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Antioxidant properties and antimicrobial activities of *Bacillus mojavensis* I4 lipopeptides and application in the biocontrol of potato dry rots caused by *Fusarium solani*.

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Running Title: Biocontrol of potato dry rots using I4 biosurfactant

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Abstract

Lipopeptides are diverse metabolites produced by various bacterial and fungal genera known for their antimicrobial and surfactant activities with diverse environmental, pharmaceutical application and also agronomic applications as biocontrol agents. In this study, the presence of NRPS genes in *Bacillus mojavensis* I4 was confirmed by PCR and this bacterial strain could produce diverse lipopeptides which belonged to fengycin, and surfactin families. The antioxidant activity of I4 biosurfactants was determined through four different *in vitro* assays. Furthermore, antimicrobial activity assays indicated that I4 lipopeptides exhibited marked inhibitory activity against several bacterial and fungal strains. The I4 lipopeptides were effective in the biocontrol of *Fusarium solani* that causes potato dry rot disease. Preventive treatment decreased the fungi penetration by almost 80 % after 15 days. This finding suggested promising application of I4 lipopeptides in potato tuber storage.

Keywords: Lipopeptide; Potato dry rots; Biocontrol; Biological activities; NRPS genes.

Introduction

Potato (*Solanum tuberosum* L.) ranks fourth among the most important food crops in the world after wheat and rice in terms of world food production and as an energy source [1] According to FAO, potatoes production is estimated about 376 million tons in 2016 and classified as the fourth crops production after corn (1060 MT), wheat (794 MT) and rice (740MT) worldwide. Potato tubers, the edible part of the plant, are affected by many plant diseases caused by bacteria, fungi, viruses and nematodes [2]. Fungal pathogens are the principal causes of crop losses before and after harvest [3]. *Fusarium solani* is one of the main soil-contaminating fungi that causes *Fusarium* wilt, which is a dry rot of tubers. This fungus penetrates in the potato tubers through wounds and causes drying and hardening. They form cavities lined with whitish mycelia. Dry rot of potatoes causes significant losses during storage.

Several effective fungicides had been used against pathogens, but as a long-term solution they were not effective owing to the expense, exposure risk, fungicide residue and environmental hazard concerns [4]. Therefore, the use of biocontrol based on environment-friendly microorganisms represents an attractive alternative for plant disease management [5]. Many research reports are available regarding, the use of *Bacillus* species as biocontrol agents [6] and their derived biomolecules including lipopeptides to inhibit fungal spore germination [6]. Recently, lipopeptides were employed as suitable alternatives to synthetic medicines with lower toxicity, biodegradability and ecological compatibility. Indeed, Antioxidant, antimicrobial as well as antitumoral properties of lipopeptides are equally exploited in cosmetic and pharmaceutical fields [7]. Biosurfactants are composed of 7 or 10 α-amino acids linked to one unique β-amino or β-hydroxy fatty acid with several families such as surfactin, iturin and fengycin [8]. Lipopeptides produced by *Bacillus* genus was synthesized by multi-enzyme templates know as non-ribosomal peptides synthetases (NRPSs). The

present study aims to evaluate the antioxidant and antimicrobial activities of the lipopeptides produced by *Bacillus mojavensis* I4 and prove their potentiality in potato tuber rot biocontrol against *Fusarium solani*.

Materials and Methods

Plant material

Potato tubers (*Solanum tuberosum* L) cv. Spunta produced and stored in our laboratory were selected free of wounds / rots and homogeneous in maturity / size, as much as possible.

Culture conditions and lipopeptides extraction

The bacterial strain, previously identified as *Bacillus mojavensis* I4, was isolated from a soil sample collected from Sfax city, Tunisia [9]. The production of I4 lipopeptides was performed in a liquid medium containing : glucose (30 g/L), glutamic acid (6 g/L), KH_2PO_4 (0.5 g/L), K_2HPO_4 (1 g/L), KCl (0.1 g/L), $MgSO_4$ (0.5 g/L), $FeSO_4$ (0.008 g/L), $CaCl_2$ (0.05 g/L) and 1mL of trace elements solution (4.4 mg/L $ZnSO_4$, 3.3 mg/L $MnSO_4$, 0.1 mg/L $CuSO_4$ and 1 mg/L NaBr) at pH 7 in 250 mL Erlenmeyer flasks containing 25 mL of culture medium and maintained for 48 h at 37 °C and 180 rpm. Under these conditions, *B. mojavensis* I4 was able to produce approximately 2.1 g/L of lipopeptides, estimated gravimetrically [10]. The lipopeptides were recovered from the culture broth by centrifugation and acid precipitation [11].

PCR detection and cloning of NRPS genes

Five pairs of degenerate primers (Table 1) were used to amplify the genes encoding for surfactin synthetase, fengycin synthetase, bacillomycin synthetase, mycosubtiline synthetase and kurstakine synthetase as previously described [12]. The PCR products were excised from Agarose gel and purified using the Pure Link Quick gel extraction Kit (Invitrogen) and cloned into the pGEM-T Easy vector (Promega) following the manufacturer instructions. Subsequently, cloned PCR products were sequenced using M13 universal sequencing primers

and the obtained sequences was analyzed by BLASTX against the non redundant protein database on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Antioxidant activity

DPPH radical-scavenging capacity

The DPPH radical-scavenging capacity of I4 biosurfactants was evaluated as described [13]. Briefly, the aliquots of the different concentrations of the lipopeptides (1 to 10 mg/mL) were mixed with 0.02 % DPPH in ethanol. The mixture was shaken vigorously, stood at room temperature for 60 min in the dark and the absorbance was measured at 517 nm. A lower absorbance of the reaction mixture indicated higher DPPH free radical-scavenging activity. BHA was used as positive control and the DPPH radical scavenging capacity was calculated using the following formula:

$$\text{Radical} - \text{scavenging activity } (\%) = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the sample) and A_{sample} is the absorbance of sample reaction (with the DPPH solution). The experiment was carried out in triplicate with similar results and mean values were reported.

