGP potentiality			
			P.a	*S.e*	*B.c*	*S.a*	*L.m*	GABI index*	*F.o*	*A.a*	*Bo.c*	*Py.a*	GAFI index*	Si	Phs	Pr	Li
FME1	**E1**	Summer	–	–	+	–	–	1	+	+	+	+	4	+	–	–	–
FMH2	**E4**	Winter	–	+	+	+	–	3	+	+	+	+	4	+	+	+	n.d
FMP5	**E3**	Spring	–	–	–	–	–	0	+	+	+	+	4	+	–	–	–
FMH6	**E1**	Winter	+	+	+	+	+	4	–	–	–	–	0	–	+	–	–
FME7	**E1**	Summer	–	–	–	–	–	0	+	+	+	+	4	+	+	–	–
FMH9	**E3**	Winter	–	+	+	+	+	4	–	+	+	+	4	+	+	+	+
FMH10	**E2**	Winter	–	+	+	+	+	4	+	+	+	–	4	–	+	+	+
FMH11	**E4**	Winter	+	+	+	–	+	4	+	+	+	+	4	+	–	+	+
FMH12	**E3**	Winter	–	+	+	+	+	4	+	+	+	+	4	+	+	+	–
FMH28	**E3**	Winter	–	+	+	+	+	4	–	+	+	+	4	+	–	–	–
FMH45	**E3**	Winter	–	+	+	+	–	3	+	+	+	+	4	+	+	+	+
FMH54	**E5**	Winter	–	+	+	–	+	3	+	+	+	+	4	+	–	+	–
FMH77	**E4**	Winter	–	+	+	–	+	3	+	+	+	+	4	+	+	+	+
FMH6v	**E1**	Winter	–	+	+	+	+	4	–	–	–	–	0	–	+	–	+
FMH 3z	**E1**	Winter	–	+	+	+	+	4	–	–	–	–	0	–	+	+	+
Pathogen/Substrate susceptibility (%)			13	80	87	60	75		75	80	80	73		73.3	66.6	60	46.6

+: active strain; -: non-active strain; n.d : not determined; ***P.a***: *Pseudomonas aeruginosa*; ***S.e***: *Salmonella enteritidis*; ***B.c***: *Bacillus cereus*; ***S.a***: *Staphylococcus aureus*; *L.**m***: *Listeria monocytogenes*; ***E.c***: *Escherichia coli*; ***F.o***: *Fusarium oxysporum*; ***A.a***: *Alternaria alternata*; ***Bo.c***: *Botrytis cinerea*; ***Py.a***: *Pythium aphanidermatum*, **GABI:** Global antibacterial activity index; **GAFI:** Global antifungal activity index, *:Activity index (total of active strains/total of tested strains); **0:** No inhibition; **1:** low inhibition (0 < GABI\GAFI < 25); **2:**medium inhibition (25 ≤ GABI\GAFI < 50); **3:** moderate inhibition (50 ≤ GABI\GAFI < 75); **4:** high inhibition (GABI\GAFI≥ 75). **Si:** siderophores production; **Phs:** Phosphate solubilization ; **Pr:** Protease production; **Li:** lipase production.

Table 3
Taxonomic characterization of selected isolates.

Strains	Identification	GenBank accession number
FME1	*Bacillus velezensis*	KX821761
FMH2	*Bacillus velezensis*	MG254519
FMP5	*Bacillus velezensis*	KX821759
FMH6	*Halobacillus dabanensis*	KX821762
FME7	*Bacillus flexus*	KX821760
FMH9	*Oceanobacillus picturae*	KX821763
FMH10	*Nesterenkonia halotolerans*	KX821764
FMH11	*Bacillus velezensis*	KX821767
FMH12	*Oceanobacillus kimchii*	KX821768
FMH28	*Bacillus thuringiensis*	KX821757
FMH45	*Bacillus subtilis subsp. spizizenii*	MG254520
FMH54	*Halomonas janggokensis*	KX821765
FMH77	*Bacillus clausii*	KX821758
FMH6v	*Idiomarina zobellii*	KX821769
FMH 3z	*Bacillus toyonensis*	KX821766

be classified into two groups based on their salt requirement. The first one **is** formed by 13 isolates able to be cultured in salt concentration ranging from 0 to 10% with an optimal growth conditions ranging between 0 and 5% of salt (Fig. 2). Consequently, they could be designated as halotolerant bacteria according to Margesin and Schinner (2001) classification. The second group harbors strains *Halobacillus kuroshimensis* FMH6 and *Idiomarina zobellii* FMH6v, requiring salt to grow and their optimal growth salinity was around 5%. These two strains could be classified as moderately halophilic bacteria as described by Kushner (1978). Based on those results, and as the glycophitic plants could not grow in presence of a salt stress exceeding 12 g L^{-1} (Brady and Weil, 2002), the salinity of 1.5% (w/v) has been adopted to study the potentiality of the 15 bacterial strains to produce plant beneficial substances.

3.5. Effect of heavy metal on bacteria growth

Minimum inhibitory concentration (MIC) of four different heavy metals (Cu (II), Co (II), Cr (VI), and Ni (II)) was examined at a concentration ranging from 1.25 to 160 mM. Tested strains showed an important resistance towards the four tested metals with a varied degree (Table 4). The six strains FME1, FMH2, FMH11, FMH28, FMH45 and FMH6v showed highest tolerance to heavy metals with MIC values ranging from 20 to 80 mM for Cr and Co and crossed 160 mM for Cu and Ni.

3.6. Biosurfactant, biofilm and EPS production

Biosurfactant production of studied strains was verified using two different methods: surface tension measurement and emulsifying activity evaluation (Fig. 3). Among the 15 tested strains, four strains FMH2, FMH11, FMH12 and FMH45 exhibited the lowest surface tensions which were statistically similar to the surface tension of the synthetic surfactant Triton X-100 (Fig. 3(a)). The screening of emulsifier potential indicated the presence of such activity in the majority of the isolates. Nine strains (FME1, FMH2, FMH6, FMH9, FMH11, FMH12, FMH28, FMH6v and FMH3z) exhibited emulsification index ≥ 40% against oils (Fig. 3(b)) indicating that the isolates produced a significant amount of emulsifier.

