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Biofilm formation is determinant in tomato rhizosphere colonization by *Bacillus velezensis* FZB42

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Abstract In this work, the behavior in tomato rhizosphere of *Bacillus velezensis* FZB42 was analyzed taking into account the surfactin production, the use of tomato roots exudate as substrates, and the biofilm formation. *B. velezensis* FZB42 and *B. amyloliquefaciens* S499 have a similar capability to colonize tomato rhizosphere. Little difference in this colonization was observed with surfactin non producing *B. velezensis* FZB42 mutant strains. *B. velezensis* is able to grow in the presence of root exudate and used preferentially sucrose, maltose, glutamic, and malic acids as carbon sources. A mutant enable to produce exopolysaccharide (EPS^-) was constructed to demonstrate the main importance of biofilm formation on rhizosphere colonization. This mutant had completely lost its ability to form biofilm whatever the substrate present in the culture medium and was unable to efficiently colonize tomato rhizosphere.

Keywords Rhizosphere · Colonization · *Bacillus* · Lipopeptides · Root exudates · Biofilm

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Introduction

Plant growth-promoting rhizobacteria (PGPR) provide beneficial effects to host plants and they contribute to increase yields of crops (Saharan and Nehra 2011). Direct mechanisms involved in the beneficial effects of these PGPR are biofertilization (increase of nutrient supply), stimulation of root development (production of phytohormones), and improvement of abiotic stress tolerance (Saharan and Nehra 2011; Glick 2012). PGPR can also indirectly favor plant growth and health by reducing the impact of diseases caused by phytopathogens via three main mechanisms that are competition for space and nutrients, antagonism toward infectious microbes, and elicitation of plant defense reactions (a phenomenon called "induced systemic resistance" (ISR)) (Van Loon and Bakker 2005; Choudhary and Johri 2009; Lugtenberg and Kamilova 2009; Kloepper et al. 2004). It is well established that to provide their beneficial effects, PGPR have to reach minimal population densities in the rhizosphere (Lugtenberg and Kamilova 2009; Das and Dkhar 2011). Thus, an efficient colonization of rhizosphere is a key step for providing both growth-promoting effect and disease control activity. The main hypothesis currently mentioned in literature to explain colonization efficiency is the strain abilities (i) to move toward the place to colonize, (ii) to use carbon and nitrogen sources (Bertin et al. 2003) provided by root exudates, (iii) to withstand plant response reaction (Budiharjo et al. 2014), and (iv) to form a biofilm at the root surface.

Bacillus amyloliquefaciens and *Bacillus subtilis* are well recognized PGPR. *B. amyloliquefaciens* strains are able to produce auxin (Chen et al. 2007; Idris et al. 2004) or can contribute to plant growth promotion under conditions of phosphate limitation by excreting phytase in phytate presence (Idris et al. 2002; Makarewicz et al. 2006).

In addition, some *Bacillus amyloliquefaciens* strains such as S499 or FZB42, now *Bacillus velezensis* FZB42 (Fan et al.

2017), have an impressive capacity to produce secondary metabolites having antimicrobial activities (Scholz et al. 2014; Molinatto et al. 2016). Besides *B. velezensis*, *B. subtilis* strains have been reported to synergistically increase plant nitrogen phosphate accumulation when co-inoculated with mycorrhiza *Glomus intraradices* (Kohler et al. 2007). *Bacillus* sp. strains are also able to synthesize a set of different secondary metabolites. Among the metabolites produced by both species *B. subtilis* and *B. velezensis*, cyclic lipopeptides (CLPs) belonging to the surfactin, iturin, and fengycin families have been well studied (Ongena and Jacques 2008). In vivo, fengycins and iturins display antifungal activities and inhibit the growth of several plant pathogens. Surfactins are poorly antifungal but may have some synergistic effects on biological activity of iturins (Deravel et al. 2014) and fengycins (Maget-Dana et al. 1992; Ongena and Jacques 2008). Surfactins, fengycins, and iturins are able to stimulate Induced Systemic Resistance (ISR), by playing a role of elicitor, in some plant species such as bean, tomato, melon, and grapewine (Ongena et al. 2007; Garcia-Gutierrez et al. 2013; Farace et al. 2015). Interestingly, the expression of the genes involved in the biosynthesis of some of these compounds is increased in the presence of root exudates (Fan et al. 2012).

Due to their surfactant and tension surface-lowering activities, it has been suggested that these lipopeptides, especially surfactin, may contribute to the root colonization process by the producing strains. This hypothesis is mainly supported by in vitro data (Leclere et al. 2006; Ongena and Jacques 2008), but also by studies demonstrating such a role *in planta* (Bais et al. 2004; Fan et al. 2011; Dietel et al. 2013). Using confocal laser microscopy and GFP-labeled strains, Fan et al. (2011 and 2012) have also demonstrated the ability of FZB42 strains to colonize roots of *Lemna minor*, *Arabidopsis thaliana*, and *Zea mays*.