Ferric reducing antioxidant power (FRAP) assay

The ability of I4 lipopeptides to reduce iron was determined as previously described [14] with slight modifications. 0.5 mL of I4 lipopeptides at different concentrations (from 1 to 10 mg/mL) were mixed with 1.25 mL of 0.2 M potassium phosphate at pH 6.6 and 1.25 mL of 1 % (w/v) potassium ferricyanide solution. The reaction mixtures were incubated for 20 min at 50 °C and stopped by the addition of 0.5 mL of 10 % trichloroacetic acid. After centrifugation at 2700 g for 10 min, 1.25 mL of the supernatant solution were mixed with 0.25 mL of 0.1 % ferric chloride and 1.25 mL of distilled water. After incubation at room temperature for 10

min, the absorbance was measured at 700 nm. The higher reducing power was indicated by the higher absorbance of the reaction mixture. Experiments were done in triplicates.

Iron (Fe^{2+}) chelating activity

For iron chelating activity, 0.1 mL of lipopeptides with a concentration range of 1 to 10 mg/mL was mixed with 0.45 mL of deionized water and 50 µl of 2 mM ferrous chloride. After 5 min of incubation at room temperature, the reactions were initiated by the addition of 200 µl of ferrozine solution (5 mM). The mixture was incubated for 10 min at room temperature and the absorbance measured at 562 nm. Control tube was prepared with the same manner with substituting the lipopeptides by water. EDTA was used as a positive control and the percentage of inhibition of ferrozine–Fe^{2+} complex formation was determined as follows:

$$\text{Chelating rate (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Where $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of biosurfactants. Experiments were done in triplicates.

DNA nicking assay

DNA nicking assay was evaluated using empty pGEM-T easy plasmid (Promega). A mixture of 10 µL of I4 lipopeptides (1 or 4 mg/mL) and 0.5µg of plasmid DNA were incubated for 10 min at room temperature followed by the addition of 10 µl of Fenton's reagent (30 mM H_2O_2, 50 µM L-ascorbic acid and 80 µM $FeCl_3$). After incubation for 5 min at 37 °C, the mixtures were analyzed by agarose gel electrophoresis.

Antimicrobial activity analysis

The antibacterial activities of I4 lipopeptides were tested against three Gram-negative (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883 and *Salmonella enterica* ATCC 43972) and four Gram-positive (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 43251 and *Micrococcus luteus* ATCC

4698) bacterial strains. The antifungal activities were evaluated against *Rhizoctonia solani*, *Fusarium solani* and *Botrytis cinerea.*

Antimicrobial activity was investigated as previously described [15]. A culture suspension (200 µL) of the tested microorganisms (10^8 spores/mL and 10^6 cfu/mL of fungal and bacterial strains, respectively) was spread on potato dextrose agar medium and Luria-Bertani (LB) agar, respectively. Subsequently, 60µl of lipopeptides solution (20 mg/mL) were added to each well (5 mm diameter), punched in the agar layer, allowed to diffuse and then incubated for 3 hours at 4 °C. Cultures were carried for 24 h at 37 °C or 72 h at 30 °C for bacterial and fungal strains, respectively. The diameters of growth inhibition zones were measured for the evaluation of antimicrobial activity. Moreover, Ampicillin (20 mg/mL) and carbendazim (0.5 mg/mL) were used as positive controls for bacterial and fungal strains, respectively. All tests were done in triplicates and the results were averaged.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of I4 lipopeptides was determined by liquid growth inhibition assay in a microtiter plate well [16]. MIC is defined as the lowest concentration of lipopeptides that completely inhibits the tested strain growth. Indeed, 0.1 mL of cell suspension were distributed in a microtiter plate well and then an equal volume of serial dilution of lipopeptides, made from 20 mg/mL solution, was added. The cells were enumerated on nutrient agar medium before and after incubation at 37 °C for 24 h.

Effect of I4 lipopeptides on *F. solani* mycelial growth and morphology

To evaluate the effect of I4 lipopeptides on the growth of *F. solani*, 10 ml of PDA medium containing I4 lipopeptides at different concentrations (0.5, 3.0 and 5.0 mg/mL) were spread on Petri dishes (diameter 6 cm). After solidification, an agar disk (6 mm) containing the mycelium of *F. solani* was deposited in the center of each Petri dish. After 10 days of

incubation at 30 °C, the mycelial growth inhibition (MGI) was calculated according to the following formula:

$$\text{MGI (\%)} = \frac{\text{diameter of fungal colony in control} - \text{diameter of fungal in treatment}}{\text{diameter of fungal colony in control}} \times 100$$

To investigate the effect of I4 lipopeptides on the mycelium morphology, a microscopic observation of mycelium near the zone of inhibition was carried (× 40 magnifications). Non treated mycelium was used as control.

Application of I4 lipopeptides in potato tuber rots biocontrol

To evaluate the capacity of I4 lipopeptides in the biocontrol of *F. solani* dry rot, potato tubers were surface sterilized in sodium hypochlorite (0.5 %) for 10 min, washed using sterile distilled water several times and then dried at room temperature. A volume of 0.1 mL of spore suspension containing 10^7 spores/mL of *F. solani* was poured into a well perforated in the tubers with a sterile cork borer. The treatment of potato tubers with different concentrations of I4 lipopeptides was evaluated by two different methods. The preventive method which consisted on the addition of 0.1 mL I4 lipopeptides (3 and 5 mg/mL) in the wells 24 h before the infection by fungi while in the curative method the lipopeptides solutions were added at 24 h post infection. Sterile distilled water and the commercial fungicide carbendazim were used instead of I4 lipopeptides as negative and positive control, respectively. Tubers were incubated in sterile plastic bags at 30 °C for 15 days at high relative humidity. Meanwhile, the rot development was evaluated by measuring the diameter of *F. solani* spreading lesions. After treatment, tubers were longitudinally cut and the parameters of dry rot induced (maximal width (w) and depth (d)) were recorded. The penetration of the pathogen into tubers was measured as follow [17].

$$\text{Penetration (mm)} = (\frac{w}{2} + (d - p))/2$$

Where:

199 *w* width of dry rot (mm)

200 *d* depth of the dry rot (mm)

201 *p* depth of the inoculation well (mm)

202 **Statistical analysis**

203 The data reported here were mean values with standard deviation. Mean values of three
204 biological replicates obtained from each experiment were compared using the Tukey's HSD
205 tests. Moreover, Pearson's correlation coefficient was employed to calculate the correlations
206 between data. All analyses were carried using JMP software version 10 (SAS INSTITUTE).