Biofilm formation varied from a strain to another (Fig. 4(a)). Statically, 8 strains (FMH1, FMH2, FMH6, FMH11, FMH12, FMH45, FMH77 and FMH6v) showed an important biofilm formation (OD_{595} ≥ 4.7) in presence of 15 g L^{-1} NaCl according to Duncan test. To confirm biofilm formation ability, EPS produced by the studied strains were extracted and **their** contents in proteins and carbohydrates were estimated. FMH2 and FMH45 could be considered as good producer of EPS since their EPS fraction contained the highest concentration of protein (7.28 and 6.25 µg mL^{-1}, respectively) and of carbohydrate (273.82 and 219.11 µg mL^{-1}, respectively) (Fig. 4(b) and (c)).

3.7. Detection of plant growth promoting substances in-vitro

Bacteria, which combine antimicrobial activities and PGP abilities, may contribute to the development of sustainable agricultural systems. For such purpose, in addition to their secretion of antimicrobial metabolites, the established halotolerant/halophilic selected bacteria were analyzed, *in-vitro,* for their capability to produce siderophores, protease and lipase, and to solubilise phosphate (Table 2). Only 66% of the tested strains, belonging to different genera, were able to solubilise phosphate. Around 60% and 47% of the active strains displayed *in-vitro* protease and lipase activities, respectively. Over 70% of strains exhibited a potential of ferrous chelation and produced siderophores.

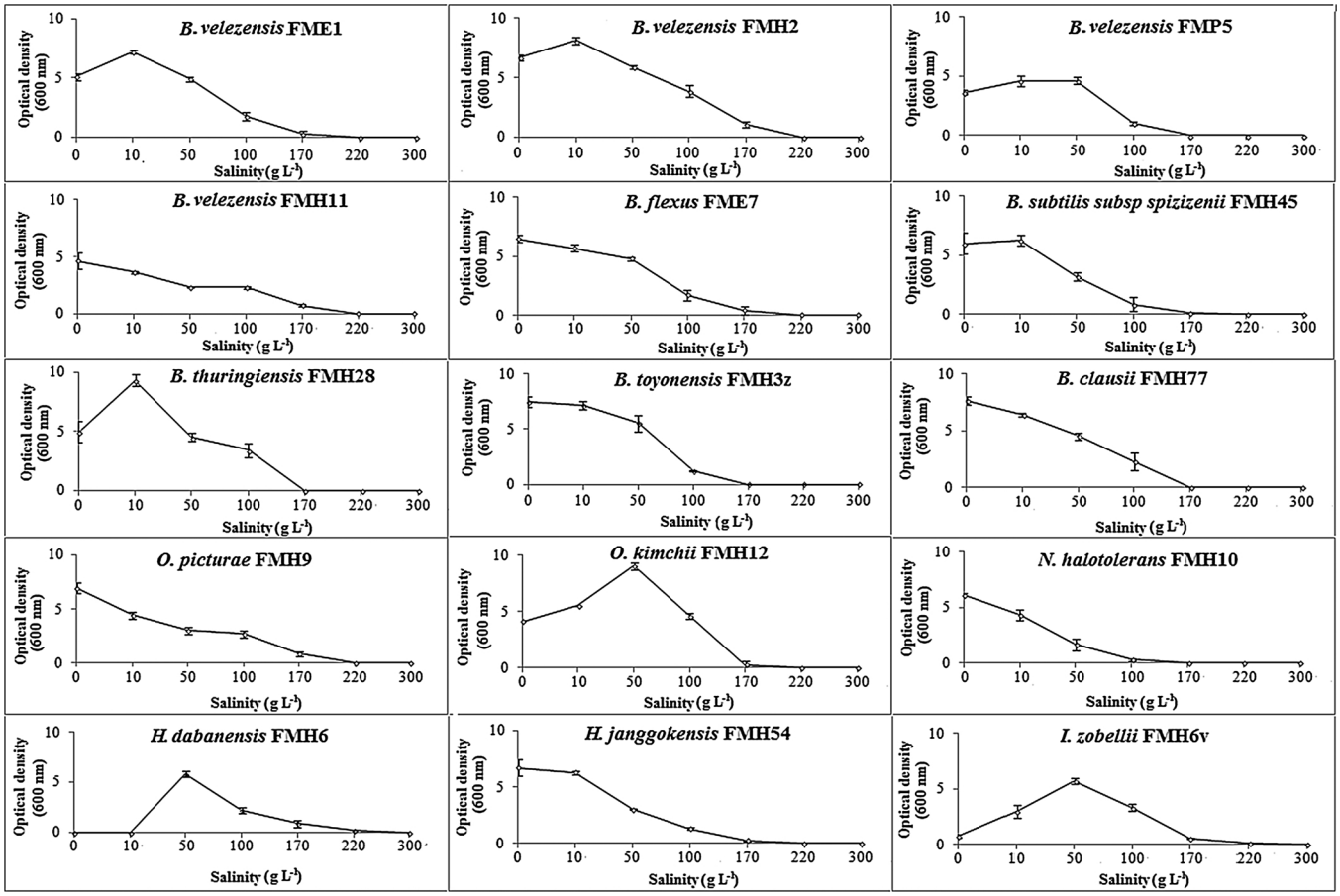

Fig. 2. Bacterial growth according to salinity concentration.

Table 4
MIC values of different metals against the 15 selected antimicrobial strains.

