Full Length Research Paper

Flow cytometry approach for studying the interaction between *Bacillus mojavensis* and *Alternaria alternata*

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Tomato, *Solanum lycopersicum* is one of the most important vegetable crops consumed in Algeria. Tomato crops are often infected by *Alternaria alternata,* which causes early blight disease. Chemical pesticides are intensively used to protect this plant, which lead to environmental pollution that might endanger animal and human health. The main objective of this study is to select potential biocontrol agents from arid soil as an alternative to chemical products. The phytopathogenic fungus which was isolated from infested tomato leaves, stems and fruits cultured in Constantine-Algeria, was identified as *Alternaria cf. alternata*. Thirty five bacteria isolates were obtained from arid soil in the south of Algeria. Three of the isolates inhibited the growth of *A. alternata*. However, the most potent isolate, E1B3 reached a 75% inhibition rate. The molecular identification of this isolate showed that it was closely related to *Bacillus mojavensis* (KC977492). This strain does not produce chitinase, but does produce lipase, protease and lipopeptides. The interaction between *A. alternata* and *B. mojavensis* was investigated for the first time in this work by flow cytometric analysis. In conclusion, *B. mojavensis* strain was antagonistic to *A. alternata* which could possibly be exploited as a biopesticide in tomato crops management.

Key words: Tomato, *Bacillus mojavensis,* early blight, *Alternaria alternata,* flow cytometry.

INTRODUCTION

Tomato, *Solanum lycopersicum* is among the essential economical and nutritious vegetable crops in the world (Peralta et al., 2005). In Algeria, the land cultivated of tomato represents 12173 Ha which covers 57.36% of the global cultivated areas. It production is ca 10 MQx of

which 3.8 MQx is for industrial use (that is equivalent to 95% of the total industrial culture production) (Snoussi, 2009). In spite of the economic importance of tomato, there are major yield losses due to plant diseases. Early blight of tomato is an important and widely distributed

disease throughout the world. This disease is caused by the fungi *Alternaria solani* and *Alternaria alternata*. *Alternaria* diseases appear usually as leaf spots and blights, but they may also cause damping-off of seedling, stem rots and tuber and fruit rots (Agrios, 1997).

Pesticides have increased crop resistance for over four decades; nonetheless, the emerging, re-emerging and endemic plant pathogens are still challenging crop safety worldwide (Berg, 2009; Gilbert and Haber, 2013). Furthermore, chemicals may leave residues on grains, fruits, vegetables and soil that may be harmful to the ecosystems and human health (Rocha et al., 2014). The development of environmentally friendly crop management practices for combating diseases represents a difficult task. The use of bacterial antagonists such as *Bacillus* (Baysal et al., 2013), *Pseudomonas* and *Streptomyces* (Palazzini et al., 2007; Etcheverry et al., 2009) is a biological alternative solution, commonly called "biocontrol". This culture management strategy is one of the most rational practices in the integrated management program reducing pesticide use in the environment (Xue et al., 2009).

Bacillus species, including the ubiquitous soil bacterium *Bacillus subtilis*, play an important role in the degradation of soil organic polymers (Emmert and Handelsman, 1999). They produce spores that are resistant to desiccation, heat, UV, irradiation and organic solvents. *Bacillus* spp. are used to manage a wide range of fungal diseases by operating as an antagonist to plant pathogen growth through their production of antibiotics (e.g. iturin, surfactin and fengycin), enzymes that degrade fungal structural polymers (e.g. chitinase and 1,3 glucanase) and production of antifungal volatiles (Fiddaman and Rossall, 1993; Knox et al., 2000; Pinchuk et al., 2002; Leelasuphakul et al., 2006).

Flow cytometry (FCM) is becoming increasingly important in the field of microbial population and community assessment (Müller and Caron, 2010), in medical applications (Walberg et al., 1996; Gauthier et al., 2002), the dairy industry (Rault et al., 2007), alcoholic beverage production (Boyd et al., 2003), and environmental and water system control (Czechowska et al., 2008). It can be combined with cellular markings as carboxyfluorescein diacetate (CFDA, a marker for intracellular esterase activity) (Bunthof et al., 2000; Tanaka et al., 2000; Nguefack et al., 2004; Flint et al., 2006).

CFDA is a lipophilic, non-fluorescent dye, used primarily for the evaluation of cellular enzymatic activity; in fact, it diffuses across the cell membranes, and is converted by unspecific esterases into a membrane-impermeant fluorescent compound, carboxyfluorescein (CF), which is retained in viable cells with intact cytoplasmic membranes (Petit et al., 1993). Single cell analysis in biotechnological applications gives a high-resolution view on a whole cell culture with regards to cellular states of viability, metabolic activity and

productivity in addition to cell concentration (biomass) estimation (Hewitt and Caron, 2001).

In this study, an arid region of south Algeria (Sahara) was chosen as a source of new bacterial isolates with potential biotechnological properties. The aim of the present study was to isolate and identify potential biocontrol agents from the Bacillus genus, in order to use them as an alternative for chemical control. The work was carried out using several steps, a) Isolation of *Bacillus* strains antagonistic to *A. alternata*; b) Identification of the most potent isolate, c) Testing for antifungal substances production; d) Study of the effect of *B. mojavensis* strain (E1B3) on *A. alternata* by flow cytometry analysis using the fluorescent dye (CFDA).

MATERIALS AND METHODS

Isolation, identification and pathogenicity test of the pathogenic fungus

Standard tissue isolation technique was followed to obtain the fungal isolates. Three month old tomato plants showing typical early blight symptoms were collected from greenhouses in Hamma Bouziane (Constantine-Algeria). The infected leaves were cut into pieces measuring 2 mm and were surface sterilized in sodium hypochloride solution (0.1%) for 2 min and washed with water. These sections were transferred into Petri dishes containing 15 ml of potato dextrose agar (PDA) and incubated at 27°C for 7 days. Cultures were subcultured onto fresh PDA.

Fungal identification was based on morphological and microscopic tests. For the morphological study of this fungus, PDA medium was used. To confirm the identification, sequencing of the *ITS* region of the nuclear ribosomal operon and a segment of the *Tef I-α* gene was carried out at the Belgian coordinated collections of microorganisms, UC of Louvain (Belgium).

Pathogenicity test of the *Alternaria* isolate were carried out under greenhouse conditions in 2011–2012 year. The inoculum was prepared by culturing the isolate on PDA medium at 27°C for 10 days. Ten milliliters of sterile distilled water was added to each plate and the colonies were scraped with a sterile needle. The resulting conidial suspension was adjusted to 5×10^6 spores/mL and used for the inoculation of tomato plants using an atomizer. After the inoculation, the plants were covered with polyethylene bags for 48 h to promote the entrance of the pathogen and maintain high humidity conditions. After 48 h, bags were removed for the aeration and the entry of light and plants were kept under greenhouse conditions (greenhouse temperature ranged from 26-28°C). Two weeks after inoculation, disease symptoms and severity were recorded.