In this work, we confirmed the role of surfactin in tomato root colonization, we characterized the influence of tomato root exudates on *Bacillus velezensis* growth and surfactin production, and we highlighted, for the first time, the main role played by the biofilm formation in the *B. velezensis* FZB42 colonization process. This last result was obtained by comparing the behavior of an exopolysaccharide non-producer mutant strain with the wild-type.

Materials and methods

Microorganisms and plants

Tomato seeds *Solanum lycopersicum* (Merveille des Marchés cultivar) and four bacterial strains (Table 1) were used in this study: *B. amyloliquefaciens* S499, *B. velezensis* FZB42, and two FZB42 derivatives: AK3 and CH1 (kindly provided by Dr. Rainer from Humboldt University, Berlin, Germany). The *Bacillus* strains were long-term preserved at − 80 °C in glycerol (40%) and routinely grown at 37 °C on Luria-Bertani (LB) broth.

Invasive growth assays

Nine centimeter diameter Petri dishes containing 25 mL of LB medium with 0.7% bacto agar (Julkowska et al. 2004) were prepared 90 min before inoculation and dried during 15 min lid open in a laminar flow hood. The center of the LB medium plate was inoculated with a drop of 3 μL of diluted culture in LB medium ($OD_{600} = 0.1$). The plates were incubated at 30 °C and the colonization was evaluated after 3 days. Each experiment was repeated at least three times.

Preparing tomato seeds for germination

Tomato seeds were surface sterilized with ethanol 75% during 2 min and in sodium hypochlorite 4.5% for 15 min and rinsed with sterile water. Then, they were put in Petri dish containing filter paper wetted with Hoagland solution and then left for germination during 4 days at 21 °C. After germination, they were used for the experiments of rhizosphere colonization, kinetic of bacterial growths, and surfactin production. They also were used for hydroponic experiments to collect root exudates.

Bacterial colonization of tomato rhizosphere

Bacterial strains were grown at 37 °C in LB medium and the bacterial cells were prepared for inocula by diluting them in the solution of 0.01 M of $MgSO_4$ until a 1×10^5 CFU mL^{-1} concentration. Surface-sterilized and pregerminated tomato seeds were soaked for 10 min in such a diluted bacterial cell suspension and placed into a sterilized glass tube containing 2 g of perlite and 9 mL of Hoagland solution (final volume = 14 cm^3). Tomato plantlets were grown at 21 °C in a culture room with a 16:8 (light/dark) hours of photoperiod. After 21 days of cultivation, three tubes were randomly chosen, aerial parts were removed, and 10 mL of trypton salt was added to each tube. These tubes were vortexed at 2500 rpm for 5 min and series of dilutions were released for bacterial plate count on LB agar. Results are expressed as total CFU per cubic centimeter of perlite.

Kinetic of rhizosphere colonization and surfactin production

B. velezensis FZB42 strain was used for kinetic study. Tomato seeds were prepared and grown as described earlier. Every 3 days, two samples of two treatments (without inoculum and inoculated with *B. velezensis*) were randomly taken. One sample was used for plate count and one for surfactin

Table 1 *Bacillus* strains used in this study. + = surfactin producer; − = non-surfactin producer

Bacillus strains	Surfactin production	References
Bacillus amyloliquefaciens S499	+	Jacques et al. 1999
Bacillus velezensis FZB42	+	Koumoutsi et al. 2004
Bacillus velezensis AK3	+	Koumoutsi et al. 2004
Bacillus velezensis CH1	−	Koumoutsi et al. 2004

quantification. For plate count, aerial parts were removed and 10 mL of trypton salt was added to each tube, these tubes were vortexed for 5 min and series of dilutions in trypton salt were released for bacterial plate count on LB agar. Results are expressed as total CFU per cubic centimeter of perlite. The sample for surfactin extraction was also randomly selected and the aerial parts were removed. Nine milliliters of acetonitrile/formic acid 0.1% (*V*/*V*) and 2 g of glass beads were added to each tube. These tubes were first vortexed for 5 min and then incubated overnight at 30 °C under agitation (140 rpm). The tubes were centrifuged at 10,000 rpm during 10 min. Surfactin was recovered by loading the supernatant on Solid-Phase Extraction Cartridges C18 (Alltech Maxi-Clean). The cartridges were washed with water and the surfactin was eluted with a solution of acetonitrile. The solutions were vacuum dried (Speed Vac Plus, SC 110A, Savant, GMI, Ramsey, USA). Dried residues were suspended in 200 μL of acetonitrile/water/formic acid 80/20/0.1 (*V*/*V*/*V*) and analyzed by HPLC (Online Degaser, 717 Autosample, 660S Controller, 626 Pumps, 2996 PhotoDiode Array; Waters Corporation, Milford, MA, USA). The column used was a C-18 (5 μm, 250 × 3 mm, VYDAC 218 TP53; Grace-Davison, Deerfield, Illinois, USA). The liquid phase was a gradient of acetonitrile (0.1% trifluoroacetic acid) in double distilled water (0.1% trifluoroacetic acid) (Table 2), the volume of injection was 20 μL and the flow rate was 0.6 mL min^{-1}.