Strains	MIC values (mM)			
	Cr	Cu	Co	Ni
FME1	40	> 160	20	> 160
FMH2	20	80	40	> 160
FMP5	5	40	5	10
FMH6	20	> 160	10	160
FME7	5	20	5	40
FMH9	10	> 160	5	80
FMH10	5	40	10	40
FMH11	40	> 160	40	> 160
FMH12	10	40	10	40
FMH28	20	> 160	80	> 160
FMH45	20	> 160	40	> 160
FMH54	10	> 160	20	40
FMH77	5	5	5	80
FMH6v	20	> 160	40	> 160
FMH 3z	20	> 160	20	160

Interestingly, the two strains *B. velezensis* FMH2 and *B. subtilis subsp. spizizenii* FMH45 presented simultaneously the four tested PGP capabilities which indicated their possible role as biocontrol and biofertilizer agents.

3.8. Tomato fruit bioassay

The studied halotolerant bacteria were tested on harvested tomatoes for their effectiveness against *B. cinerea* causative agent of grey mould disease. For such purpose, tomatoe fruits inoculated with the phytopathogenic fungus and treated with bacterial spores were compared to untreated or treated fruits with the commercial biofungicides “Prevam” and “Biobac” (Fig. 5). Eight strains established a reduction in disease symptoms ranging between 50 and 90%. This result proved that those stains exhibited interesting biocontrol activities when compared to the commercial biofungicide “Prevam” and “Biobac” which inhibited only 40% and 41%, respectively of decay’s proliferation (Fig. 5(b)).

3.9. Ability of selected strains to stimulate germination and growth of tomato seeds under stressful conditions

The effects of the different selected strains on seed germination and root growth under different stressful conditions were studied and vigor indexes (VI) were calculated. The results of all treatments presented significant differences in VI compared with the control (Fig. 6). Majority of studied strains presented an important phytostimulation properties and the highest VI were recorded after treatment with the strains FMH2, FME7, FMH10, FMH45, FMH54 and FMH6v (Fig. 6(a)). In the presence of salt stress, seeds treated with FMH2 and FMH45 presented the most improved VI (134 and 166, respectively) while that of the untreated seeds exhibited a VI of 87.33 (Fig. 6(b)). After exposure to a high concentration of a mixture of heavy metals (0.5 mM), seeds treated with the strains FMH2, FMH28 and FMH45 showed the highest seed vigor index and could be considered more vigorous (Fig. 6(c)).

3.10. Principal component analysis

Loading plot of Principal component analyses showing that the majority of *in-vitro* variables (antifungal activity, biofilm formation, EPS production, PGP traits) are highly correlated with the *in-vivo* variables (severity decay reduction, phytotoxicity, germination in presence of salt or heavy metals) and define the first axis, while the

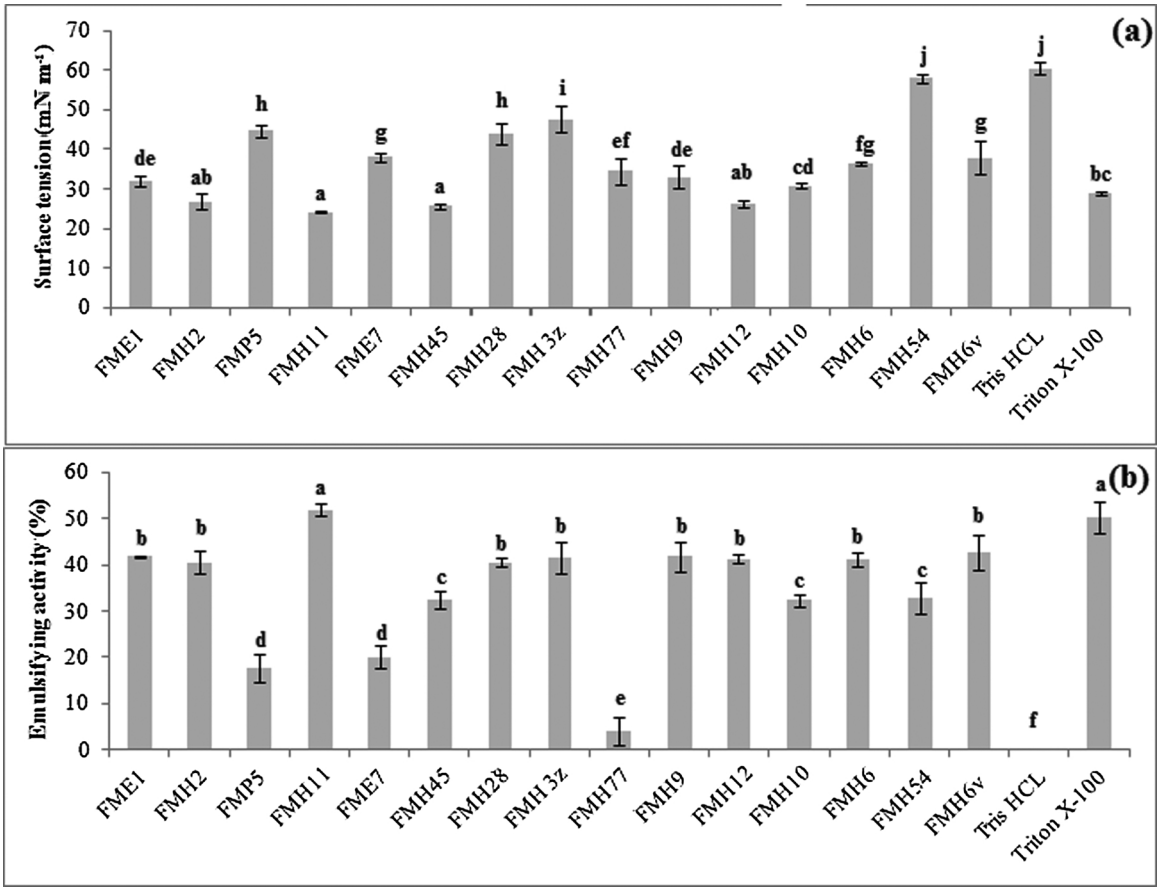

Fig. 3. Potentiality of the 15 isolates to produce biosurfactant. (a): Surface tension reduction; **b:** Emulsifying activity. Bars with the same letters indicate no significant differences between means at the confidence level 99.9% according to the Duncan's multiple range test (at $p \leq 0.05$). Errors bars indicate ± SE.

variables of antibacterial activity, MIC of heavy metals and emulsifying activity define the second axis (Fig. 7(a)). Globally, the two axes explain 56.5% of the variability divided into 36.1% of total variation for the first axis and 20.2% of variation for the second one. Plotting individuals showed that the two strains FMH2 and FMH45 are located in the right in the same area of the first axis indicating the important potential of those both strains in the protection of plant against biotic and abiotic aggressors (Fig. 7(b)).