Isolation of antagonistic bacteria

Bacterial strains were originally obtained from the soil of "Tolga" in Biskra, located in the south of Algeria (Sahara). The isolation was based on the technique which was described by Aneja (2003). One gram of soil sample was suspended in 9 ml of NaCl (0.85%), and decimal dilutions were prepared. 1 mL in ten of each dilution (from 10^{-1} to 10^{-6}) was plated on nutrient agar (NA) and incubated at 28°C for 24 h. Finally, the isolates were stored in inclined agar tubes at 4°C.

Screening bacteria as antagonist of *Alternaria*

The antagonistic effect of bacteria isolated against *Alternaria* sp.

was tested by the dual plate assay. Bacteria were streaked on the PDA and Czapek Dox Agar plate edge (this medium is prepared according to the formula developed by Thom and Church, 1926) and a mycelial plug of *Alternaria* (5 mm) was deposited in the center, approximately 3.5 cm far from the bacteria. Control plates that were not inoculated with bacteria were also prepared. Plates were incubated at 28°C until the fungal growth of control plates reached the edge of the plate. Mycelia growth inhibition was expressed as the percentage of reduction of mycelium expansion when compared with the control plates (without bacteria) (Toure et al., 2004).

Molecular identification of the screened bacteria

The isolate (E1B3) developing the most important antagonist effect against *Alternaria* sp. on PDA medium was screened for further experiments in this work. This bacterium was identified by 16S r-DNA and gyraseA analysis. For that, the total DNA was extracted from bacteria (Potential biocontrol strains) liquid cultures by the wizard Genomic DNA purification kit (Promega), using the manufacturer's instructions. The primers used for the PCR amplification were the universal primers 16S P0 (GAA GAG TTT GAT CCT GGC TCAG) and 16S P6 (CTA CGG CTA CCT TGTTAC GA) for the *DNA-16S* gene, *gyr-A.f* (CAG TCA GGA AAT GCG TAC GTC CTT) and *gyr-A.r* (CAA GGT AAT GCT CCA GGC ATT GCT) for the *gyr-A* gene (Izumi and Aranishi, 2004). The purification of the PCR products was achieved by using the GFX PCR DNA and Gel Band purification Kit. The amplified genes were sequenced using the same primers sited above and the obtained sequences were corrected by the Bio-edit program. The sequences were deposited in Genbank database. To identify bacteria isolates, the DNA sequences were compared with those previously published in Genbank using the BLASTN program.

Cell-wall degrading enzymes

Chitinolytic activity

Chitinolytic activity of the antagonistic strain was evaluated on chitin-agar (CA) plates. The CA agar (per liter) contains the following minerals: K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$ $7H_2O$, $FeSO_4$, $ZnSO_4$, $CaCl_2$ (0.8 g, 0.3 g, 0.3 g, 0.5 g, 0.1 g, 0.001g and 0.1 g, respectively), in addition, it contains; 3 g colloid chitin, 8 g yeast extract, 20 g agar, with pH 6.5. The CA plates inoculated with the bacteria isolates were incubated at 28°C for 5 days. After that, the width (mm) of every clear halo around the antagonistic isolate was measured and recorded as indicator of chitinolytic activity (Nihorimbere et al., 2013).

Protease activity

The protease production was tested by using the casein skim milk as a carbon source (Larpent and Larpent, 1985). The culture medium is composed of 9 g/L casein peptone, 9 g/L yeast extract, 9 g/L of skim milk and 14 g/L agar. An overnight colony was deposited on the agar plate centre, and then incubated at 28°C for one week. Protease activity was highlighted by the presence of a transparent halo around colonies (Nihorimbere et al., 2013).

Lipase activity

The ability of isolate to produce the lipase enzyme was determined by testing the tributyrin hydrolysis. This test was performed by the method described by Larpent and Larpent (1985) with some

modifications. An overnight colony was deposited on the centre of the agar plate. After one week of incubation at 28°C, the lipase activity was confirmed by the formation of a transparent halo around the colonies. The lipase production was measured as the ratio of diameters of clearing zone to that of the colony (Nihorimbere et al., 2013).

Lipopeptides production

The lipopeptides (LPs) were analyzed by mass spectrometry. The *Bacillus* strain**s** were grown in agitated flasks (180 rpm) containing the optimum medium at 30°C for 72 h. Cultures were centrifuged at 15000 rpm for 20 min. The supernatant samples were loaded on C18 solid-phase extraction cartridges (900 mg, Alltech) and lipopeptides were desorbed with 100% acetonitrile ACN. The resulting samples were analyzed by reverse phase HPLC coupled with single quad mass spectrometer (HPLC Waters Alliance 2695/diode array detector, coupled with Waters SQD mass analyzer) on a X-terra MS (Waters) 150 9 2.1 mm, 3.5 lm column as previously described by Nihorimbere et al. (2013). In this work, a single elution gradient allowing the simultaneous measurement of all three lipopeptides families was used. Water acidified with 0.1% formic acid (A) and acetonitril (ACN) acidified with 0.1% formic acid (B) were used as a mobile phase. The flow rate was maintained at 0.5 ml/min and the column temperature at 40°C, with a gradient of 35 min (43–80%, vol/vol ACN in 18 min; 100%, vol/vol ACN for 9 min, and 43%, vol/vol ACN in 8 min). Compounds were identified on the basis of their retention times ad compared to the purified standards. The identity of each homologue was confirmed on the basis of the masses detected in the SQD by setting electrospray ionization conditions in the MS as source temperature, 130°C; desolvation temperature, 250°C; nitrogen flow, 500 l/h; cone voltage, 70 V. The positive ion mode was used for analyzing the three families.

Effect of *Bacillus mojavensis* on *Alternaria alternata*

Microbial suspension preparation

A. alternata was cultured on PDA plates, from which, the colony surface was gently scraped off and transferred to a tube containing 50 mL of sterile distilled water. Spores were counted with a hemocytometer, and the concentration was adjusted to 10^6 spores/mL. On the other hand, *B. mojavensis* was cultured in Erlenmeyer flasks containing 250 mL of optimum liquid medium as described by Jacques et al. (1999) and incubated at 30°C on a rotary shaker (100 rpm) for 72 h. The cultures were centrifuged at 12 000 rpm for 30 min at 4°C.