207 **Results and discussion**

208 **Detection of NRPS genes in *B. mojavensis* I4**

209 PCR was used to detect the presence of NRPS genes in the I4 strain. Positive amplification at
210 expected size was obtained for the tested primers suggesting the presence of five NRPS genes
211 (Fig. 1). BLASTX analysis of each amplified NRPS gene sequences (Table 2) showed that the
212 fragment amplified by As1-F/ Ts2-R primers pair showed 91 % identity with surfactin
213 synthase gene from *Bacillus subtilis*, which is characterized as surfactin-producing strains
214 [18]. Similarly, amplified fengycin DNA fragment (primers Af2-F/ Tf1-R) shared high
215 similarity (95 %) with fengycin synthetase from *B. subtilis* (Table 2). Moreover, PCR
216 fragments corresponding to Bacillomycins, Mycosubtilin and Kurstakin showed significant
217 homology with different peptide synthetases with a homology higher than 88% (Table 2).
218 These founding suggested that I4 strain could produce surfactin and fengycin. Indeed,
219 previous HPLC analysis revealed that surfactin is the major compound in the lipopeptides
220 mixture produced by I4 strain [19]. In this sense, the detection of NRPS genes is correlated
221 with the nature of produced lipopeptides and their biological activities. the *Bacillus* sp. strain
222 III4 which produced diverse antifungal lipopeptides against *Botrytis cinerea*, *Fusarium*
223 *oxysporum* and *Rhizoctonia solani* also displayed positive PCR results for the presence of
224 NRPS genes encoding to bacillomycin, surfactin, fengycin and mycosubtilin [20]. Besides,

mass spectrometry analysis of lipopeptides from *Bacillus* strains SS-12.6 showed the presence of a lipopeptide compound corresponding to surfactin which was linked to the presence of PCR products related to genes involved in synthesis of iturins and surfactins [21]. Furthermore, *Bacillus amyloliquefaciens* An6 lipopeptides exhibited an antioxidant and antimicrobial activities produced multiple lipopeptides belonging to surfactin, iturin, and fengycin families [22]. The nature of produced lipopeptides by An6 and their biological activities are in correlation with the positive detection of surfactin, fengycin, and bacillomycin genes in this strain was also reported [22].

Antioxidant activity

DPPH radical scavenging activity

As a stable free radical, DPPH has been widely employed to evaluate the free radical scavenging capacity of natural compounds by giving hydrogen to form a stable DPPH molecule [23]. DPPH radical scavenging activities of I4 lipopeptides and BHA (used as positive control) as showed in Fig. 2A were concentration-dependent. In fact, at 1 mg/mL, I4 lipopeptides showed a potential scavenging effect of 69.25 % which is higher than the scavenging ability of lipopeptides from *B. mojavensis* A21 which achieved a DPPH radicals scavenging activity of 65 % [24] and similar to the DPPH radical-scavenging activity of lipopeptides from *Bacillus subtilis* SPB1 which was 70.4 % at the same concentration [25]. Moreover, the IC_{50} value of I4 lipopeptides was 0.36 mg/mL. The IC_{50} obtained here is similar to that reported for lipopeptides from *Bacillus methylotrophicus* DCS1 which achieved a DPPH scavenging potential of 80.6% with an IC_{50} of 0.357 mg/mL [26]. The results suggested that I4 lipopeptides possessed the capacity to donate hydrogen which could be explained by the presence of some active residues in the peptide ring, which acted as electron donor and could react with free radicals of DPPH [26].

Ferric reducing antioxidant power (FRAP) assay

The reducing powers of I4 lipopeptides was determined and compared to BHA at different oncentrations were showed in Fig. 2B. The ability of I4 lipopeptides to reduce Fe^{3+} to Fe^{2+} was concentration dependent and the reducing power of I4 lipopeptides increased significantly until a concentration of 6 mg/mL beyond which reducing power seemed to be constant. However, the I4 lipopeptides showed lower reducing power activities compared to BHA at the same concentrations. The reductive capacity of I4 lipopeptides reached 2.0 (A700 nm) at a concentration of 8 mg/mL which was is slightly higher than those of *Bacillus mojavensis* A21 lipopeptides which showed the same reducing activity at a concentration of 10 mg m/ mL [24]. Furthermore, compared to others lipopeptides from bacillus species, the Ferric reducing power of I4 lipopeptides was lower than those reported for *Bacillus methylotrophicus* DCS1 [26] and B. subtilis RW-I [27] which reached and reducing ability of 2.0 (A700 nm) at a concentrations of 1.3 and 2.5 mg/ mL, respectively. It was reported that the reducing power of lipopeptides is correlated with the presence of functional groups in their structure which act as electron donors such as hydroxyl groups that could react with free radicals to convert them into more stable products [27].

Iron (Fe^{2+}) chelating activity

Iron chelating activity determination showed a dose dependent chelating effect of I4 lipopeptides (Fig. 2C). Indeed, at a concentration of 10 mg/mL, the ferrous-chelating effect of I4 lipopeptides was 76.21% against 98.25% obtained with EDTA (positive control). The IC_{50} values for crude biosurfactant and EDTA were estimated at 0.4 ± 0.03 and 0.29 ± 0.01 mg/mL, respectively. This activity was lower than those reported by Zouari et al. (2016) which found that the chelating effect of biosurfactant of *B. subtilis* SPB1 and EDTA were 80.32% and 98.89%, respectively, at a concentration of 1 mg/mL and those from *Bacillus methylotrophicus* DCS1 lipopeptides which exhibited a ferrous-chelating activity of 79.8% at 4 mg/mL [26]. This difference in Iron chelating activity observed among different bacillus

species could be explained by the structural differences of lipopeptides produced by each species. Indeed, the differences in the sequence of amino acids and carbon atoms in the fatty acids provide biosurfactants with diverse chemical structures and physiochemical properties [29].