4. Discussion

Biotic and abiotic stresses might induce metabolic imbalance in nutrient uptake, and affect plant growth and yield in many crops. However, these effects can be reduced by the presence of efficient PGPB strains in the rhizosphere which may exert a direct and/or indirect stimulation and protection on plant growth and development (Paul and Lade, 2014; Hayat et al., 2010; Lugtenberg and Kamilova, 2009). Bacterial strains, exhibiting tolerance to grow in saline and heavy metal contaminated soils, are able to produce several kinds of biological metabolites which could be considered as promising keys to sustain plant growth and protection under different stressful conditions. In this context and during this study, we tried to isolate bacterial strains endowed with multiple biological activities to promote plant growth under salt stress and in presence of phytotoxic concentration of heavy metals and to protect crops from fungus pathogens.

Fifteen active strains were selected, identified and classified into three groups according to their antifungal (GAFI index) and antibacterial (GABI index) activities. The first class included 3 bacteria (*B. velezensis* FME1 and FMP5 and *B. flexus* FME7) which were able to inhibit only fungi proliferation (GAFI > 3 and GABI ≤ 1). The second group included also 3 bacterial strains *B. toyonensis* FMH3z, *H. kuroshimensis* FMH6 and *I. zobellii* FMH6v. This group was characterized by a high antibacterial activity (GABI ≥ 3) and weak or absent antifungal one (GAFI ≤ 1). The last group included nine strains with a broad antibacterial and antifungal spectrum (*B. velesenzis* FMH2 and FHH11, *O. picturae* FMH9, *N. halotolerans* FMH10, *O. kimichi* FMH12, *B. thuringiensis* FMH28, *B. subtilis subsp. spizizenii* FMH45, *H. janggokensis* FMH54 and *B. clausii* FMH77).

Previous works mentioned that *B. toyonensis* had an antimicrobial activity against plant pathogens and several pathogenic gram-positive bacteria (Mahmoodi, 2015; Jayachandran et al., 2016). Some species belonging to the *Halobacillus* genus were shown to be active against several plant pathogens (Li and Yu, 2015). Moreover, several *Bacillus* strains, obtained from saline environment, were described as efficient agents in the eradication of phytopathogenic microorganisms. For example, Stabb et al. (1994) described a *B. thuringiensis* strain as a good producer of insecticidal metabolites as well as antimicrobial ones. Vijayaraghavan et al. (2012) and Sadfi-Zouaoui et al. (2007) reported a *B. subtilis* strain with antifungal activity. However, few studies reported the antifungal activity of *B.subtilis subsp. spizizenii* (Wen et al., 2015; Palande et al., 2015). Several studies described also members of the *Oceanobacillus* genus as promising sources for bioactive substances, able to prevent pathogens proliferation (DH and Dhundale, 2013; Jadhav et al., 2013). Pakpitcharoen et al. (2008) proved the inhibitory effect of *O. picturae* against *Fusarium sp.*

From the other side, our work reported for the first time the antimicrobial effect of *B. flexus* FME7, *I. zobellii* FMH6v, *H. janggokensis* FMH54 and *N. halotolerans* FMH10. In fact, Priyadarshini et al. (2013) reported that *B. flexus* were not endowed with antimicrobial activity. Singh et al. (2015) mentioned that *Idiomarina* genus did not exhibited