Flow cytometry analysis

In the FCM assay, microbial suspensions of *A. alternata* and *B. mojavensis* were used at 10^6 spores/mL and 10^7 cell/mL, respectively. About 50 μL of *B. mojavensis* biomass was centrally inoculated on PDA medium using a micropipette. After inoculum adsorption, 50 μL of *A. alternata* was centrally inoculated on each PDA medium Petri plate. The cytometry experiments were conducted with three replicates after 3, 5 and 7 days of incubation at 30°C (Rocha et al., 2014 method modified). After the necessary incubation time, cultures were removed from the agar by scraping, transferred into 1 mL Eppendorf containing sterile distilled water and Tween 80 (2 drops/100 mL water), and centrifuged at 10000 rpm for 15 min. The cells were rinsed with the Tween 80/water solution twice and resuspended in 1 mL of phosphate-buffered saline (PBS; 136 mM NaCl, 1.4 mM KH_2PO_4, 8.09 mM Na_2HPO_4,
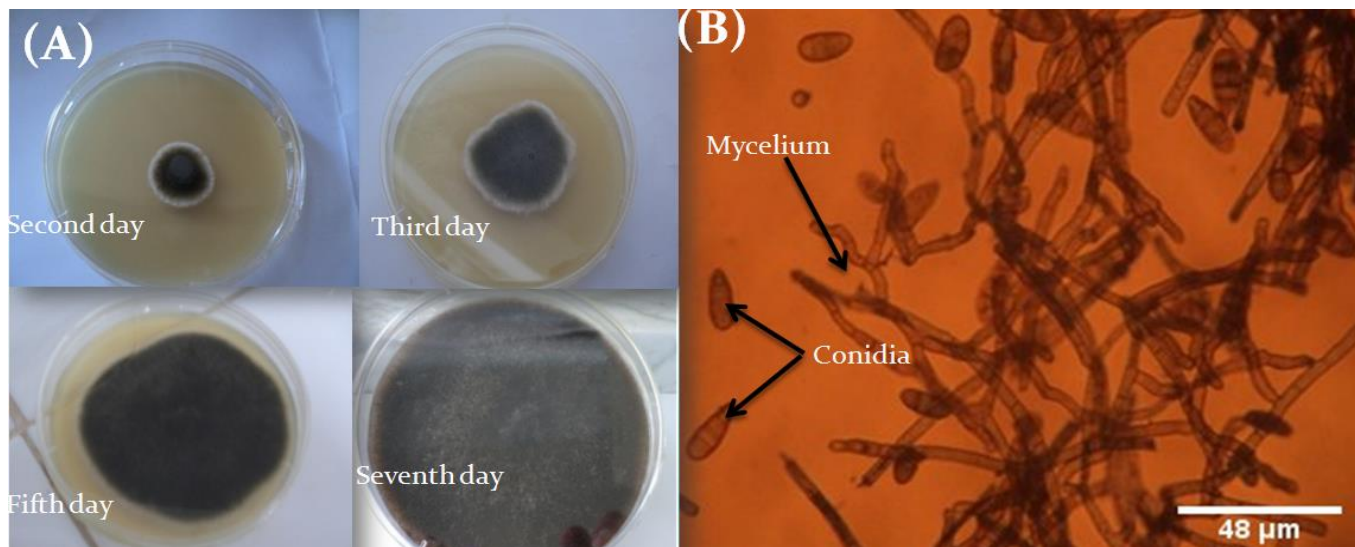
Figure 1. Morphology of *Alternaria* sp. isolated from diseased tomato plants leaves, (A) aspect of macroscopic observation from the third day to seventh, (B) observation of mycelium and conidia under optic microscope (GX40).

12 H_2O and 2.6 mM KCl, pH 7.2). The cell solution was stained with 10 µL of CFDA (a working solution of 100 µg. mL^{-1} was prepared in (PBS) and stored at 4°C in the dark). The mixture was incubated in the dark for 15 min before FCM analysis. The samples were centrifuged at 10000 rpm for 15 min and resuspended in 1 mL of sterile PBS. After a second centrifugation, the final pellet was resuspended in 1 mL of sterile PBS and stored in ice until the analysis. To analyze the influence of thermal treatments (control), 10^6 spores/mL of the *A. alternata* were heated at 90°C for 12 min in a water bath. Cell viability of the heat treated solution was analyzed by FCM after staining with CFDA. One milliliter of a 10^6 spores/mL of *A. alternata* solution was analyzed before and after heat treatment to confirm that dead cells cannot absorb CFDA and also to distinguish between the death and live cells GFI. The cells were incubated with CFDA in the dark for 15 min and stored in ice until the analysis. The same manipulations were carried on bacteria to estimate the effect of heat treatment on cells viability.

The samples were analyzed on a FACScan flow cytometer with an argon laser operating at 488 nm. Green fluorescence of cells stained with cFDA was collected in the FL1 channel (525 ± 20 nm). 40,000 events were collected per sample. The experiment was repeated twice.

Fluorescence microscopy

The same microbial cultures on PDA plates prepared for flow cytometry test as described before, were used for studying the effect of E1B3 on *A. alternata* growth at intracellular and intercellular levels, by fluorescence microscopy using a Zeiss, AXIOSCOP 2MOT.

RESULTS AND DISCUSSION

Isolation, identification and pathogenicity test of pathogenic fungus

One fungus was isolated and purified on PDA plates.

This isolate had green black colonies with a very thin white margin and cottony texture (Figure 1A). Conidiophores appear under optic microscope straight, with a brown color and bearing light brown conidia with a short beak at the tip.

The comparison of the fungus, *ITS* and *Tef I-α* genes sequences with those previously published in Genbank showed that this isolate was *A. cf. alternata.* The results of pathogenicity test indicated that *A. alternata* strain was able to infect tomato plants causing typical early blight symptoms (Figure 2).

Isolation, identification and screening bacteria as antagonism of Alternaria

Strains of *Bacillus* spp. are among the most commonly reported biofertilizer and biopesticide bacteria. The characterization of this genus for their antifungal effect has been carried widely on *Bacillus* strains isolated from the rhizosphere of agricultural crops (Beneduzi et al., 2008; Pamela et al., 2010). However, this study is, from the authors' knowledge, one of the few studies exploring *Bacillus* strains isolated from arid soil.