Samples preparing for microscopic root observation

The microscopic observation of root was carried out on tomato plant roots after 21 days of cultivation. The dye used was acridine orange 0.01% (*w*/*v*) prepared in 0.1 M acetate buffer, pH 4 (36 mL of 0.1 M sodium acetate mixed with 164 mL of 0.1 M acetic acid). Several plants were randomly chosen, the aerial parts were removed, and the roots were submerged in acridine orange for 5 min. Samples were protected from light during this treatment. The roots were then fixed between slide and cover slip. The observation was performed under a fluorescence microscope, Nikon EFD-3, using oily lens with 100X magnification. The images were obtained by using a Nikon DS-1 Fi camera connected to a computer.

Root exudates collection

After 4 days of germination, sterilized seeds were put in sterile tubes containing Hoagland solution. The germinated seeds were left for growth at condition of 8:16 (dark/light) hours of photoperiod and at room temperature (21 °C). After 21 days, the root exudates were collected by recovering all the solution from the hydroponic experiment. Solution was sterilized by passing through a filter (0.22 μm) and then 50 times vacuum-concentrated and stored at − 20 °C.

Kinetic study for bacterial growth in root exudates and different carbon sources

A kinetic study was performed to elucidate the demeanor of bacterial growth during 72 h in root exudates and in different carbon sources. BioLector system was used as a simple and efficient high-throughput screening tool to follow microbial kinetics. By this tool, 48 samples can be studied in the same time under different conditions. The conditions of cultures, temperature, and agitation are controlled and pH, biomass, oxygen, and GFP are continuously measured by sensors supplied in BioLector system. One thousand two hundred microliters of root exudates and each carbon source in minimum medium were loaded in each well of BioLector microplates at 21 °C and 160 rpm for 72 h. Growth kinetics were followed by online measurement of optical density. The results were expressed as optical density (600 nm).

Table 2 Isocratic gradient for surfactin, fengycin, and iturin quantification by HPLC. ACN/TFA, acetonitrile with 0.1% trifluoroacetic acid; Water/TFA, water with 0.1% trifluoroacetic acid

Time/min	ACN/TFA	Water/TFA
0	40	60
20	40	60
35	65	35
40	80	20
55	80	20
56	100	0
61	100	0
62	40	60
70	40	60

B. velezensis FZB42 growth and surfactin production on different carbon sources

Ten milliliters of concentrated root exudates and a set of five milliliters solutions containing an equivalent of 2 g of carbon were prepared with one of the following carbon sources: glucose, sucrose, fructose, maltose, or xylose as sugars and glutamic, malic, succinic, fumaric, citric, or oxalic acids, dissolved in 1 L of minimal medium composed of ($Na_2HPO_4 \cdot 2H_2O$ 33.7 mM, KH_2PO_4 22.0 mM, NaCl 8.55 mM, $MgSO_4 \cdot 7H_2O$ 1 mM, $CaCl_2 \cdot H_2O$ 0.3 mM, thiamin-HCl 0.003 μM, biotin 0.004 μM, EDTA 0.17 mM, $FeCl_3 \cdot 6H_2O$ 0.03 mM $ZnCl_2$ 0.0062 mM, CuCl2·$2H_2O$ 0.76 μM, $CoCl_2 \cdot 2H_2O$ 0.42 μM, H_3BO_3 1.62 μM, and MnCl2-4H2O 0.08 μM. pH was adjusted to 7 and the (C/N) ratio was (8:1). All these media and root exudates were inoculated by 1×10^5 CFU of *B. velezensis* FZB42 and were incubated at 21 °C under agitation (160 rpm) for 72 h. After 72 h, the population of bacteria was determined by using bacterial plate count. The bacterial suspensions were taken and centrifuged at 10,000 rpm through 10 min. The supernatants were prepurified and analyzed by HPLC as described above.

Biofilm assay

To quantify *B. velezensis* FZB42 biofilm formation, the procedure described by Hsueh et al (2006) was used. The strains were grown in LB medium until mid-log phase, and the cells were collected by centrifugation at 10,000 rpm during 10 min and resuspended in minimum medium supplemented with the different carbon sources as described previously. The initial biomass of media containing different carbon sources and concentrated root exudates inoculated with *B. velezensis* FZB42 was 1×10^5 CFU mL^{-1}. All cultures were incubated at 21 °C without shaking for 72 h. The contents of each well were then removed and the well was washed five times with PBS buffer and air-dried. Biofilm cells were stained with 1% crystal violet (CV) solution in 33% (*v*/*v*) acetic acid for 20 min. Excess CV was then removed with water for five times. The bound CV was solubilized in 200 μL of 33% acetic acid and the absorbance measured at 590 nm.