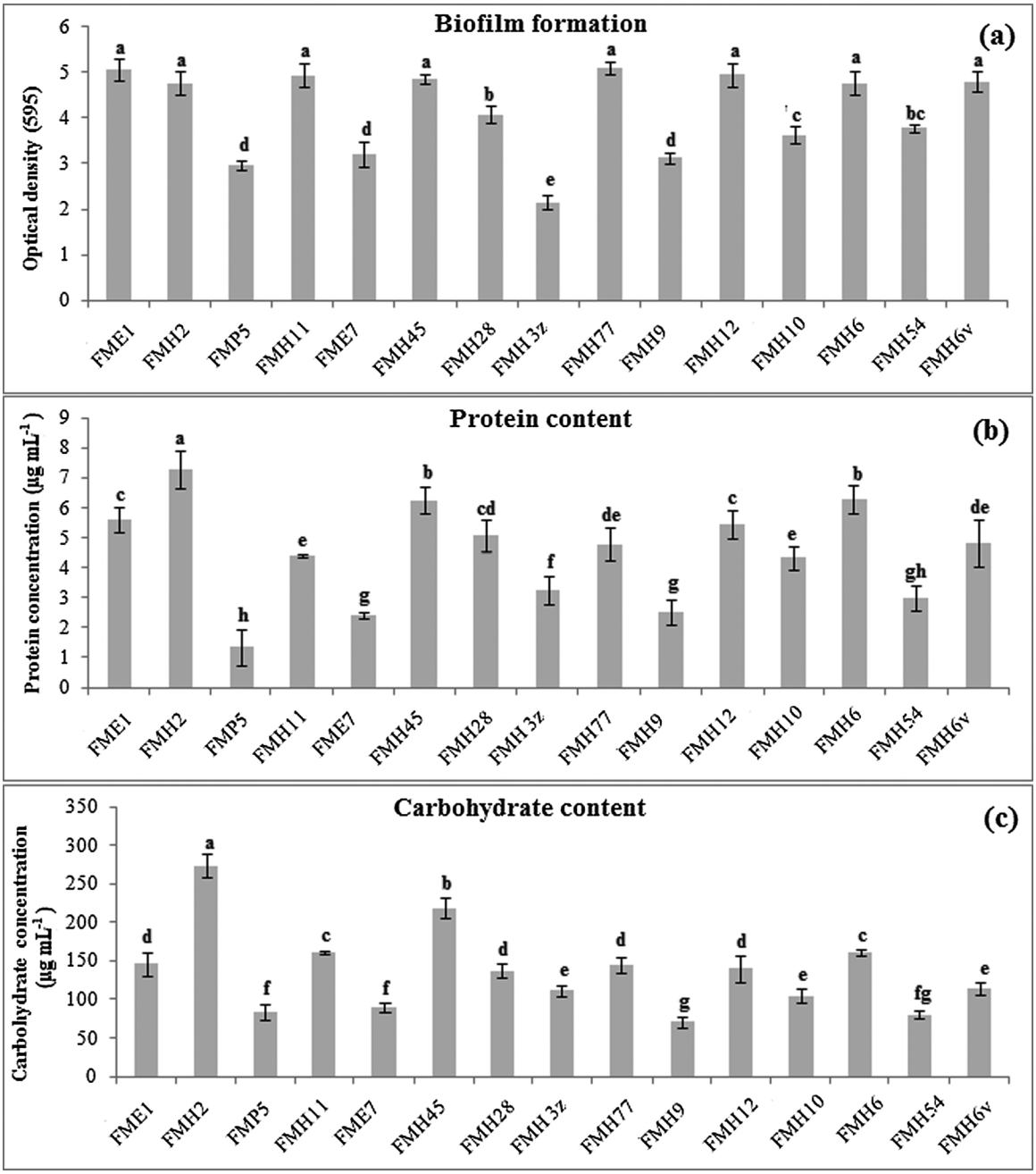

Fig. 4. Potentiality of the 15 isolates to produce biofilm and EPS. (a): Biofilm formation; (b): Protein content of the EPS extract; (c): Carbohydrate content in the EPS extract. Bars with the same letters indicate no significant differences between means at the confidence level 99.9% according to the Duncan's multiple range test (at $p \leq 0.05$). Errors bars indicate ± SE.

any antimicrobial ability. Moreover, the *Halomonas* genus was described as a hyperactive genus, which exhibited antibacterial, antifungal, antiviral, and anticancer metabolites (Bitzer et al., 2006; Donio et al., 2013) but no data concerning the antimicrobial activity of *H. janggokensis* could be found. The *Nesternkonia* genus was described only as producer of protease and ammonia (Soussi et al., 2015). This discordance could be due to the variation in geographical location, meteorological and seasonal variation (Chen et al., 2010).

Among the fifteen isolated strains, *H. kuroshimensis* FMH6 and *I. zobellii* FMH6v were considered as halophilic bacteria. Brettar et al. (2003) and Hua et al. (2007) confirmed these findings. They described also those two strains as moderately halophilic bacteria requiring around 6% NaCl for an optimal growth. Our results were also consistent with those reported by previous studies mentioning that strains endowed with antimicrobial belonged mostly to halotolerant or moderately halophilic class and exhibited a high potential to grow in different range of salinity crossing 100 g L^{-1} (Sadfi-Zouaoui et al., 2007; Chen et al., 2010).

Heavy metals are anthropogenic factors in the environment and their high level in agriculture soil upset the ecological balance (Chakraborty et al., 2015; Mallick et al., 2018). Multi-heavy metal resistant bacteria support the development of the resistance mechanisms of plant (Mallick et al., 2018). The 15 isolated strains showed a potential resistance to different heavy metals starting from 5 mM and up to 160 mM. Thus, the heavy metal tolerance of our strains would be an advantage for their better survival in salt and heavy metals co-contaminated sites. As reported in previous studies, numerous halotolerant and moderately halophilic bacteria possess metal tolerance thanks to