Thirty five bacteria were isolated from arid soil in the south of Algeria (Sahara). Three isolates inhibited the growth of *A. alternata*. However, the most potent strain was the coded bacteria (E1B3) which reached 75% inhibition rate (Figure 3). The antagonism test was performed on PDA and Czapek Dox Agar plates which is a semi synthetic medium, containing sucrose as the sole source of carbon while sodium nitrate as the sole source of nitrogen. Dipotassium phosphate buffers the medium; Magnesium sulphate, potassium chloride, ferrous
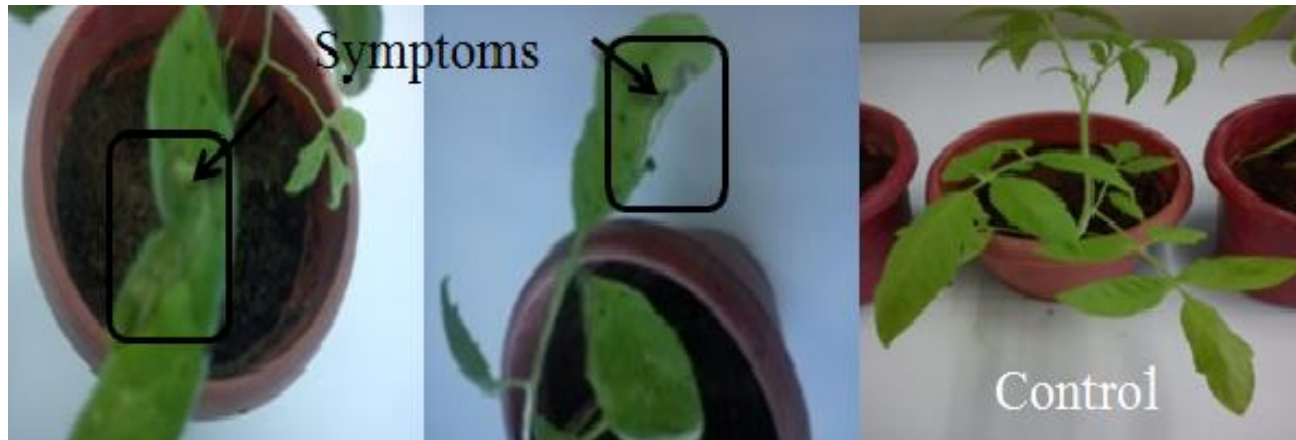
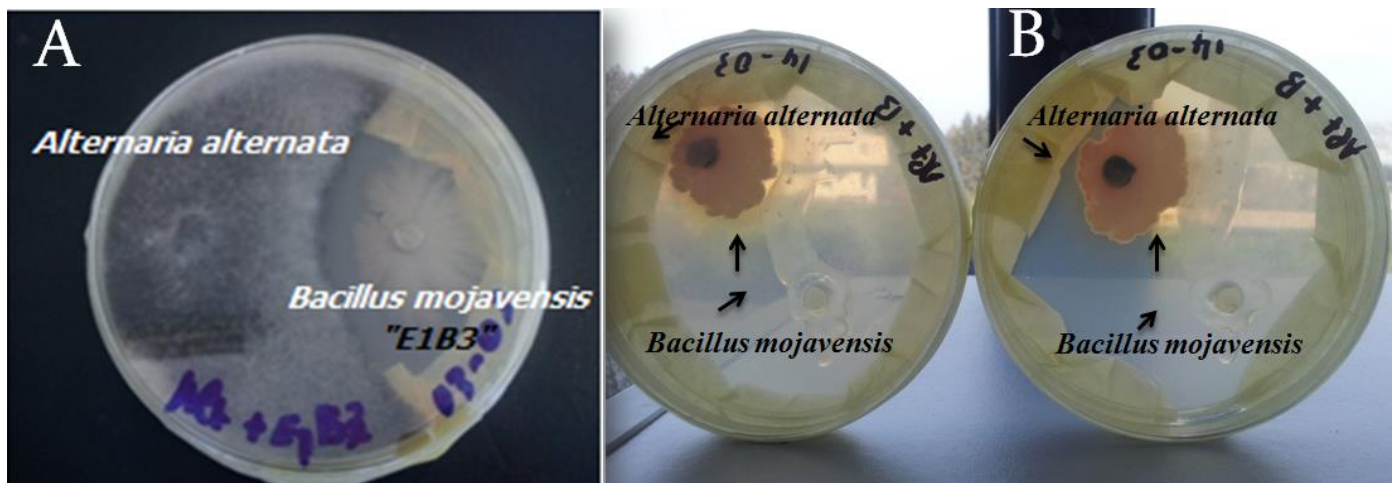**Figure 2.** Pathogenicity test of *A. alternata* on tomato.

Figure 3. Inhibition of *A. alternata* by the bacterium (E1B3). The antagonism test was performed on PDA (A) and Czapek Dox Agar (B) plates. The bacteria and the fungi were inoculated at the same time and the antagonism was scored after 2-5 days of incubation at 28°C.

sulphate as sources of essential ions. The production of small conidia was observed when compared with the normal growth (Figure 4). The molecular identification of the screened strains (E1B3) by *16S r-RNA* sequences analysis showed that it is closely related to a *Bacillus subtilis* group including several species. However, *gyraseA* sequences provided more precise identification and classify it among members of *Bacillus mojavensis* (KC977492) species.

A relative antifungal performance against the same or other fungi were widely investigated previously by diverse *Bacillus* species in the case of *in- vitro* and *vivo* tests. In fact, for *in vitro* tests, Pengnoo et al. (2006) found that 16 isolates of *Bacillus* spp. had the ability to inhibit mycelia growth of *Rhizoctonia solani*, causal agent of leaf blight of bambara groundnut. Among these isolates, *Bacillus firmus* had the greatest activity in anti-microbial tests against *Rhizoctonia solani*. In the other hand, in the case

of *in vivo tests*, some data can be presented as the control of early leaf spot of peanut (Kokalis et al., 1992), yam leaf spot (Michereff et al., 1994), grey mould of strawberries, and post-bloom fruit drop of citrus (Sonoda et al., 1996).

The growth inhibition ability of *B. mojavensis* on other plant pathogens (*Ambrosiella macrospora*, *Botrytis cinerea*, *Fusarium oxysporum*, *Fusarium moniliforme*, *Macrophomina phaseolina*, *Mucor rammanianus*, *Alternaria solani*, *Phytophthora meadii*, *Pythium aphanidermatum* and *Rhizoctonia solani*), was demonstrated in previous studies (Nair et al., 2002; Youcef-Ali et al., 2014). This showed the possibility of using *B. mojavensis* strain to manage the production of several crops sensible to the above cited fungi. It has been shown that the suppressive effect of *B. mojavensis* on *A. alternata* could also be correlated with secreting inhibitory molecules.
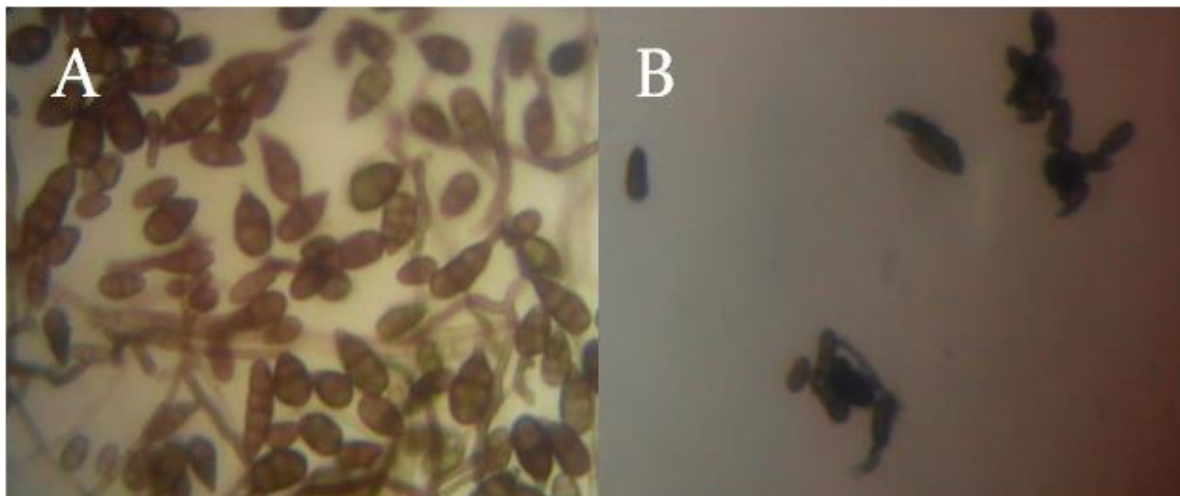
Figure 4. Effect of E1B3 on "intracellular and/or intercellular" growth areas of the pathogen *A. alternata.* (A) Fungus growth in absence of E1B3 and (B) in the presence of E1B3.
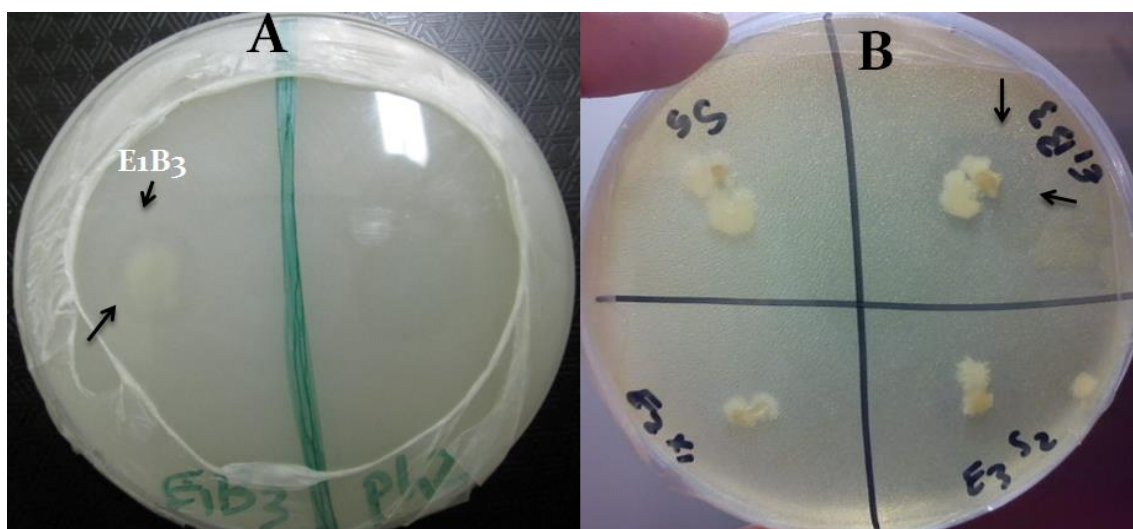