DNA nicking assay

The DNA nicking assay was used to investigate the scavenging ability of I4 lipopeptides against hydroxyl radical generated by the Fenton reaction. Results (Fig. 2D) showed that the plasmid DNA was completely degraded in the presence of hydroxyl radicals generated by the Fenton reaction (Lane 3) compared to the native plasmid (Lane 4). The migration profiles of the plasmid pre-incubated with the I4 lipopeptides at 1 mg/mL (Lane 1) and 4 mg/mL (Lane 2) is similar to the control plasmid (Lane 4). This founding suggested that I4 lipopeptides could inhibit the oxidation of DNA by hydroxyl radicals. This results were similar to those reported by Ben Ayed et al. (2016) who showed that lipopeptides from *B. amyloliquefaciens* An6 counteract the oxidative stress produced by Fenton reaction and allowed protection of plasmid DNA against oxidation using the same test [22].

Antimicrobial activity of I4 lipopeptides

The antibacterial activities of I4 lipopeptides were tested against Gram-positive (*S. aureus*, *L. monocytogenes*, *E. faecalis* and *M. luteus*) and Gram-negative (*E. coli*, *P. aeruginosa*, *K. pneumoniae* and *S. enterica*) bacteria according to method of double diffusion in the agar. Ampicillin was used as a positive control. The antibacterial activity was evaluated by the determination of the diameter of growth inhibition zone and the MIC values. Results showed that I4 lipopeptides at a concentration of 20 mg/mL had antibacterial activity against all strains tested (Table 3). The inhibition zones of I4 lipopeptides were between of 8–16 mm and 7–15 mm with Gram-positive and Gram-negative bacteria, respectively. The most resistant strains were *E. coli*, *E. faecalis* and *L. monocytogenes* with DZI of 7, 8 and 10 mm,

300 respectively while the most sensitive strains were *S. aureus*, *M. luteus*, *S. enteric* and *K.*
301 *pneumonia* with a diameter of the inhibition zones of 15, 16, 17 and 15 mm, respectively.
302 Moreover, the strains with the largest inhibition zones showed the lowest MIC values (Table
303 3). Similarly, Ben Ayed et al. (2016) showed that the lipopeptide of *B. amyloliquefaciens*
304 An6 exhibited a high antibacterial activity against *B. cereus*, *S. aureus* and *E. coli* at a
305 concentration of 2 mg/mL with DZI of 20, 18 and 17 mm, respectively. Regarding antifungal
306 activity, the I4 biosurfactants inhibited the growth of *F. solani*, *R. solani and B. cinerea*
307 (Table 3) with inhibition zones of 24 for *F. solani*, *R. solani* and 23 mm for *B. cinerea*.
308 We determined Pearson's correlation coefficient to establish the relationship between the
309 antioxidant and the antimicrobial activites. The obtained results have shown significant
310 correlations between the antioxidant activity and the antimicrobial activity of I4 lipopeptides
311 ($0.95 \leq R^2 \geq 0.99$, $P < 0.01$). Indeed, the highest correlation was obtained between MIC value
312 and Ferrous ion chelating ($R^2 = 0.99$, $P < 0.01$). Similarly significant correlation was observed
313 between antimicrobial activity and DPPH scavenging activity ($R^2 = 0.95$, $P < 0.01$). These
314 correlations could be explained by the capacity of lipopeptides to neutralization of the free
315 radicals by acting as an electron donors and their ability to permeate the cell membrane of the
316 target organism leading to cell death. Indeed, the major compound in the I4 biosurfactants
317 was surfactin [30]. Moreover, it was reported that the major compound in lipopetides from
318 *Bacillus subtilis* RW-I is surfactin and the crude biosurfactant showed powerful scavenging
319 properties [27]. It was reported that lipopeptides from *B. mojavensis* A21 contained surfactin
320 and fengycin as major compounds [31] and has both antimicrobial and antioxidant activities
321 [24]. Snook et al. (2009) reported that *Bacillus mojavensis* RRC 101 produced surfactin which
322 was effective in the biocontrol of *Fusarium verticillioides* in maize. Furthermore,
323 biosurfactants from *B. subtilis* SPB1, contained different lipopeptide isoforms belonging
324 surfactin, iturin, and fengycin families, was reported to exhibit potent inhibitory effects

against *Penicillium notatum*, *Penicillium italicum* and *Aspergillus niger* [33]. The observed antibacterial and antifungal activities of I4 blipopeptides corroborated the PCR detection of NRPS genes. These findings suggested the synergistic antimicrobial inhibitory effects of different antimicrobial lipopeptides produced by *B. mojavensis* I4.

The effect of I4 biosurfactants on mycelial growth of *F. solani*

Fusarium dry rot is a postharvest disease which caused severe potato tubers losses could be caused by several *Fusarium* spp among them *F. solani* remains the most prevalent [34]. In this work we are considering the use of crude I4 lipopeptides as cost effective biocontrol agent of this disease instead of chemical control. The growth inhibition assay showed that the I4 lipopeptides possessed an important antifungal activity against *F. solani* with an inhibition zone of 24 mm (Table 3). Further, the growth of *F. solani* were evaluated during 10 days in the presence of different lipopeptides concentrations (0.5, 3 and 5 mg/mL) in comparison to the mock control without biosurfactants. Results showed that the I4 lipopeptides effectively inhibited the growth of *F. solani* compared to the control (Fig. 3A). The micelial growth inhibitions (MGI) obtained here were about 25.2, 51 and 72% in the presence of I4 lipopeptides at 0.5, 3 and 5 mg/mL, respectively. It was reported that *Bacillus subtilis* strain Bs-Cach harbour *in vitro* antagonism with an inhibition of mycelial growth between 50.33 and 51.93 % against tow *F. solani* strains (ICA-3 and ICA-4) which was correlated with the synthesis of lipopeptides belonging to the family of iturins and also the positive detection of presence of iturin (ItuA), surfactin (SrfC), and fengycin (FenD) genes in this strains [35]. *Bacillus subtilis* SPB1 biosurfactants, reported contain different lipopeptide compounds such surfactin, iturin and fengycin, totally inhibits the growth of *F. solani* at 3 mg/mL and *Rhizoctonia solani* at 4 mg/mL [36]. To affirm the antifungal potency of I4 lipopeptides, mycelium of *F. solani* was microscopically observed near the zone of inhibition (Fig. 3B). In fact, the non-treated control showed articulate hyphae with normal mycelium and smooth