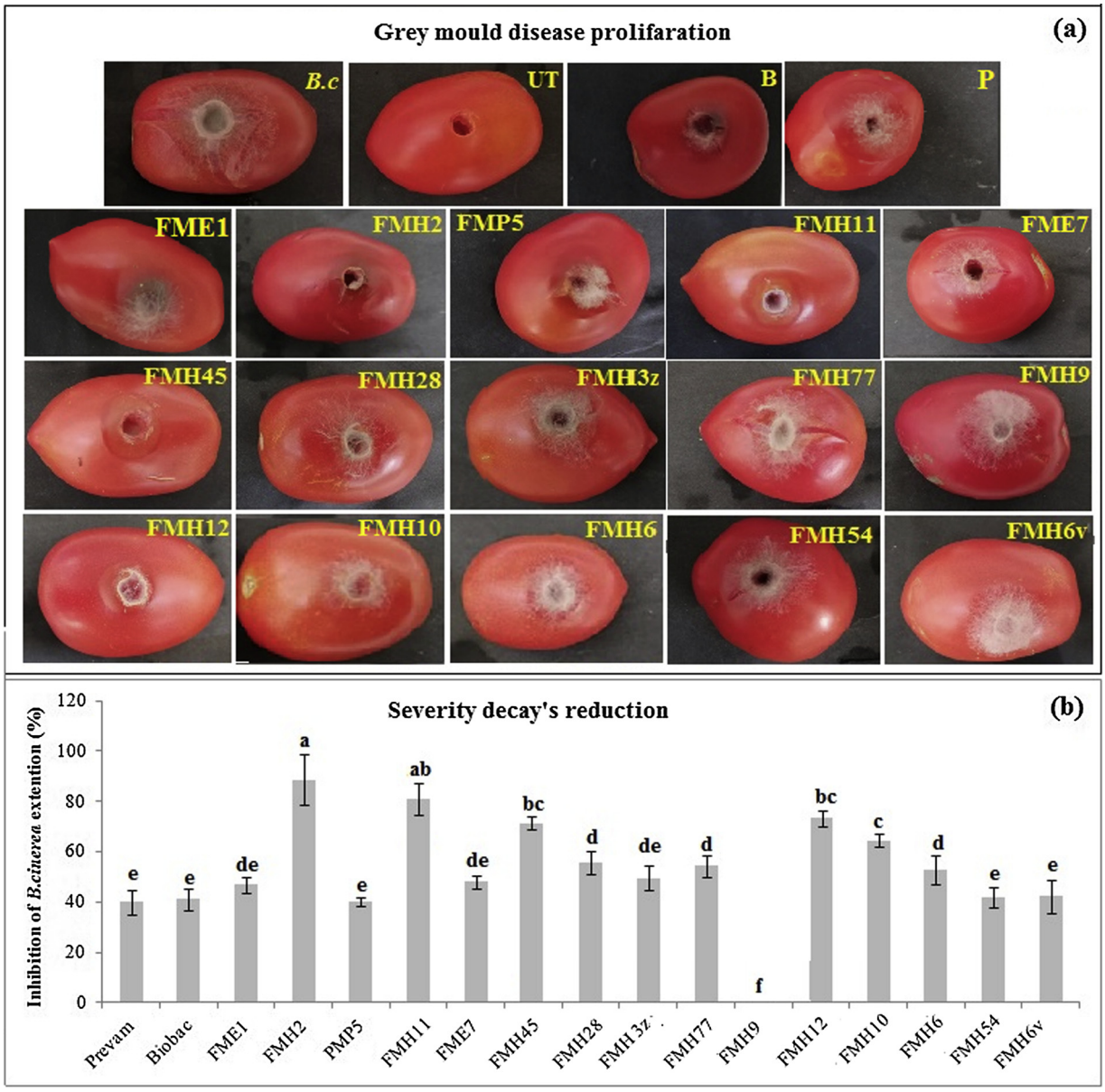

Fig. 5. Potentiality of the halophilic strain to protect harvested tomatoes from the grey mould disease. (a): Disease proliferation after treatments with studied strains and commercial biofungicides. ***B.c***: fruits inoculated only with *B. cinerea*, **UT:** untreated fruits nor with fungus neither bacteria; **B:** fruits inoculated with *B. cinerea* and the commercial biofungicide "Biobac"; **P:** fruits inoculated with *B. cinerea* and the commercial biofungicide "Prevam". (b): Reduction of the decay's severity in the harvested tomatoes (%). Bars with the same letters indicate no significant differences between means at the confidence level 99.9% according to the Duncan's multiple range test (at $p \leq 0.05$). Errors bars indicate ± SE.

their excellent sorption capacity and their numerous potential active chemosorption sites on the cell wall (Samanta et al., 2017; Voica et al., 2016). These bacteria are also known by their high-added values biomolecules production (biosurfactants, biofilm, EPS, *etc.*) playing vital role in motility and improving the agricultural soil quality by soil remediation (Sachdev and Cameotra, 2013; Mishra et al., 2017).

The biosurfactants action lies in their accumulation at the interface of immiscible compounds which decreases the interfacial surface energy and solubilises pollutants to improve degradation and desorption from soils (Batista et al., 2006). Extracellular polysaccharides (EPS) possess substantial quantity negatively charged residues prone and help to sequester heavy metal cations in a non-specific manner (Poli et al., 2011; Ayangbenro and Babalola, 2017). Moreover, bacterial EPS induce biofilm formation which enhances tolerance of bacteria to various environmental stresses by forming a protective layer and transforming toxic metal into non-toxic forms (Flemming and Wingender, 2001; Ayangbenro and Babalola, 2017).

For such purpose, it has been proposed to study the potentiality of the selected strains to produce biosurfactant and EPS as well as their ability to form biofilm. Candidates' strains showed important abilities to produce such biomolecules. Among the studied halotolerant strains, the production was found to be the highest using *B. velezensis* FMH2 and *B. subtilis subsp. spizizenii* FMH45 strains. Shekhar et al. (2015) mentioned that *Bacillus* species could be considered as good producers of biosurfactants/bioemulsifiers. Lu et al. (2018) reported the important role of EPS produced by *B. velezensis* FZB42 to colonize roots and induce systemic drought tolerance in Arabidopsis. Moreover, Altaf et al. (2017) mentioned that *Bacillus* species provide an opportunity for efficient use as bioinoculant as they could survive better under different stress conditions compared to other PGPR.

Bacteria exhibiting PGP abilities may offer, at the same time, an untapped resource for compounds to deal with the alarming ascent of