Figure 5. A) Detection of protease activity developed by *B. mojavensis* KC977492. B) Detection of lipase activity developed by *B. mojavensis* KC977492.

Cell-wall degrading enzymes and LPs production

A number of antifungal compounds, including polypeptides that interact with the fungal membrane, are produced by *Bacillus* species (Rocha et al., 2014). This process of fungal growth inhibition was attributed to many mechanisms, including the production of antimicrobial molecules, the secretion of hydrolytic enzymes, the competition for nutrients, or the combination of mechanisms (Compant et al., 2005; Rocha et al., 2014). Lipopeptides, that is, surfactin, iturin and fengycin, were described previously as major classes of *Bacillus* antibiotic peptides. These molecules have a great

potential for diverse biotechnological applications (Peypoux et al., 1999; Ait Kaki et al., 2013). *B. subtilis* group including *B. mojavensis* is recognized as a safe organism (GRAS) by the United States Federal Drug Administration (FDA) (Sanders et al., 2003).

In this work, the screening of antagonist bacterium, *B. mojavensis* showed a negative result in the enzymatic activities test of chitinase, while, there is an important positive production of lipase and protease (Figure 5) with a clear halo on medium reaching 0.5 cm. On the other hand, it has been shown that *B. mojavensis* produce three LPs families, iturin, fengycin and surfcatin after interpreting mass spectra corresponding to mass
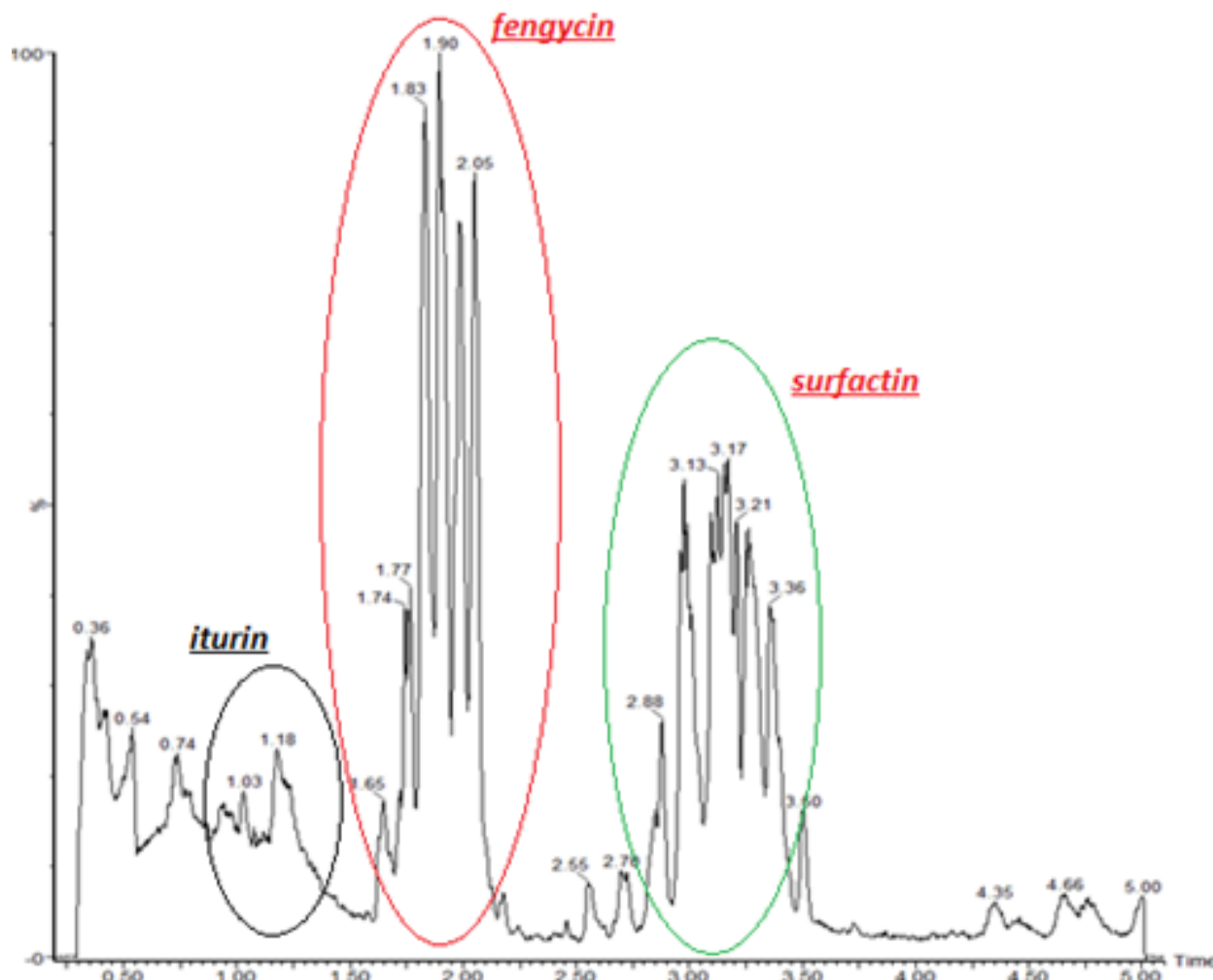
Figure 6. MS profile of *B. mojavensis*. MS analysis showing the production of three LPs families at different retention time, iturin (80s to 1.20 min), fengycin (1.7 to 2.2 min) and surfactin (2.7 to 3.40 min).

spectrometry (MS) peaks (Figure 6).