EPS$^-$ mutant construction

Escherichia coli JM109 and *B. velezensis* FZB42 were routinely cultured in LB liquid medium at 37 °C and 160 rpm or on LB agar plate at 37 °C. When appropriate, ampicillin (Ap; 100 μg mL^{-1} for *E. coli*) and erythromycin (Em; 20 μg mL^{-1} for *B. velezensis* FZB42) were added to the medium. The vectors used in this study were pGEM-T Easy and pMUTIN-GFP$^+$. Firstly, an *epsA* amplicon was amplified using polymerase chain reaction procedure (denaturation temperature, 94 °C for 2 min, annealing temperature, 55 °C for 45 s, and elongation temperature, 72 °C for 2 min; during 35 cycles). The forward primer sequence was 5′GGTACCCT TTTCTTCTGCGG′3, whereas the reverse primer was 5′ CGGCCGGCTTAAGAC′3. These primers were designed by both Primer3 (Version 4.0) and Amplifix programs. The PCR product was introduced in *E. coli* JM109 using pGEM-T Easy vector according to the instructions of the supplier (Promega Corp, Madison, WI, USA). The transformation mixture was spread onto LB medium containing the required antibiotic and the plates were incubated at 37 °C for 24 h. The pGEM-T Easy containing the *epsA* fragment was extracted using Gene Jet Plasmid Miniprep Kit (Thermo Scientific Fermentas, Vilnius, Lituania). A sufficient amount of *epsA* fragment was digested by selected restriction enzymes (Thermo Scientific Fermentas) and then transferred in *E. coli* JM109 after ligation within pMUTIN-GFP$^+$ previously digested by the same enzymes. The resulting hybrid plasmid was transferred into *B. velezensiss* FZB42 using electroporation following two procedures (Zhang et al. 2011; Cao et al. 2011): an overnight LB culture of the FZB42 cells was diluted 100-fold in NCM fresh medium. When the optical density reached 0.5, the cell walls were weakened by adding 3.89% glycine and 1.06% DL-threonine. After 1 h of shaking, the cells were cooled on ice for 20 min and then collected by centrifugation at 4 °C and 8000×*g* for 5 min. Cells were washed four times with ice-cold ETM buffer (0.5 M sorbitol, 0.5 M mannitol, and 10% glycerol), containing KH_2PO_4, K_2HPO_4, and $MgCl_2$ at 0.25, 0.25, and 0.5 mM, respectively (pH adjusted to 7.0). The electro-competent cells were resuspended in 1/100 volume of the original culture. One hundred microliter of this suspension was mixed with 100 ng of column-purified pMUTIN-GFP$^+$ plasmid carrying the *epsA* amplicon. The mix was loaded into a prechilled 1 mm gap electroporation cuvette which was briefly incubated on ice and was shocked by a single 2.1 kV cm^{-1} pulse generated with resistance and capacitance set at 200 Ω and 25 μF, respectively. The cells were immediately diluted into 1 mL of recovery medium (growth medium containing 0.38 M mannitol and 0.5 sorbitol), following warming in a water bath at 46 °C for 6 min. Then, the cells were gently shaken for 3 h at 37 °C. Aliquots were spread onto LB medium agar plate supplemented with erythromycin (20 μg mL^{-1}). After subculture in LB Em_{20}, genomic DNAs were extracted from transformants using Wizard® Genomic DNA Purification Kit (Promega Corp.).

Interruption of *epsA* in *B. velezensis* FZB42 using fusion with GFP marker

A fragment from the *eps* operon was amplified by polymerase chain reaction (PCR) using the primers forward: 5′ GGTACCCTTTTCTTCTGCGG′3 and reverse: 5′

CGGCCGGCTTAAGAC′3 designed by both Primer3 (Version 4.0) and Amplifix programs, and chromosomal DNA from *B. velezensis* FZB42 as template. The PCR product was cloned in pGEM-T Easy and the ligation mixture was transformed into *E. coli* JM109 using a thermal shock procedure. Transformants were grown overnight in LB medium containing 100 μg mL^{-1} ampicillin. Then, the purified hybrid plasmid with the *epsA-epsC* fragment was extracted, purified, and cut using the restriction enzymes *Kpn*I and *Xma*III. The *epsA-epsC* amplicon was ligated to pMUTIN-GFP^+ cut with the same enzymes. The ligation mixture served to transform *E. coli* JM109 as above, with a selection by resistance to 20 μg mL^{-1} erythromycin. After overnight growth of transformants in LB medium + Em_{20}, the purified pMUTIN-GFP^+::*epsA-C* was used to transform *B. velezensis* FZB42 using electroporation method with Em resistance selection. Transformants were grown overnight in LB medium and samples were analyzed by fluorescence microscopy. The results showed that the fusion gene *eps-gfp* was expressed in all cells as compared with the wild-type strain. To ensure that *epsA* was integrated within the corresponding chromosomal locus of FZB42, a fragment of 1972 bp was designed as above, with the primers forward: 5′ ACTCATCTTCCGTGTCTCC′3 and reverse: 5′ GTCTTGTAGTTCCCGTCATC′3. This fragment consisted of a part of *slr*, *epsA*, *epsB* and a part of *gfp* genes and was amplified using chromosomal DNA from both strain FZB42 and its Em-R fluorescent derivative. After agarose gel electrophoresis analysis, the 1972-bp amplicon was observed only in the Em-R transformant (data not shown).

