Université Lille 1

Sciences et Technologies

Thèse

Présentée par

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Pour L'obtention du titre de

Docteur de l'Université Lille 1 Sciences et Technologies

Spécialité : Ingénierie des Fonctions Biologiques

Characterization of *Bacillus amyloliquefaciens* FZB42 and *Bacillus subtilis* BBG131 properties responsible for their ability to colonize tomato rhizosphere

Préparée à l'Institut Charles VIOLLETTE, UPRES-EA 1026

Soutenue le 2 Juin 2016 à Université de Lille 1, France,
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Résumé

Les bactéries qui promeuvent la croissance de plantes (PGPR) constituent une partie indispensable du biote de la rhizosphère et lorsqu'elles sont cultivées en association avec les plantes hôtes elles peuvent stimuler la croissance de ces dernières. Les PGPR favorisent la croissance de plantes directement soit en facilitant l'acquisition de ressources (azote, phosphore et des éléments essentiels) soit en modulant le niveau d'hormone de plante, ou indirectement en diminuant les effets inhibiteurs de différents agents pathogènes sur la croissance des plantes. Plusieurs espèces de *Bacillus* appartiennent aux PGPR et ont la capacité de produire des lipopeptides cycliques d'origine non ribosomique tels que la surfactine, la fengycine et la bacillomycine. Les lipopeptides cycliques peuvent être produits *in vitro* et *in vivo,* tels que dans la rhizosphère. La production de ces lipopeptides dans la rhizosphère joue un rôle important pour réprimer les agents pathogènes des plantes et améliorer la relation entre PGPR et plantes hôtes.

La rhizosphère est la région complexe en contact étroit avec les racines des plantes. Les principaux partenaires biologiques de la rhizosphère sont la plante hôte, les microorganismes délétères et les microorganismes bénéfiques. Dans cette région, la colonisation représente le critère le plus important pour les facteurs biotiques en particulier pour les agents de biocontrôle. Plusieurs propriétés des microorganismes peuvent influencer leur potentialité à coloniser la rhizosphère comme leurs capacités de formation de biofilm et de production de lipopeptides. Ces deux propriétés peuvent, elles-mêmes être modulées par la composition des exudats racinaires.

Dans cette étude, nous avons décidé d'étudier ces trois paramètres avec différentes souches de *Bacillus sp*. en relation avec la rhizosphère de la tomate.

Les deux souches principalement étudiées ont été : *B. amyloliquefaciens* FZB42, une souche sauvage productrice des trois familles de lipopeptides (surfactine, fengycine et bacillomycine) et qui représente un potentiel élevé comme agent de contrôle des maladies fongiques des plantes et *B. subtilis* BBG131 qui est un microorganisme génétiquement modifié qui surproduit de la surfactine. Les résultats ont montré un comportement différent entre *B. amyloliquefaciens* FZB42 et *B. subtilis* BBG131. Après 21 jours de colonisation de la rhizosphère, la biomasse de *B. amyloliquefaciens* FZB42 était 25 fois plus élevée que celle de *B. subtilis* BBG131, tandis que la production de surfactine était 5 fois plus faible que celle de *B. subtilis* BBG131. Cultivées sur des exsudats racinaires, les deux souches montrent également des comportements différents : *B. amyloliquefaciens* FZB42 produit plus de biomasse que *B. subtilis*

BBG131 mais moins de surfactine. Nous avons également analysé l'effet de différentes sources de carbone sur la production de surfactine par ces deux souches et montré, par exemple, une production de surfactine par unité cellulaire avec du saccharose de 425 μg 10^{-8} cellules pour *B. subtilis* BBG131 contre 63 μg 10^{-8} cellules pour *B. amyloliquefaciens* FZB42

Les résultats sur la formation de biofilm présentent également des différences significatives entre *B. amyloliquefaciens* FZB42 et *B. subtilis* BBG131. *B. amyloliquefaciens* FZB42 a montré une capacité importante à former des biofilms alors que *B. subtilis* BBG131 a montré le contraire. Un mutant de *B. amyloliquefaciens* FZB42 Eps^- (incapable de produire l'exopolysaccharide indispensable à la formation de biofilm) a été obtenu et son comportement a été comparé à la souche mère. Les résultats présentent clairement le rôle essentiel de la formation de biofilm dans la colonisation. Un comportement similaire de *B. amyloliquefaciens* FZB42 Eps^- et *B. subtilis* BBG131 a été observé pour la formation de biofilm qui se reflète dans leur colonisation dans la rhizosphère.

Globalement, notre travail représente la première étude comparative du comportement dans la colonisation de deux souches de *Bacillus* appartenant à des espèces différentes. Nous avons mis l'accent sur les facteurs importants influençant la colonisation de la rhizosphère. Ainsi, la colonisation de la rhizosphère est affectée par des éléments différents (biotiques et abiotiques) et la formation de biofilm a un impact essentiel.