surfaces (Fig. 3B) while the treated mycelium showed irregular shape with excessive lyses and destructed spores. Moreover, no fungal growth was observed with 5 mg/mL of lipopeptides which suggested total destruction of the spores ensuring high efficacy of antagonistic activity. This observation suggested that I4 biosurfactants acted on the cell surface by the potent permeabilizing activity leading to cellular death. Similarly the cell lysis of the pathogenic fungi *F. solani* when treated by SPB1 biosurfactants was earlier reported [6].

Application of I4 biosurfactants in potato tuber rots biocontrol

The efficacy of I4 biosurfactants in the treatment of potato tuber rot caused by *F. solani* was investigated *in vivo* (Fig. 4). For this purpose, both curative and protective treatments were carried out. Commercial antifungal agent carbendazim was used as positive control (Fig. 4A). Results showed that I4 lipopeptides were effective in the biocontrol of the disease at all tested concentrations in both curative and protective treatments (Fig. 4A and 4B). Penetration values for controls and the treated tubers revealed that the lipopeptides reduced the penetration of the pathogen up to 80% at 5 mg/mL in preventive treatment while in curative one the reduction was almost 67% (Fig. 4C). Moreover, significant differences were observed between the tested concentrations in both the treatments. Indeed, the reduction of the fungus penetration was significantly more effective with 5 mg/mL than 3 mg/mL. Besides, at the same concentration (5 mg/mL), the preventive treatment was more efficient than the curative one ($P \leq 0.05$). However, lipopeptides from *B. mojavensis* I4 was less effective compared to the commercial antifungal agent as it reduced 90 % of the fungus penetration using the preventive treatment. This result is in accordance with the previous report that preventive treatment was more effective than preventive treatment in the biocontrol of *F. solani* in potato tuber using biosurfactants from *B. subtilis* SPB1 [6]. Moreover, lipopeptides, mainly fengycin, surfactin and iturin, from *Bacillus* species act as biocontrol agents against several phytopathogens [37]. Fengycin from *Bacillus subtilis* M4 was effectively used in the biocontrol of *Pythium ultimum*

and *Botrytis cinerea* that caused bean root rot and apple mold disease, respectively [38]. Similarly, lipopeptides from *Bacillus subtilis* NCD-2 effectively reduced the cotton seedling damping-off disease caused by *R. solani* [39]. Besides, FPLC analysis revealed that the antifungal compound of *Bacillus subtilis* NCD-2 was fengycin homologs witch was positively correlated with the PCR detection of fenC gene in *B. subtilis* NCD-2 [39]. Torres MJ,Brandan CP,Sabate DC,Petroselli G,Erra-Balsells R, Audisio MC [40] showed that lipopeptides from *Bacillus amyloliquefaciens* PGPBacCA1 harbour antifungal activity against several phytopathogen fungi. These authors also reported that PGPBacCA1 lipopeptides inhibited the growth of *Rhizoctonia solani* up to 73% and also reported that surfactin, iturin and fengycin were identified as the main responsible for the antagonistic effect. Moreover, crude biosufactants from *Bacillus licheniformis* significantly reduced the incidence of root rot in faba bean caused by *Rhizoctonia solani* AG-4 [41].

In conclusion, in the present study we explored the *in vitro* antioxidant and antimicrobial activities of lipopeptides from *B. mojavensis* I4 and its efficiency in the biocontrol of potato dry rot caused by *F. Solani*. The results of our work demonstrate that *B. mojavensis* I4 could produce different type of lipopeptides according to the positive detection of five NRPS genes. I4 lipopeptides were found to exhibit strong antioxidant and antimicrobial activities Moreover, I4 lipopeptides effectively reduced the potato tuber dry rots caused by *F. solani* using preventive treatment. Considered together, results obtained here suggested the potential application of I4 lipopeptides in potato tubers treatment before their storage.

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Table 1: List and characteristics of primers used to detect lipopeptide biosynthesis genes.

Primer name	Sequence of primer	Expected fragments size (bp)	lipopeptides
As1-F	CGCGGMTACCGVATYGAGC	419, 422, 425, 431	Surfactins
Ts2-R	ATBCCTTTBTWDGAATGTCCGCC		
Af2-F	GAATAYMTCGGMCGTMTKGA	443, 452, 455	Fengycins
Tf1-R	GCTTTWADKGAATSBCCGCC		
Am1-F	CAKCARGTSAAAATYCGMGG	416, 419	Mycosubtilin
Tm1-R	CCDASATCAAARAADTTATC		
Abl1-F	GATSAWCARGTGAAAATYCG	428, 431, 434	Bacillomycins
Tbl1-R	ATCGAATSKCCGCCRARATCRAA		
Aks-F	TCHACWGGRAATCCAAAGGG	1125, 1152, 1161, 1167, 1173	Kurstakins
Tks-R	CCACCDKTCAAAKAARKWATC		

Using IUPAC DNA code: Y = C or T, M = A or C, K = G or T, W = A or T, D = G, A or T, S = G or C, B = G, T or C, R = A or G

561 **Table 2:** Blast results of sequence fragments obtained by PCR amplification with degenerate
562 primers