Results and discussion

Rhizosphere colonization by different bacterial strains

B. amyloliquefaciens S499 and *B. velezensis* FZB42 and two derivatives (AK3 and CH1) were compared for their capacity to colonize tomato rhizosphere. The results obtained after 21 days of colonization are shown in Fig. 1. The results indicated similar colonization performance of the strains *B. amyloliquefaciens* S499, *B. velezensis* FZB42 and AK3 with respectively 3.8×10^7, 3.4×10^7, and 3.6×10^7 CFU cm^{-3} and a weaker result for the surfactin non-producing derivative CH1 with 2.2×10^7 CFU cm^{-3}. This significant difference in colonization observed between the surfactin producers and the non-surfactin producer confirmed that surfactin production might promote the rhizosphere colonization. Nevertheless, this difference is weak (around 30% difference in biomass) indicating that other factors have to be considered.

Kinetic of bacterial growth and surfactin production during rhizosphere colonization

The kinetic of bacterial growth and surfactin production in the tomato rhizosphere of *B. velezensis* FZB42 was followed during 21 days. The results are presented in Fig. 2.

The bacterial population of *B. velezensis* FZB42 in the rhizosphere continuously increased from an initial population of 1×10^5 to 2×10^8 CFU cm^{-3} at the end of experiment (21 days). The surfactin production followed the bacterial growth and final surfactin concentration was 6 μg cm^{-3}. The surfactin production was harmonious with the biomass. Nevertheless, the specific production of surfactin was low compared to this obtained with a non-colonizing surfactin overproducing *Bacillus* strain (data not shown). This result confirmed that other parameters than surfactin are determinant for efficient root colonization.

Effect of root exudates on bacterial growth and surfactin production

In order to verify the role of root exudates on bacterial growth and surfactin production, root exudates from tomato roots were used as culture medium for *B. velezensis* FZB42.

B. velezensis FZB42 population reached value of 2.5×10^8 CFU mL^{-1} and surfactin production was 0.4 μg per 10^8 CFU. These findings are consistent with what has been obtained from the results of kinetic and this demonstrates the importance of root secretions to explain the rhizosphere colonization. Makarewicz et al. (2006) also found that the root exudates from tomato plant supported bacterial cell division and enhance the growth of *B. amyloliquefaciens*.

Root exudates include a diverse array of carbon sources like primary metabolites such as phenolic acids, organic acids, sugars and amino acids and secondary metabolites compounds (Badri and Vivanco 2009; Emmert and Handelsman 1999; Kohler et al. 2007). They provide the growth factors as well as nutrient sources for bacterial growth of *B. velezensis* FZB42.

The root exudates contain different organic compounds (Vancura and Hovadik 1965; Vancura and Hanzlikova 1972) and these compounds are indeed necessary to bacterial growth. Nevertheless, it is very complicated to get a good characterization of root exudates in terms of composition. Then, individual carbon sources (sugars and organic acids) were tested for bacterial growth and surfactin production.

Effect of different carbon sources on bacterial growth and surfactin production

As the root exudates contain many organic compounds that could affect growth and surfactin production, different sugars and organic acids were individually tested in this assay.

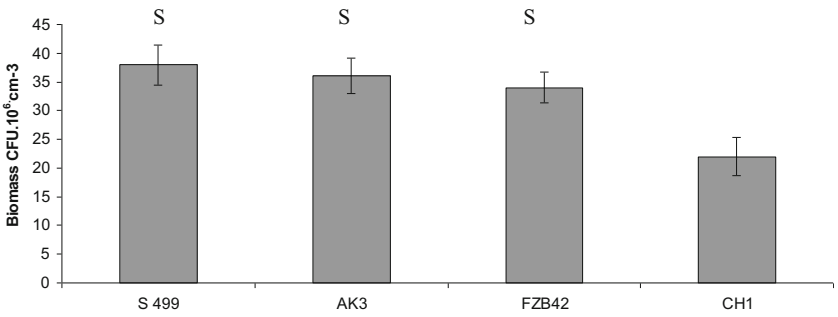

Fig. 1 Rhizosphere colonization by different strains of *Bacillus* spp.: *B. velezensis* S499, *B. velezensis* AK3, *B. velezensis* FZB42, and *B. velezensis* CH1. Tomato plants were planted in room culture at 21 °C, 16:8 h (light/dark) photoperiods during 21 days. Three replicates were used for bacterial count. S = strain producing surfactin

The effect of sugars and organic acids was tested using BioLector system. The results of growth are presented in Figs. 3 and 4. For sugars, the highest biomass was observed when maltose was used as a carbon source followed by sucrose, glucose, fructose, and xylose. These results indicated a non-negligible influence of the sugars used for bacterial growth of *B. velezensis* FZB42. Concerning organic acids, the highest biomass was obtained with glutamic acid, followed by malic, fumaric, succinic, citric, and oxalic acids. Oxalic acid had a negative role by inhibiting the bacterial growth; this observation was in agreement with the report of Rudrappa et al. (2007). The same observation occurred with *Pseudomonas polymyxa* SQR-21 (Ling et al. 2011).

The biomass was around two to three times higher with glutamic and malic acids than with citric and oxalic acids indicating also a great influence of the organic acids used as carbon sources for *B. velezensis* FZB42 growth. These results on different carbon sources generally present in root exudates indicate that root exudates composition has an influence on bacterial growth.