In comparison, Youcef-Ali et al. (2014) showed that *B. subtilis* and *B. mojavensis* produce enzymes such as cellulase and protease, but not chitinase in *B. mojavensis*. The same results were obtained in lipopetide production by members of *B. subtilis* group. In fact, Yu et al. (2002), Fernandes et al. (2007) and Ait kaki et al. (2013) reported that *B. amyloliquefaciens* B94, *B. subtilis* and *B. subtilis spizezenii* produced lipopeptides families' iturin, fengycin and surfcatin. The capacity of lipopetides production is restricted on members of *Baciillus subtilis* group. Indeed, *P. polymyxa* (18SRTS) did not produce any type of lipopeptides as mentioned by Ait kaki et al. (2013).

Flow cytometry analysis

The antagonistic effect of the *Bacillus* strain against *A. alternata* was investigated here and previously in other studies by dual culture technique (Ait Kaki et al., 2013). However, to the authors' best knowledge, the study of the antagonistic effect of *B. mojavensis* against *A. alternata* by using flow cytometry analysis was investigated for the first time in the present work. This technique has emerged as a high-resolution technology that supports the characterization of individual cell types within mixed populations. It provides an analysis of a large number of cells and can identify changes within a population and between different populations (O'Donnell et al., 2013). Carboxyfluorescein diacetate (CFDA) is a lipophilic, non-fluorescent dye, used primarily for the evaluation of a cellular enzymatic activity and for the fluorescence labeling. The ester bonds are hydrolyzed by enzymes with esterase activity, yielding the green fluorescent dye molecules because enzyme activity is needed for hydrolysis, and membrane integrity is required for the retention inside cells. It is supposed that viable cells
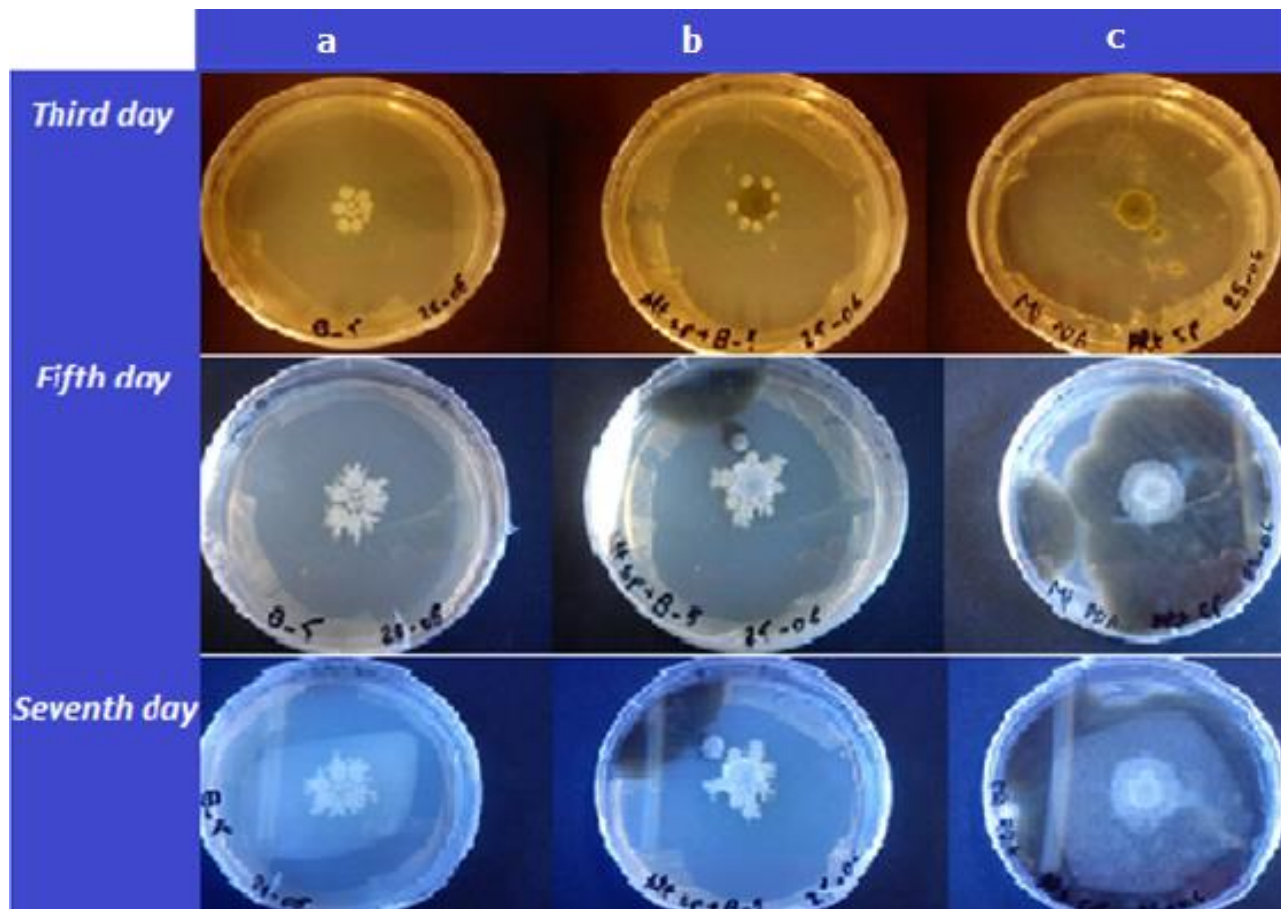
Figure 7. Effect of *B. mojavensis* on the mycelial growth of *A. alternata* during 7 days growth on potato dextrose agar (PDA). The effect in the third, fifth and seventh day are shown in this figure. (A) PDA plates inoculated with *B. mojavensis* strain, (B) PDA plates inoculated centrally by *B. mojavensis* and *A. alternata* strain and (C) PDA plates inoculated with *A. alternate.*

accumulate fluorescein (derivatives) but dead cells are not able to do so (Rotman and Papermaster, 1966).

In this study, the effect of *B. mojavensis* on *A. alternata* growth at intracellular and intercellular levels was carried by FCM analysis. The growth evolution of *B. mojavensis* and *A. alternata* centrally cultured on PDA plates showed a high inhibition of the fungus isolate by the bacterium *B. mojavensis* during the incubation time, from the third to the seventh day (Figure 7). Figure 8 shows a good microbial (*B. mojavensis* and *A. alternata* cultred on PDA plates) staining with a fluorescent product CFDA in FCM analysis. Furthermore, FCM profiles of dead and alive *B. mojavensis* and *A. alternata* cells was verified before launching analysis on PDA plates where fungal and bacterial strains were centrally cultured.