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Primer names	Target NRPS gene	Product size (bp)	BLASTX hit	Genbank accession	Identity (%)	e-value
As1-F Ts2-R	Surfactins	419	Surfactin synthetase (*Bacillus subtilis*)	P27206	91	1e-80
Af2-F Tf1-R	Fengycins	443	Fengycin synthetase (*Bacillus subtilis*)	AAB80955.2	95	2e-88
Abl1-F Tbl1-R	Bacillomycins	428	Peptide synthetase (*Bacillus mojavensis*)	WP_010332989	88	2e-70
Am1-F Tm1-R	Mycosubtilin	416	Peptide synthetase (*Bacillus subtilis*)	AMA51368.1	85	1e-58
Aks-F Tks-R	Kurstakins	1152	Peptide synthetase (*Serratia marcescens*)	WP_060443815.1	96	9e-135

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Table 3: Antimicrobial activity of I4 lipopeptides biosurfactants from *B. mojavensis*

	Microorganisms	Inhibition Zones diameter (mm)	MIC (mg/ mL)	Ampicillin Inhibition zones diameter (mm)
Gram +	*S. aureus*	15 ± 0.91 (c)	10	35 ± 2.24
	E. faecalis	8 ± 1.04 (a)	20	27 ± 1.13
	M. luteus	16 ± 1.29 (c)	1.25	28 ± 2.19
	L. monocytogenes	10 ± 0.98 (b)	20	32 ± 2.29
Gram-	*E.coli*	7 ± 0.86 (a)	20	25 ± 2.09
	S. enteric	17 ± 1.6 (c)	2.5	35 ± 2.6
	K. pneumonia	15 ± 0.9 (c)	20	24 ± 1.92
				Carbendazim Inhibition zones diameter (mm)
Fungi	*F. solani*	24 ± 1.2 (de)	nd	30 ± 2.15
	B. cinerea	23 ± 1 (de)	nd	25 ± 1.11
	R. solani	24 ± 1.1(de)	nd	32 ± 2.21

nd: non determined, column with different letters indicates that the difference between two means is statistically significant ($P < 0.05$)

Figure legends

Figure 1. PCR detection of NRPS genes in *B. mojavensis* I4. **M**: molecular weight marker GeneRuler 1 kb DNA Ladder (Thermo Scientific), Lane 1: Kurstakins primers (Aks-F/Tks-R), Lane 2: Fengycin primers (Af2-F/Tf1-R), Lane 3: Surfactins primers (As1-F/Ts2-R), Lane 4: Mycosubtilin primers (Am1- F/Tm1-R) and Lane 5: Bacillomycins primers (Abl1-F/Tbl1-R).

Figure 2. Antioxidant activities of I4 biosurfactants. (A) DPPH radical scavenging activity, (B) reducing power essay, (C) metal chelating effect and (D) gel electrophoresis pattern of the empty pGEM-T easy plasmid incubated with Fenton's reagent in the presence and absence of I4 lipopeptides. Lanes 1 and 2: Fenton's reagent + DNA + 1 mg and 4 mg/mL of I4 lipopeptides, respectively, lane 3: DNA sample incubated with Fenton's reagent and lane 4: untreated control: native pGEM-T easy plasmid (0.5µg).

Figure 3. Antifungal activity of I4 biosurfactants against *F. solani*: (A) effect of increasing concentration on the antifungal potency. (B) Effect of I4 biosurfactants on *F. solani* mycelial growth: representative microscopic pictures (10×40 magnifications) of mycelium and spores grown in medium.

Figure 4. Effect of *B. mojavensis* I4 biosurfactants lipopeptides and carbendazim treatment on potato dry soft rot development caused by *F. solani* after 15 days of incubation at 30 °C using preventive (**A**) and curative (**B**) treatment. **C**: Effect of I4 lipopeptides and carbendazim treatment on the penetration of *F. solani* into potato tubers in both preventive and curative treatments. Value reported were means ± SD, histograms highlighted with different letters indicates that the difference between two means is statistically significant ($P < 0.05$).