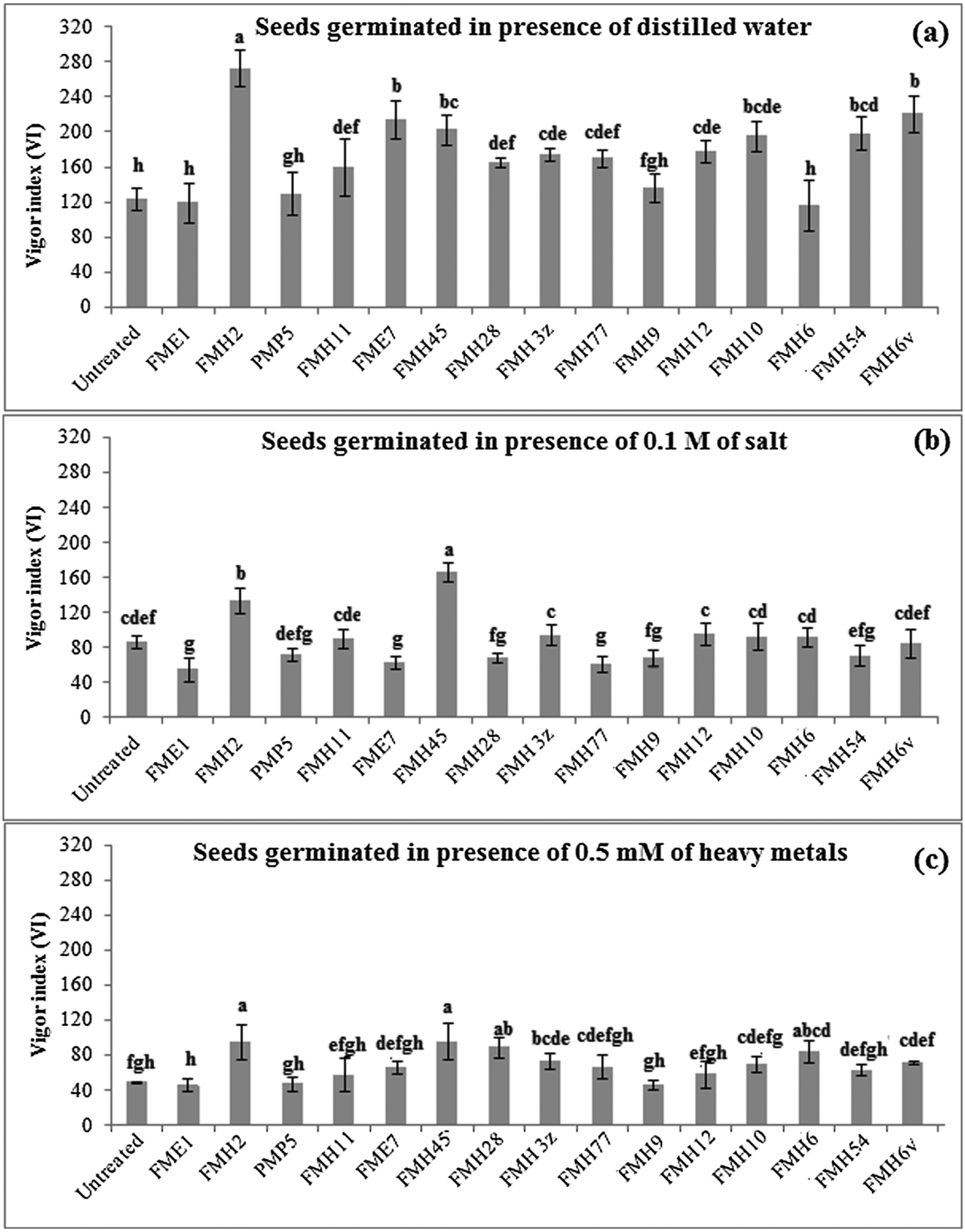

Fig. 6. Seeds germination in absence and in presence of salt and heavy metals stresses. (a): VI of germinated seeds in presence of distilled water; (b): VI of germinated seeds in presence of 0.1 M of salt; (c): VI of germinated seeds in presence of 0.5 mM of a cocktail of heavy metals. Bars with the same letters indicate no significant differences between means at the confidence level 99.9% according to the Duncan's multiple range test (at $p \leq 0.05$). Errors bars indicate ± SE.

phytopathogenic microorganisms and contribute in sustainable plant growth promotion (Hayat et al., 2010; Beneduzi et al., 2012). Our studied strains showed different production capacities of protease and lipase enzymes, siderophores, phosphate solubilization, and biological nitrogen fixation. The two strains *B. velezensis* FMH2 and *B. subtilis subsp. spizizenii* FMH45 presenting simultaneously the five tested PGP potentialities, were able to enhance plant growth. It was reported by Ahmad et al. (2008) that the simultaneous expression of different PGP traits, acting in a synergic manner, promotes plant growth. *In vivo* tests remain indispensable for checking the effectiveness of potential candidates (Fravel, 1988). Tests involving the fungal pathogen, the vegetal organs, antagonist strains and stressful conditions could be helpful to detect correlations between *in vitro* and *in vivo* traits and to select effective biological control agents against fungal diseases, salt stress and heavy metals intoxication.