Before treating microbial preparations with heat (90°C during 12 min), the green fluorescent intensity (GFI) of alive cells reached 10^4 and 10^5 MH (megahertz), respectively in the case of *B. mojavensis* (Figure 9A) and *A. alternata* (Figure 10A). However, dead cells GFI of both of them was less important varying between 10^2 and 10^3 MH (Figure 9B and C). The evolution of green

fluorescence intensity of *B. mojavensis, A. alternata* and *B. mojavensis-A. alternata* interaction plates from the third to the seventh day of time incubation was carried by FCM analysis.

After the 3rd day, the green fluorescence intensity of *B. mojavensis* reached its maximum at 10^4 MH, that of *A. alternata* reached 10^6 MH. However, this intensity was between 10^3 and 10^4 MH in the case of *B. mojavensis-A. alternata* interaction plates (Figure 11). After the 5th day, the green fluorescence intensity GFI of *B. mojavensis* decreased slightly until reaching 10^3 MH. Two peaks in 10^3 and 10^6 MH were observed in the case of *A. alternata*. However, the GFI was stable close to 10^3 MH in the case of *B. mojavensis-A. alternata* interaction (Figure 11). After seven days of incubation, approximate GFI values of the fifth day were obtained in *B. mojavensis and A. alternata* plates. Interestingly, GFI of microbial interaction plate increased reaching values between 10^4 and 10^5 MH.

In the control, the GFI values of *B. mojavensis* (Figure 9B,C) showed that not all cells were dead after 12 min at 90°C, this can be explained by the presence of spores
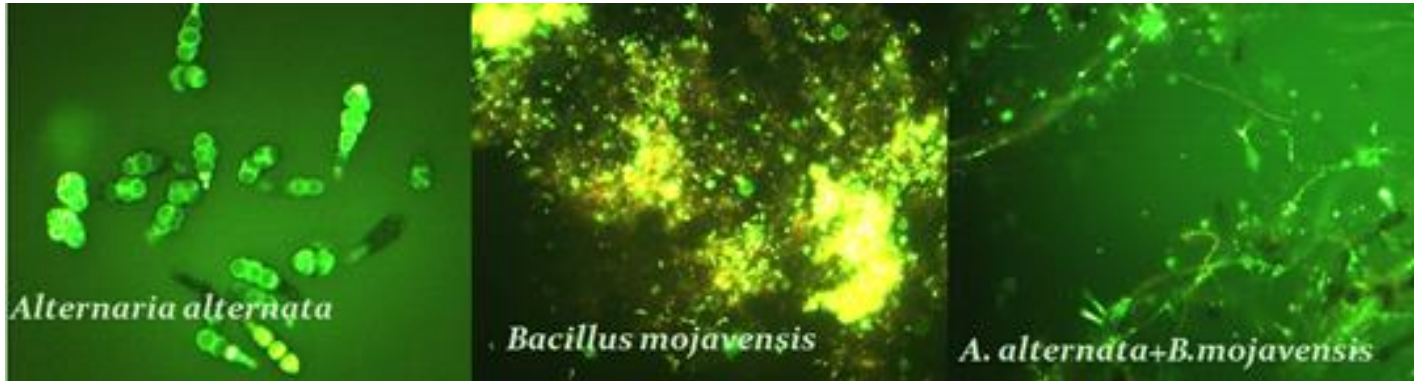
Figure 8. Interaction between *A. alternata* and *B.mojavensis* monitored by fluorescence microscopy. Cells were treated as described in the Materials and Methods section. Fungal cells were stained with CFDA (green fluorescence) microscopic observation showed in this figure was 7 days of interaction (GX 40).
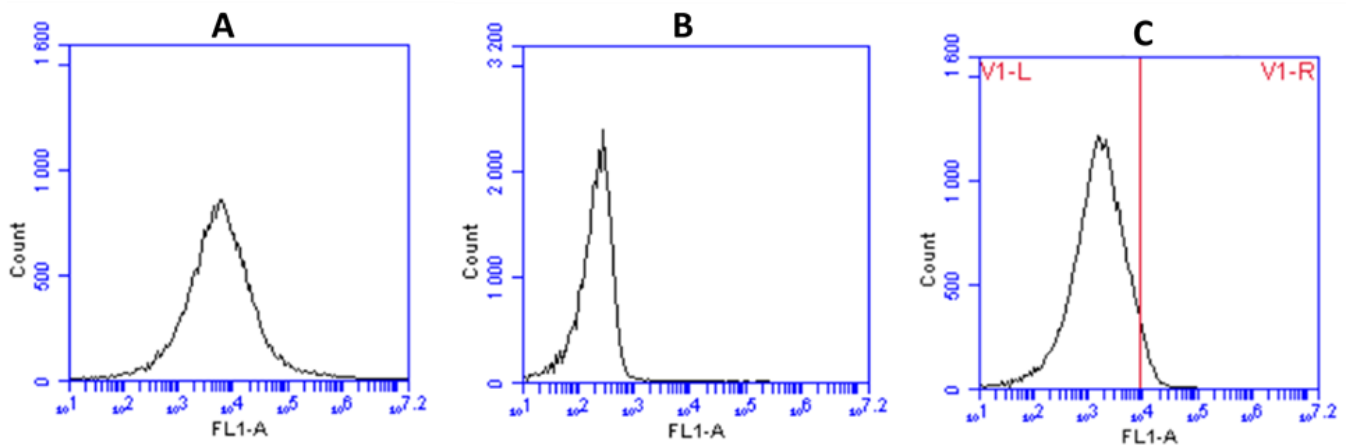

Figure 9. Flow cytometry profile of control, *B. mojavenisis* plates after 3 days of incubation. (A) bacterial cells alive before heat treatment (90°C during 12 min), (B) dead bacterial cells not colored with CFDA, after heat treatment, (C) bacterial dead cells colored with CFDA, after heat treatment.
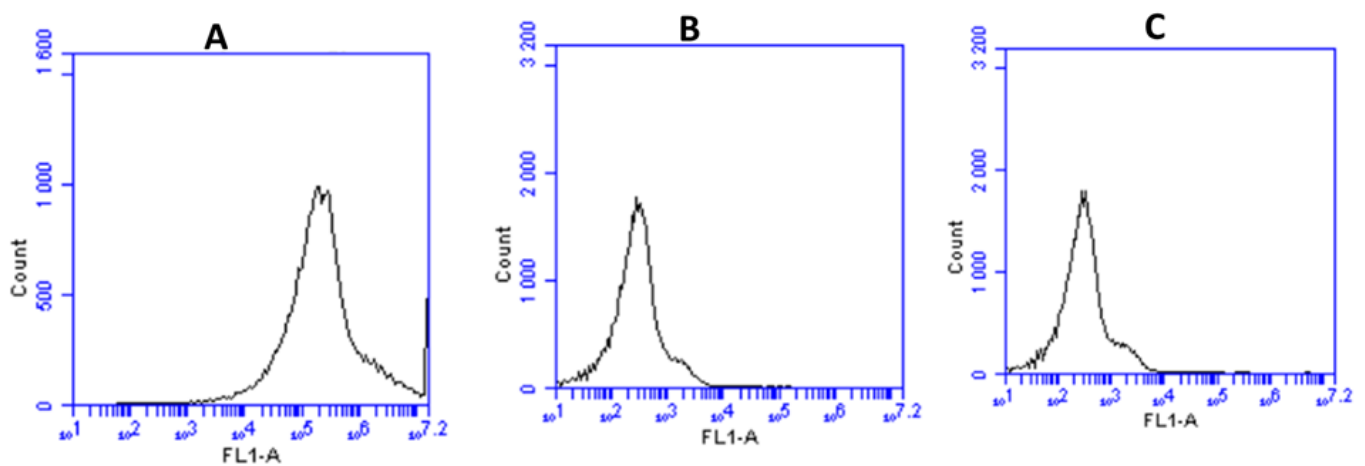