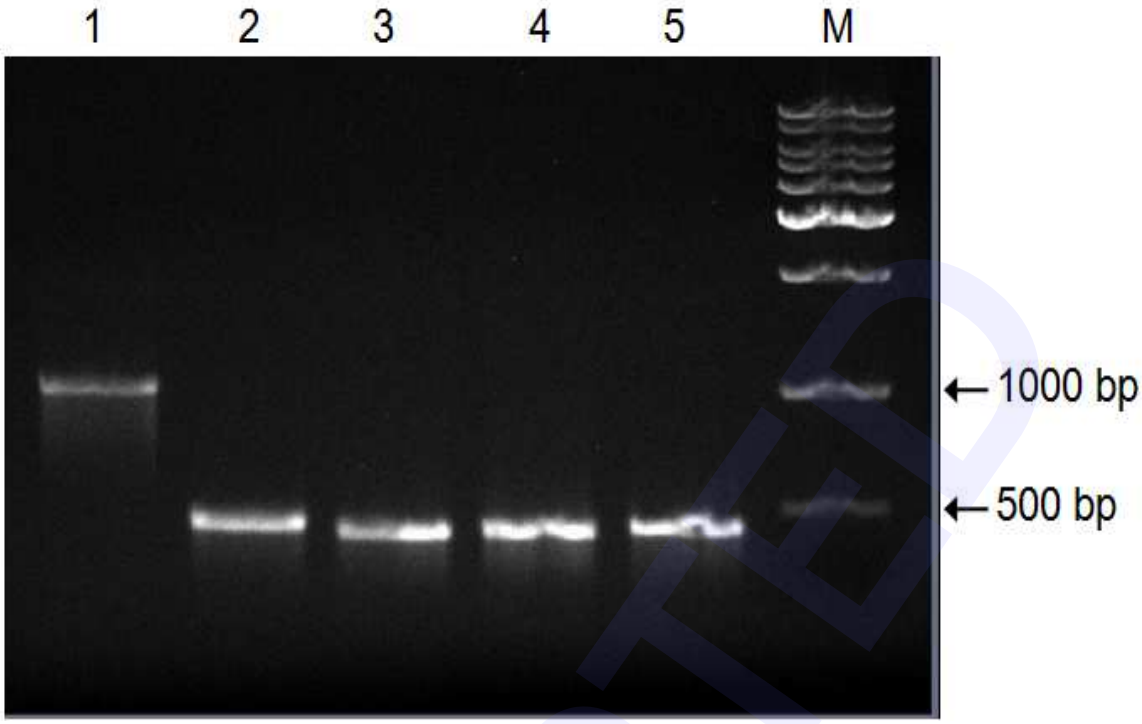

Fig.1

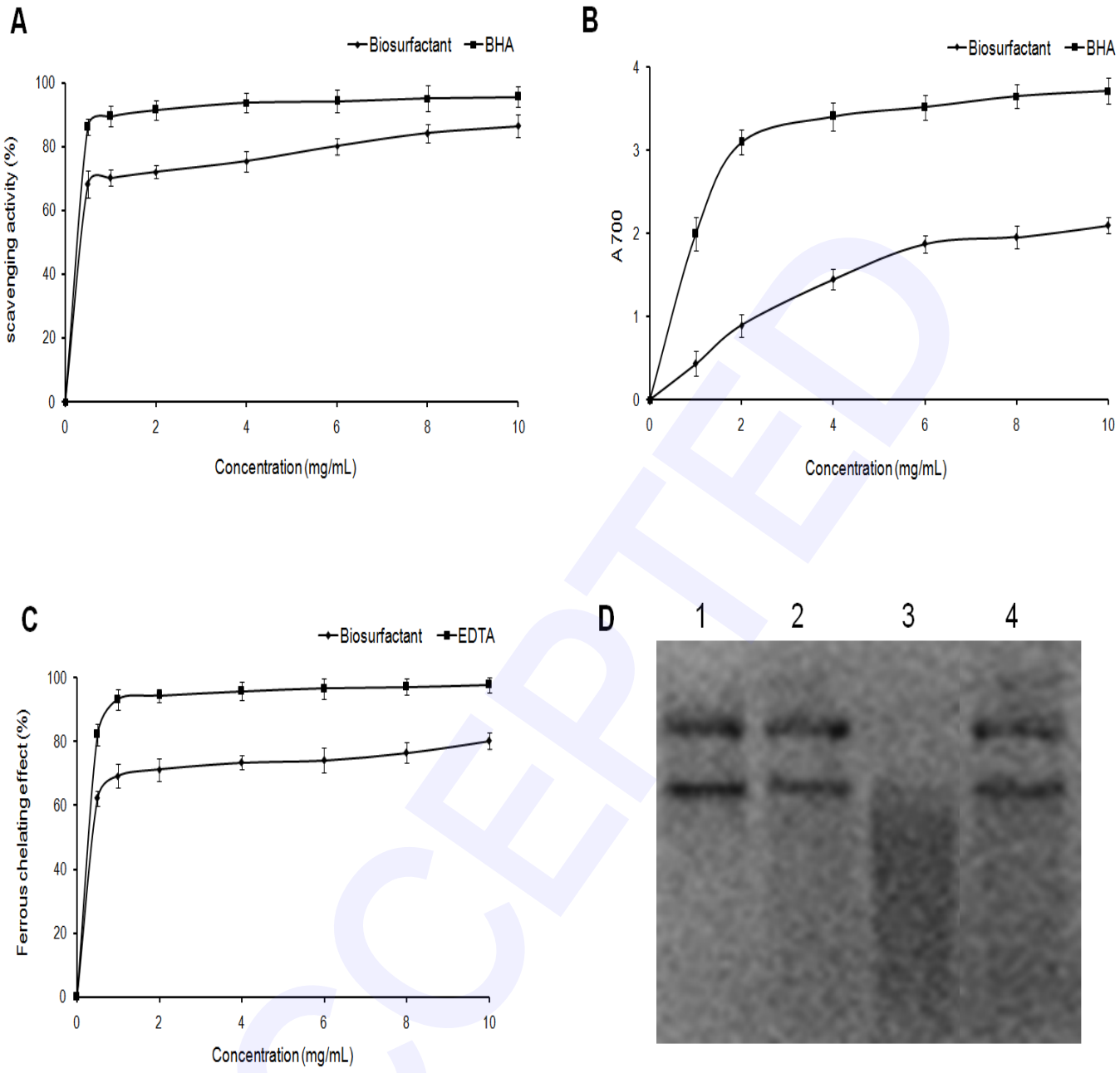

Fig.2

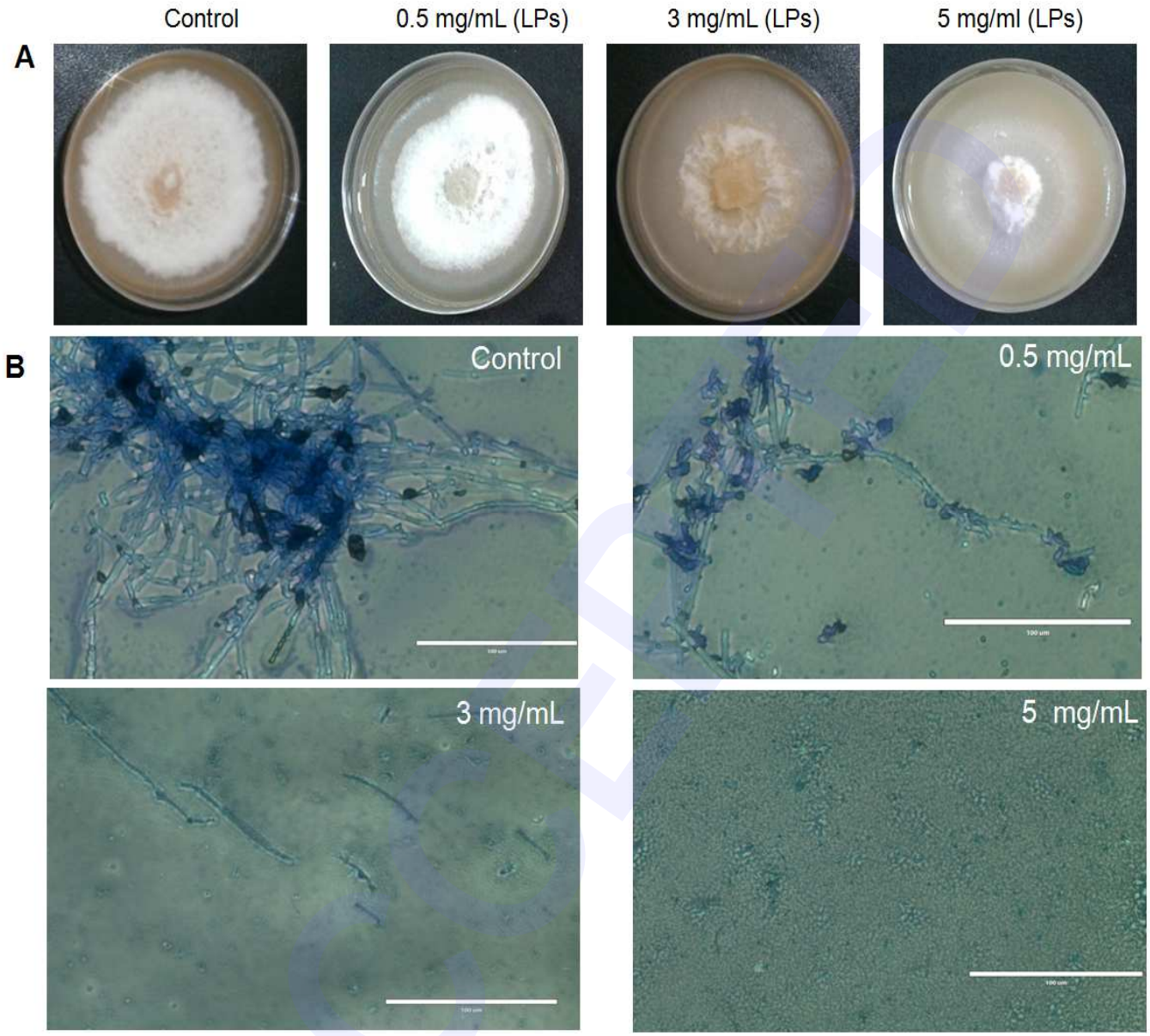