Mots clef : *Bacillus*, rhizosphère, exsudats racinaires, lipopeptides, sources de carbone, biofilm

Abstract

Plant growth promoting bacteria (PGPR) are an indispensable part of rhizosphere biota that when grown in association with the host plants can stimulate the growth of the host. PGPR promote plant growth directly by either facilitating resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth. Many of *Bacillus* species belong to PGPR and have the ability to produce different cyclic lipopeptides of non-ribosomal origin such as surfactin, fengycin and bacillomycin. These cyclic lipopeptides can be produced *in vitro* and *in vivo* such as in rhizosphere. The production of these lipopeptides in the rhizosphere play an important role to suppress the plant pathogens and it improves the relationship between PGPR and host plant.

Rhizosphere is a complex region that is close to the plant roots. The main biological parenters in the rhizosphere are the host plant, deleterious microorganisms and beneficial microorganisms. In this region, the colonisation represents the most important criterion for the biotic factors, especially for the biocontrol agents. Many properties of microorganisms may influence their potential to colonize the rhizosphere such as the capacity to form a biofilm and lipopeptides production. These two properties may themselves be modulated by the composition of root exudates.

In this work, we have decided to study these three parameters with different strains of *Bacillus* species in the relation with the tomato rhizosphere.

The two main strains studied were: *Bacillus amyloliquefaciens* FZB42, a natural wild-type strain produces three families of lipopeptides (fengycin, surfactin and bacillomycin) and which shows a high potential as biopesticide for the control of fungal plant diseases and *Bacillus subtilis* BBG131 which is a genetically engineered microorganism which overproduces surfactin.

The results showed a different behaviour of *B. amyloliquefaciens* FZB42 and BBG131. After 21 days of rhizosphere colonisation, the biomass of *B. amyloliquefaciens* FZB42 was 25 times higher than with *B. subtilis* BBG131, whereas surfactin production was 5 times less than this produced by *B. subtilis* BBG131. Grown on the root exudates, the two strains also show that same behaviours are observed after 21 days of colonisation: *B. amyloliquefaciens* FZB42 produced more biomass than *B. subtilis* BBG131but the surfactin was less than *B. subtilis* BBG131. We have also analyzed the effect of different carbon sources on the surfactin

production by these two strains and show, for example, that surfactin production expressed per cell unit with sucrose was 425 μg 10^{-8} cells for *B. subtilis* BBG131 against 63 μg 10^{-8} cells for *B. amyloliquefaciens* FZB42.

The results of biofilm formation also present a significant difference between *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131. *B. amyloliquefaciens* FZB42 showed an intense ability to form a biofilm in contrast to *B. subtilis* BBG131. A mutant of *B. amyloliquefaciens* FZB42 Eps^- (unable to produce exopolysaccharide essential for biofilm formation) was obtained and its behaviour was compared to the wild type. The results clearly demonstrate the essential role of biofilm formation in the colonisation. A similar behaviour of *B. amyloliquefaciens* FZB42 Eps^- and *B. subtilis* BBG131 was observed in the biofilm formation which is reflected to their colonisation in the rhizosphere.

Globally, our work represents the first comparative study in the colonisation of two strains of *Bacillus* belonging different species. We have focused on the important factors influencing the rhizosphere colonisation and we can conclude: the rhizosphere colonisation is affected by different elements (biotic or abiotic) and the biofilm formation plays an essentials impact.

Keywords: *Bacillus*, rhizosphere, root exudates lipopeptides production, carbon source, biofilm

Remerciement

Au cours de mon étude, j'ai rencontré beaucoup de gens qui, sans eux, ne m'auraient pas permis terminer mon étude et afin de reconnaître leur gratitude :

Je tiens à remercier le professeur ***Pascal DHULSTER*** *de m'avoir permis de travailler dans le Laboratoire Régional Charles Viollette.*

Je tiens également à remercier le professeur ***Philippe JACQUES, le directeur de thèse*** *pour son grand soutien et pour son suivi tout au long de ce travail. Je voudrais le remercier pour toutes les connaissances acquises sous sa supervision.*

Je tiens à exprimer ma profonde gratitude au Docteur ***François KRIER, mon encadrant,*** *pour son soutien tout au long de ce travail, pour m'avoir donné le bénéfice de ses connaissances et pour son précieux soutien lors de la rédaction de cette thèse.*

Je tiens à remercier un noble homme, le Docteur ***Max BÉCHET*** *pour tout ce qu'il a fait pour moi.*

Je remercie les rapporteurs et les examinateurs d'avoir accepté d'évaluer ce travail.