Figure 10. Flow cytometry profile of the control, *A. alternata* plates after 3 days of incubation. (A) fungal cells alive before heat treatment (90°C during 12 min), (B) dead fungal cells not colored with CFDA, after heat treatment, (C) dead fungal cells colored with CFDA, after heat treatment.
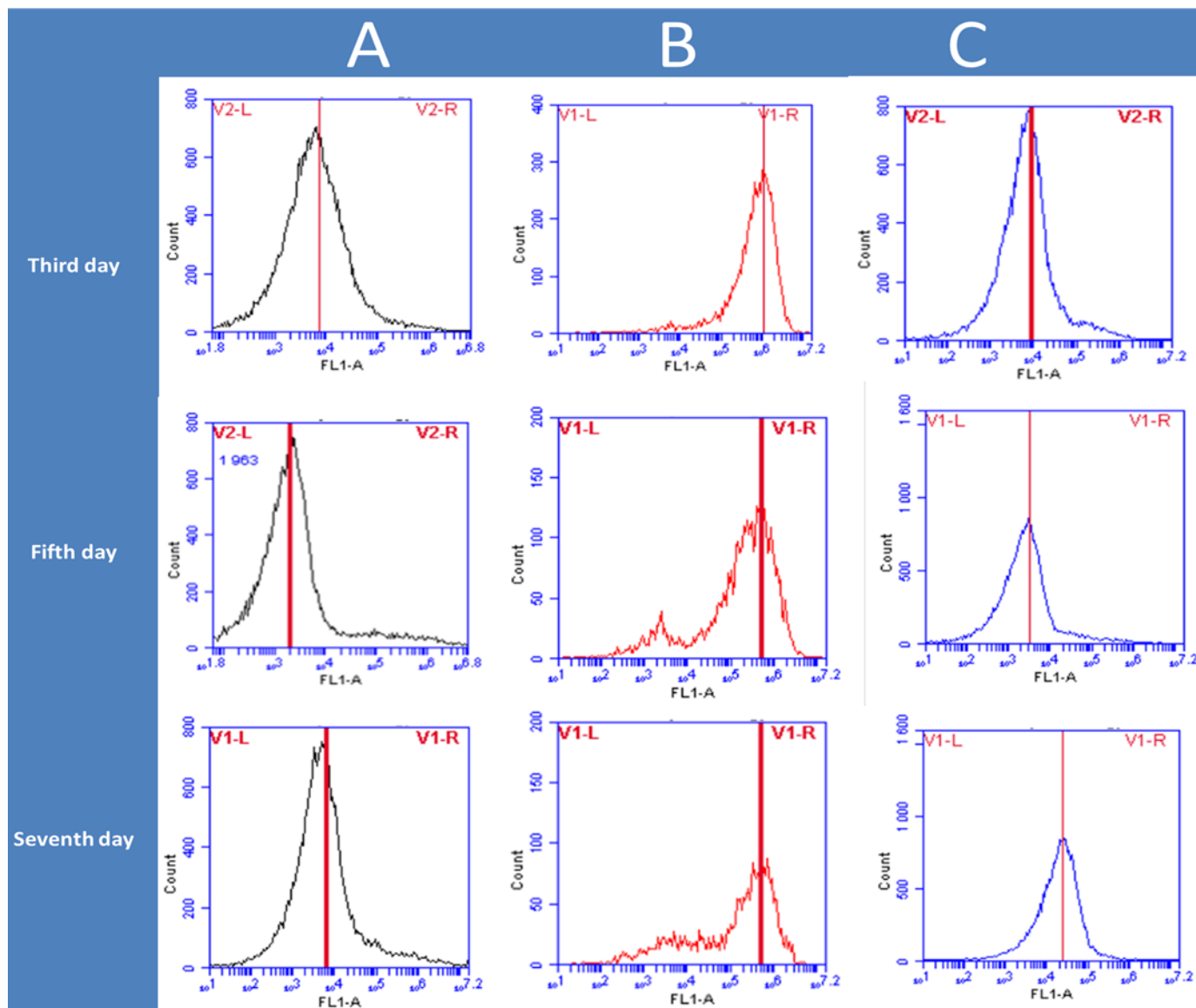
Figure 11. Flow cytometry profiles experimental of *B. mojavensis* (A), *A. alternata* (B), *B. mojavensis-A. alternata* interaction (C) after the third, fifth and the seventh day of incubation.

that are resistant to high temperatures (Rocha et al., 2014).

Cells of *A. alternata* are viable with a maximum GFI which reached 10^6 MH (Figure 11B) after the 3rd, 5th and 7th day, in comparison with the control, dead cells of *A. alternata*, which was 10^2 MH (Figure 10C). However, the GFI of the interaction between *A. alternata* and *B. mojavensis* was proximate to that of *B. mojavensis* which was between 10^3 and 10^4 MH, after the 3rd, 5th and 7th day (Figure 11A and C). These results confirm that *B. mojavensis* deteriorate fugal mycelium of *A. alternata*.

In fact, the comparison of GFI values of the FCM analysis (Figure 11) and the effect of *B. mojavensis* on *A. alternata* after the 3rd, 5th and 7th day on (PDA) plates (Figure 7) confirm the antifungal activity of *B. mojavensis* against *A. alternata*. Rocha et al. (2014) used flow cytometric for studying the interaction between *Fusarium verticillioides* and *Bacillus thuringiensis* Subsp. Kurstaki using Calcofluor White (CFW) and 7 aminoactinomycin (7-AAD). A reduction in cell numbers in the treated cultures on the third, fifth and seventh days during the FCM analysis was observed.

Fluorescence microscopy

The microscopic examination by fluorescence microscopy revealed an evident antagonistic effect in the

seventh day, in comparison with the control, *A. alternata* culture (Figure 8). The results are the same with that of Rocha et al. (2014) on interaction between *F. verticillioides* and *B. thuringiensis* subsp. *kurstaki*.

Conclusion

In conclusion, the FCM method results showed interesting antagonistic effect of *B. mojavensis* on *A. alternata* while additional green house and field experiments on tomato plants are required for the possibility of using it as a biocontrol agent.

Conflict of Interests

The authors have not declared any conflict of interests.

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