Sugars as carbon sources have also been tested for surfactin production by *B. velezensis* FZB42. The results are presented in Table 3. The production of surfactin depends on the sugar used by *B. velezensis* FZB42. Glucose and fructose are the best sugars and low production is observed with maltose and xylose.

When comparing the results of the specific production of surfactin, a difference between the different sugars was also clearly showed. For fructose and glucose, the values of specific production were almost the same but were reduced with sucrose, maltose, and xylose. The bigger contrast was observed with the maltose which allows the best biomass but with a weak production of surfactin. All these specific productions were higher than the one observed in the presence of the root exudates. However, surfactin had a very low critical micellar concentration (about 10 mg L^{-1}) and its influence on the surface tension was still effective at low concentrations (Ongena and Jacques 2008).

Construction of a *B. velezensis* FZB42 EPS$^-$ mutant

Previous reports clearly indicated that mutants which were unable to synthesize exopolysaccharide were also unable to form biofilms, even though they may still form microcolonies and attach to the surfaces in limited scope (Allison and Sutherland 1987; Watnick and Kolter 1999; Sutherland 2001). As exopolysaccharides are known to be an important factor in biofilm formation, experiments were conducted for the purpose of interrupting a gene (*eps*) implied in exopolysaccharide synthesis in *B. velezensis* FZB42 strain.

Once this EPS$^-$ mutant obtained, preliminary experiments were performed to compare the behavior of the EPS$^-$ mutant to the wild-type FZB42. Growth kinetics of the two strains in LB medium showed no significant differences between these strains (same growth and same surfactin production), indicating that there was no effect of *eps* gene interruption on the bacterial growth and surfactin production of the EPS$^-$ mutant (data not shown).

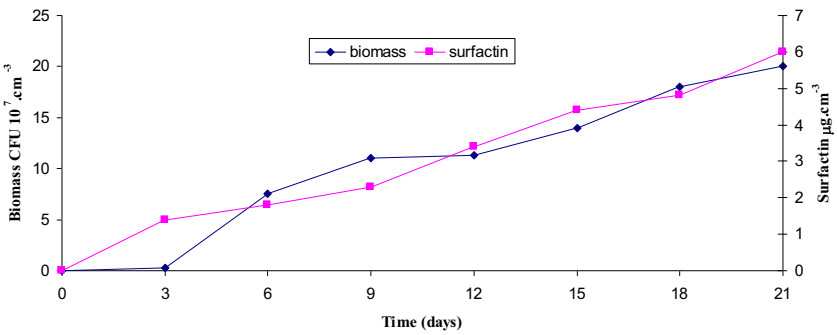

Fig. 2 Kinetic of tomato root colonization and surfactin production by *B. velezensis* FZB42. Tomato plants were planted in room culture at 21 °C, 16:8 h (light/dark) photoperiods and during 21 days. Three replicates were used for bacterial count and three others were used for surfactin quantification

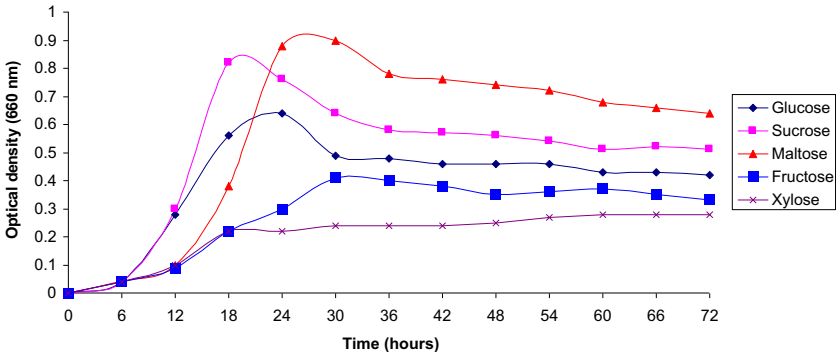

Fig. 3 Kinetic of growth of *B. velezensis* FZB42 in different sugars used as a carbon source after 72 h of incubation at 21 °C under a 160-rpm agitation. BioLector system was used with a culture volume of 1200 μL. All sugars were calculated as 2 g C L^{-1} dissolved in minimal medium

In vitro biofilm comparative assays with *B. velezensis* FZB42, and its EPS^{-} derivative

Thereafter, the two strains were grown in static cultures containing different carbon sources to investigate their ability to form a biofilm. The optical densities of biofilm stained with crystal violet were measured and the results are presented in Fig. 5.

A high difference was observed between these strains in forming a biofilm. With all tested carbon sources, the wild-type *B. velezensis* FZB42 was able to form a biofilm contrary to the EPS^{-} mutant for which the biofilm formation was very weak. These results pointed out that the production of exopolysaccharides is necessary for biofilm formation by *B. velezensis* FZB42 and confirmed previous findings (Allison and Sutherland 1987; Watnick and Kolter 1999; Sutherland 2001).

Biofilm formation by *B. velezensis* FZB42 was also carbon sources dependent. The best sources being glucose, glutamic, succininc, and malic acids. Contrary to the results obtained on the effect of the substrates on the bacterial growth (Fig. 3), maltose and sucrose allowed a high biomass but not a high biofilm formation. Concerning the tested organic acids, a good correlation was observed between growth and biofilm formation.