Je tiens à exprimer mes sincères remerciements à toute l'équipe de ProBioGEM et spécialement mes collègues de la salle de thésards. Je n'oublierai jamais que dès qu'ils font du bruit dans la salle, certains ont pris les casques pour écouter de la musique et se concentrer sur la lecture .... J'ai passé de merveilleux moment avec eux.

Et aussi, je n'oublierai pas les moments passés avec mes meilleurs amis, parfois entrecoupés de discussions sur divers aspects de la vie parfois difficiles. Je vous dis merci mes chers amis : Ahmed, Alaa, Yazin, Qassim, Yanat et Mohamad pour tout ce que vous avez fait pour moi……. et merci à tous ceux m'ont aidé dans tous les aspects de la vie.

Finalement j'ai l'occasion de dire un grand merci à ma famille en Irak pour leur encouragement. Je lève mon chapeau en l'honneur et la reconnaissance de l'excellent travail que mon épouse m'a présenté dans tous les aspects de la vie et je présente ce travail comme cadeau à mon épouse ***Abtisam*** *et à mes enfants :* ***Mustafa, Muntadher, Zakariya et Yahya***

Acknowledgement

During my study, I met many people who, without them, could not permit me to finish my study and to recognize their gratitude. I have to thank:

I would like to thank Professor ***Pascal DHULSTER*** *for allowing me to work in the laboratory.*

I would also like to thank ***Professor Philippe JACQUES,*** *for his great support and for his follow throughout this work. I would like to thank him about all knowledges I gained of him under his supervision.*

I express my deepest gratitude to ***Dr. François Krier****, for supporting me throughout this work, for giving me the benefit of his knowledge and valuable support during the writing of this thesis.*

I would like to thank a noble man, ***Dr. Max BÉCHET*** *for all he has done for me.*

Thank you to the referees to have accepted to evaluate this work.
I express my sincere thanks to the all the team of ProBioGEM, with a special mention to the PhD students. I will never forget that when they make noise in the room, some of us took headphones to listen to music and focus on reading.... "It was wonderful moments".

And also, I do not forget the times that I spent with my best friends, sometimes interspersed with discussions on various aspects of life difficulties here. I say thank you my dear friends: Ahmed Alaa, Yazin, Qassim, Yanat and Mohammad for all what they have done for me ..... and thank you to anyone who helped me in all aspects of life.

Lastly, I have the opportunity to present a big thanks to my family in Iraq for their encouragement. I take off the hat in honor and recognition of the excellent work that my wife introduced for me in all aspects of life and I present this work as a gift to my wife: ***Abtisam*** *and my children:* ***Mustafa, Muntadher, Zakariya*** *and* ***Yahya.***

Abbreviations list

Organismes:

B. subtilis*: Bacillus subtilis*

B. amyloliquefaciens*: Bacillus amyloliquefaciens*

E. coli*: Escherichia coli*

AM: Arbuscular mycorrhiza

DRB: deleterious rhizobacteria

PGPR: plant growth promoting rhizobacteria

Plant-microorganisms signals:

ACC: 1-Aminocyclopropane-1-carboxylic acid

AHL: Acyl-homoserine lactone

APX: ascorbate peroxidase

CAT: catalase

ISR: Induced Systemic Resistance

LOX: lipoxygenase

PAL: phenylalanine ammonia lyase

PO: peroxidase

PPO: polyphenol oxidase

PR: protein resistance

SAR: systemic acquired resistance

SOD: superoxide dismutase

Molecules:

2, 3-DHB: 2,3-dihydroxybenzoate

4-PP: 4’phosphopantheteinic cofactor

ACN: acetonitrile

Ala: alanine

Asn: asparagine

Asp: aspartic acid

Asx: asparagine or aspartic acid

ATP: adenosine triphosphate

CP: carrier protein

CV: crystal violet

DDT: dichlorodiphenyltrichloroethane

DHB: 2,5dihydroxybenzoic acid

CHCA: α-cyano-4hydroxycinnamic acid

DNA: deoxyribonucleic acid

EDTA: ethylenediaminetetraacetic acid

EGTA: [ethylene glycol-bis (2-aminoethylether)-N, N, N′, N′-tetraacetic acid]

EPS*:* exopolysaccharide

GFP: green fluorescent protein

Gln: glutamine

Glu: glutamic acid

Glx: glutamine or glutamic acid

IAA: indole-3-acetic acid

Ile: isoleucine

IST: induced systemic tolerance

LB: Lysogeny Broth ou Luria-Bertani

Leu: leucine

LPS: lipopeptides

MOPS: 3-(*N*-morpholino) propanesulfonic acid

NPR1: natriuretic peptide receptor

NRPS: Non-Ribosomal Peptide Synthetase

Orn: ornithine

PCP: peptidyl-carrier protein

PDA*:* potato dextrose agar

PK: polyketide synthase

PKS: polyketide synthases

Pro: proline

PVC: polyvinylchloride

RE: root exudates

RNA: ribonucleic acid

S: surfactin

Ser: serine

TFA: trifluoroacetic acid

Thr: threonine

Tris: [2-Amino-2-hydroxymethyl-propane-1,3-diol]