Control
0.5 mg/mL (LPs)
3 mg/mL (LPs)
5 mg/ml (LPs)
A
B
Control
0.5 mg/mL
3 mg/mL
5 mg/mL

Fig.3

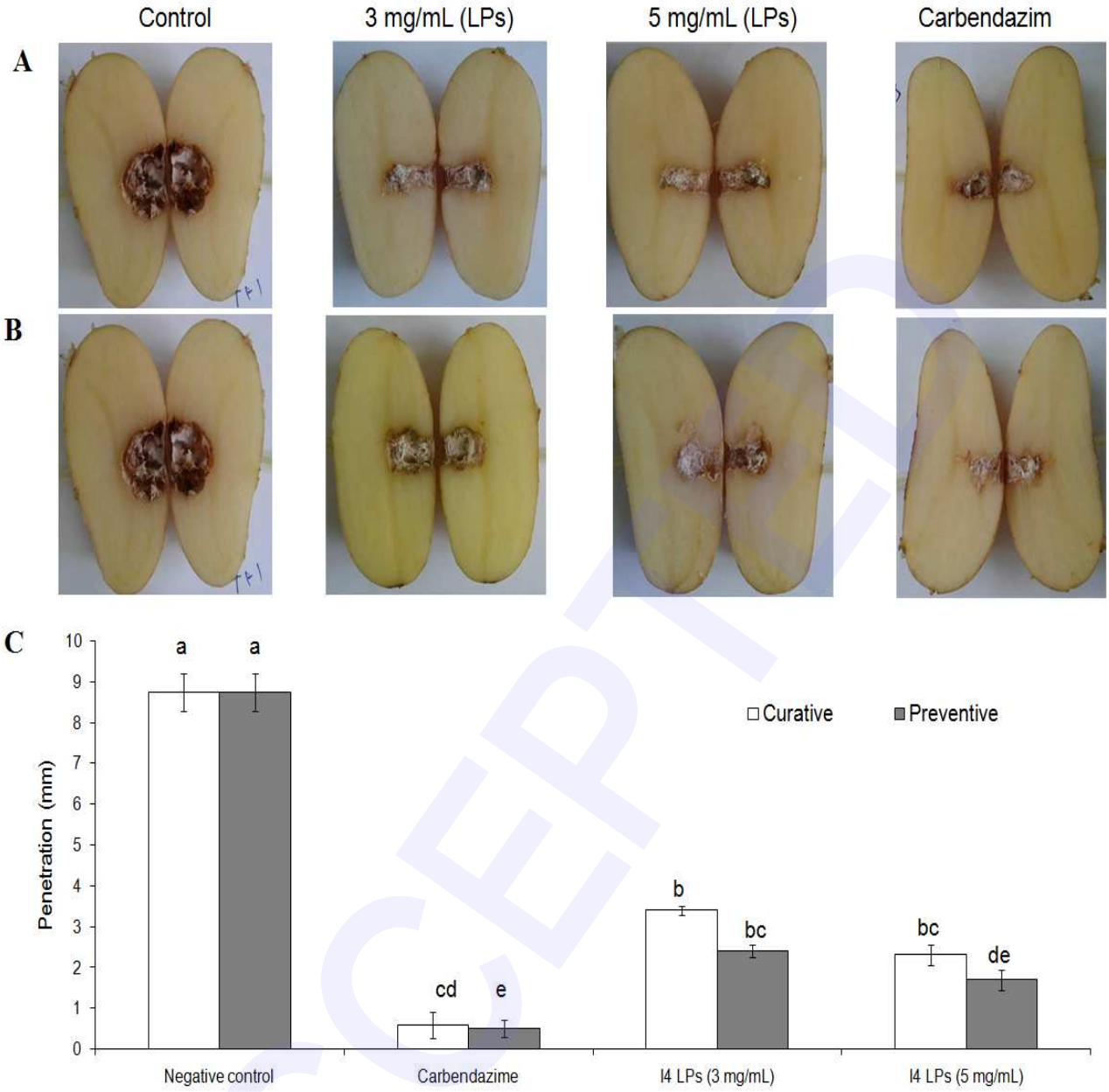

Fig.4