Biofilm formation is an important process which represents the basis of root colonization and aggregate communities on soil particle surface by rhizobacteria (Davey et al. 2003; Tan et al. 2013). Biofilm formation was lower with concentrated root exudates than with other carbon sources, due to the facts that (i) the bacteria have a tendency to live in aggregate communities as a response to environmental stress and nutrient starvation (Donlan and Costerton 2002; Leclerc 2003; Swiecilo and Zych-Wezyk 2013) and (ii) the root exudates provide the essential elements for bacterial growth (Bertin et al. 2003; Vancura and Hanzlikova 1972; Vancura and Hovadik 1965). However, the low biofilm formation observed with the concentrated tomato root exudates compared to other carbon sources can be illustrated by the lack of both environmental harsh and nutrient deficiency and starvation in this concentrated tomato root exudates.

Microscopic observation and colonization assays

For more details, a microscopic observation was performed to compare the pattern colonization of both strains on the root of tomato plantlets using fluorescent microscopy (Fig. 6). Both strains colonized the rhizoplane of tomato following specific patterns.

These assays were realized depending on both the results of biofilm formation obtained under in vitro conditions and several reports which indicated the inability to form a biofilm in the absence of exopolysaccharide compounds (Allison and Sutherland 1987; Watnick and Kolter 1999; Sutherland

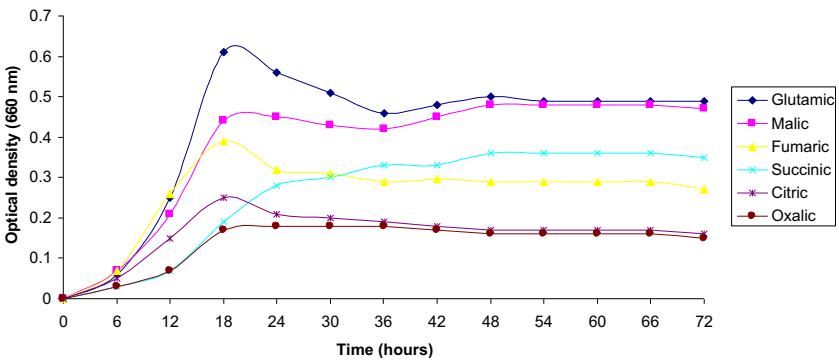

Fig. 4 Kinetic of growth of *B. velezensis* FZB42 in different organic acids used as a carbon sources after 72 h of incubation at 21 °C under a 160-rpm agitation. BioLector system was used with the culture volume of 1200 μL. All organic acids were calculated as 2 g C L^{-1} and dissolved in minimal medium

Table 3 Effect of different sugars on biomass of *B. velezensis* FZB42, surfactin production expressed as milligram per liter and specific surfactin production expressed in microgram per 10^8 cells after 72 h at 21 °C under a 160 rpm agitation in BioLector system

Carbon source	Biomass (CFU × 10^8 mL^{-1})	Surfactin (mg L^{-1})	Surfactin productivity (μg per 10^8 cells)
Fructose	1.85 ± 0.20	124.83 ± 2.20	68.14 ± 1.09
Glucose	2.04 ± 0.18	128.73 ± 6.50	63.58 ± 3.54
Sucrose	2.15 ± 0.32	67.57 ± 2.00	31.98 ± 0.94
Maltose	2.27 ± 0.35	15.05 ± 1.00	6.73 ± 0.45
Xylose	1.20 ± 0.20	2.15 ± 0.50	1.84 ± 0.39

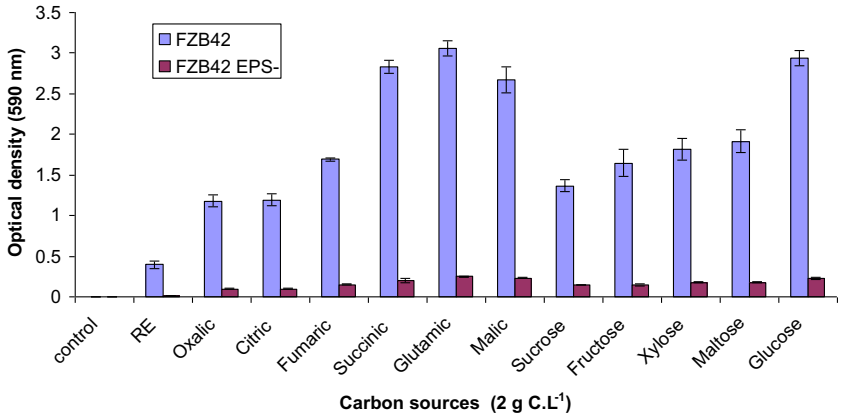

Fig. 5 Biofilm formation by *B. velezensis* FZB42 and *B. velezensis* FZB42 EPS⁻ mutant. The biofilm stained with crystal violet after 72 h of incubation at 21 °C. Control, minimum medium; RE, tomato root exudates concentrated. Carbon sources were dissolved in minimum medium at the concentration of 2 g C L^{-1}