The efficacy of the 15 strains in eradicating grey mould disease was

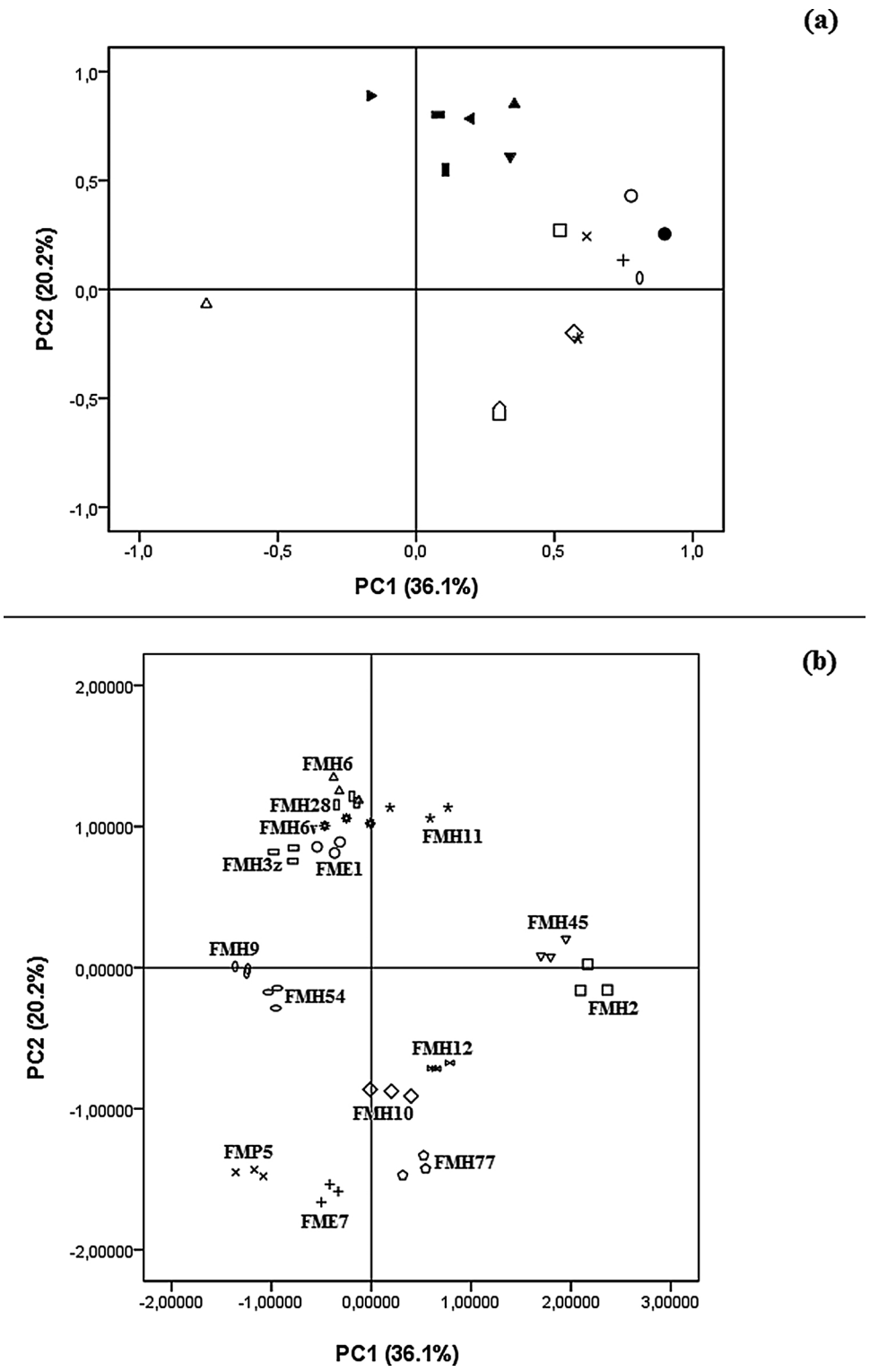

Fig. 7. Principal component analysis profiles indicating correlations between *in vivo* and *in vitro* variables and presenting strains position. (a): Plot of Principal component analyses based on the different results of the *in vitro* and *in vivo* tests. (○): protein content; (●): carbohydrate content; (×): Biofilm formation; (□): VI in presence of heavy metals; (+): VI in presence of salt: (◊): VI in presence of distilled water; (**0**): reduction of disease severity; (*): Total of PGP potentialities; (⌓): Total of antifungal activities; (▮): Total antibacterial activities; (Δ): Surface tension; (▬): Emulsifying activity; (▲): MIC $NiCl_2$; (►): MIC $CuSO_4$; (▼): MIC $CoSO_4$; (◄): MIC $K_2Cr_2O_7$. (b): Plot of the strain in the first two principal components.

determined. Eight strains, including 6 bacteria belonging to *Bacillus* genus, reduced between 50 and 90% the grey mould disease and presented a higher efficiency than the both commercial biofungicides "Prevam" and "Biobac" which inhibited only 40% and 41% respectively, of this disease. Our results confirm the observations reported by Sadfi-Zouaoui et al. (2008) and Berrada et al. (2012), that halotolerant *Bacillus* are particularly effective against *B. cinerea* on tomatoes. The promoting effects of the candidate strains on seed germination and root growth presented significant differences. Six strains showed the highest vigor indexes. Under salt or a multi-metals stresses, *B. velezensis* FMH2 and *B.* subtilis *subsp. spizizenii* FMH45 presented the most vigorous strains with an enhanced VI compared to that of the untreated seeds. Several studies reported the growth promoting effect of *B. velezensis* and *B. subtilis* species (Meng et al., 2016; Radhakrishnan and Lee, 2016; Radhakrishnan et al., 2017). However, few reports showed the efficiency of these species in the alleviation of salt or heavy metals stresses Qurashi and Sabri (2013) described *B. subtilis* RH-4 as efficient strain in improving of seed germination and plant growth in salt-injured

chickpea plants. No data was found concerning the intervention of these species in alleviation of salt and heavy metal stresses in plants. The beneficial effects of these strains could be related to its significant ability to grow and produce promoting substances, in presence of salt stress as has been proven in our *in vitro* investigation (Ndeddy Aka and Babalola, 2016).

Principal component analysis established that *in vivo* results were highly correlated with biofilm formation, EPS production, emulsifying activities and PGP features. Our results were in totally concordance with several previous studies mentioning that halotolerant bacteria, displaying simultaneously several PGP features, were found to be able to enhance germination rate, seedling emergence, root elongation and plants protection from biotic and abiotic stresses (Lugtenberg et al., 2002). These findings may be due to increased hormone synthesis and synergic activities, which would have triggered the activity of specific enzymes that promoted early germination and seedling vigor (Bharathi et al., 2004; Gholami et al., 2009). Mallick et al. (2018) reported that halotolerant bacteria able to produce EPS and form biofilm might be involved in seed germination and plant growth even in presence of important amount of heavy metals.

5. Conclusion

The 15 halophilic/halotolerant bacteria, isolated from Sfax solar saltern and selected based on their antimicrobial potentialities, exhibited simultaneously direct and indirect plant growth promoting potentialities thanks to their production of interesting biomolecules such as Biosurfactant, EPS, lipopeptides, Hydrolytic enzymes, siderophores, *etc*. The PGPB *B. velezensis* FMH2 and *B. subtilis subsp. spizizenii* FMH45 were able to protect tomato fruits from the grey mould disease and promote tomato seed germination and root elongation under salt and metal stresses. Those two strains could be considered as good agents for application in a sustainable agriculture in saline and multi-contaminated soils and their potentiality to promote plant growth and tolerance under salt stress *via* homeostasis modulation and antioxidant enzymes regulation could be the object of further studies.

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