2001). Hence, the same strains were selected to inoculate germinated tomato seeds. They were left for growth in hydroponic system for 21 days. After this period, the aerial parts were removed and the roots were prepared for microscopic observation. *B. velezensis* FZB42 was treated with acridine orange, while the EPS⁻ mutant carried the *gfp* marker. *B. velezensis* FZB42 was the best colonizer of roots as compared with its mutant (Fig. 6). A strong correlation was clearly demonstrated between the low biofilm formation and the weak colonization ability. Associated with the microscopic observation, samples of roots were used to count the number of bacteria that colonized these roots after 21 days. The results expressed as CFU per cubic centimeter were 17 × 10^7 for *B. velezensis* FZB42 and 0.60 × 10^7 for the EPS⁻ mutant. Coupling the results obtained under in vitro conditions with the results of root colonization, the important role of biofilm in colonization was highlighted and we showed that biofilm formation plays a necessary role for roots and rhizosphere colonization.

As shown in previous reports, the rhizosphere colonization by plant growth-promoting bacteria is the most important step for the biocontrol agents (Weller et al. 2002; Vessey 2003; Pii et al. 2015). Our experiments shed light to the role of biofilm formation in rhizosphere colonization. Bacteria cells physically interact with plant by various means. These interactions commonly appear as the colonization of roots and/or rhizosphere. The bacteria adhere to the surface of plant tissues as individual and aggregated cells. The latter are defined as biofilms and they display various arrangements of dimensions, locations, and compositions (Nongkhlaw and Joshi 2014). The plant microenvironment has different

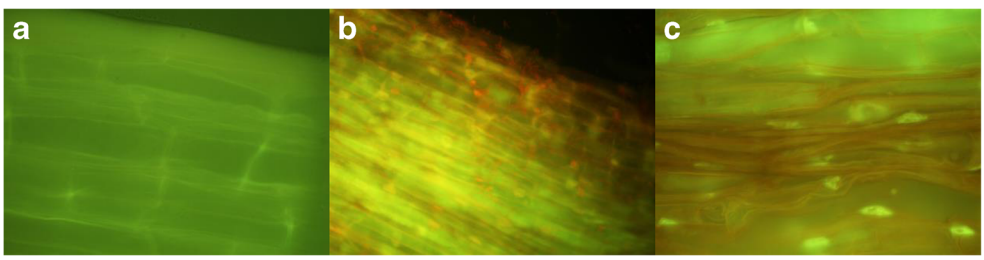

Fig. 6 Patterns of root colonization by different strains stained with the acridine orange. **a** Control; **b** *B. velezensis* FZB42; **c** FZB42 EPS⁻. The pictures were taken by fluorescence microscopy coupled with a camera connected to a computer using oily lens with x100 magnification

characterizations such as saturation levels, nutrient availabilities, and surface chemistries, which strongly influence both the form and activity of biofilms (Ramey et al. 2004).

The amount of compounds within the biofilm depending on the carbon compounds availability and the equilibrium between carbon and other nutrients (Sutherland 2001; Fang et al. 2009), differences have been observed with different carbon sources. EPS$^-$ mutant presented a low biofilm formation and a low colonization of the root and the rhizosphere due to the fact that exopolysaccharide contribute directly to the properties of the biofilms and supply mechanical stability to the biofilms (Mayer et al. 1999; Flemming et al. 2007; Flemming and Wingender 2010).

Conclusion

Previous reports indicated that the rhizosphere colonization by plant growth-promoting bacteria is the most important step for the biocontrol agents (Weller et al. 2002; Vessey 2003; Pii et al. 2015). In this work, we investigated three main factors influencing the rhizosphere colonization by *B. velezensis*. Surfactin production, composition of root exudates, and capability of biofilm formation were evaluated. Surfactin-producer strains better colonize the rhizosphere but the difference is weaker (around 30% of difference of biomass) when compared with a non-surfactin producer strain. These results indicated that surfactin plays a role on the invasion of a Petri plate and on the rhizosphere but surfactin alone cannot explain this last phenomenon. Then, tomato root exudates and their components were evaluated by measuring the growth and the surfactin production by *B. velezensis*. Sugars and organic acids allowed a bacterial growth and a surfactin production but many differences were observed in the behavior of the strain. Depending on the substrates, biomass and surfactin productions differ but here also, root exudates associated to surfactin production cannot explain the capacity of a strain to be a good rhizosphere colonizer or not. Then, we investigated the role of biofilm formation in root colonization. A non-exopolysaccaride producer *B. velezensis* FZB42 mutant was constructed and compared to the wild type. By suppressing the capacity of exopolysaccharide production, the EPS$^-$ mutant also lost his capability to form a biofilm while the wild strain *B. velezensis* FZB42 showed a high biofilm formation. We also showed that the biofilm formation was influenced by the different substrates found in root exudates. Our results led that the rhizosphere colonization by *B. velezensis* FZB42 is dependent on surfactin production and on root exudates composition but the main factor influencing a good colonization is the capability of this strain to form a biofilm.

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