Tyr: tyrosine

Val: valine

Reactions:

A: adenylation domain

C: condensation domain

T: thiolation domain

TE: thioesterase domain

Cy: cyclisation

E: epimerisation

Me: methylation

Ox: oxidation

Organizations:

CDC: The Centers for disease control

FAO: Food and agriculture organization

NCBI: National Center for Biotechnology Information

NPIC: National pesticide information center

ProBioGem: The laboratory of biological processes, enzymatic and microbial engineering

UNEP: United Nations Environment Programme

Measurement units

°C: degree celsius

CFU cm^{-3}: colony forming units per centimeter cube

CFU mL^{-1}: colony forming units per milliliter

CFU: colony forming unit

cm: centimeter

CMC: critical micelle concentration

Da: dalton

g C L^{-1}: gram of equivalent Carbon atoms per liter

g L^{-1}: gram per liter

g: gram

h: hour

kDa: kilodalton

kV: kilovolt

L: liter

M: Molar

mg L^{-1}: milligram per liter

min: minute

mL: milliliter

mM: millimolar

ng: nanogram

nm: nanometer

bp: base pair

pH: hydrogen potential

rpm: rotation per minute

s: second

Tm: Melting temperature, annealing temperature of the primers

V: Volt

v/v: volume/volume

w/v: weight/volume

µF: microfarad

µg cm^{-3}: Microgram per centimeter cube

µg mL^{-1}: Microgram per milliliter

µg: microgram

µL: microliter

µm: micrometer

Ω: ohms

Techniques:

CRD: Completely randomized design

HPLC: High-performance liquid chromatography

IEF: Isoelectric focusing

MALDI-TOF MS: Matrix-assisted laser desorption/ionization mass spectrometry

PCR: Polymerase chain reaction

TOF: Time of flight

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Chapter I

General introduction

I. General introduction

Agriculture is one of the important skills to sustain life on the planet and, in the present time, the agriculture development in terms of the increasing population and the preservation of the ecosystem from pollution is a major challenge. For these reasons, the tendency to use biocontrol in agriculture practices in recent decades increased. Among different practices in biocontrol, the plant growth promoting rhizobacteria (PGPR) have received a lot of attention. These beneficial bacteria are associated with many plant species and are commonly present in various environments and they have the ability to colonize the plant roots and promote plant growth directly or indirectly via biological control (Kloepper and Schroth 1978; Ahemad and Kibret, 2104; Ahmad *et al*., 2011).

The positive effect of PGPR on plants is interposed by different mechanisms including improvement of nutrient availability, enhancement of plant tolerance to biotic and abiotic stress, modification of root development, as well as suppression of soil-borne diseases (Glick 1995; Glick, 2012; Kloepper *et al.,* 1989). The bacterial traits involved in these activities include nitrogen fixation, phosphate solubilization, iron sequestration, synthesis of phytohormones, modulation of plant ethylene levels and control of phytopathogenic microorganisms (Gamalero and Bernard, 2011; Ahemad and Kibret, 2104; Ahmad *et al*., 2011).

Many bacterial genera including *Bacillus, Pseudomonas, Azotobacter*, *Azospirillum, Gluconacetobacter, Azoarcus, Arthrobacter, Clostridium, Enterobacter,* and *Serratia*, are belonging to PGPR and these bacteria competitively colonize the roots of plant and can act as biofertilizers and/or biopesticides or at the same time have the both actions (Fuentes-Ramirez *et al.,* 2005). Among these, species of *Pseudomonas* and *Bacillus* are the most largely studied (Muraleedharan *et al*., 2010).

Several studies have been conducted to illustrate the colonisation of rhizosphere by bacteria. Juhnke *et al*., (1987) and Milus and Rothrock (1993), have presented the successful establishment of bacteria in the wheat rhizosphere after inoculation of wheat seeds by different strains of *Bacillus*. Some of these studies preliminary compared the rhizosphere colonisation by different strains of *Bacillus.* Deravel, (2011) reported that *Bacillus amyloliquefaciens* population was higher than that one of *Bacillus subtilis* in the tomato rhizosphere at the end of experiment (17 days) (figure 1), as well as Nihorimbere *et al*., (2011) presented that the higher number of

bacterial population in the tomato rhizosphere colonized by *B. amyloliquefaciens* S499, was observed after 13 days and then it was lowering. It is well known that *B. subtilis* and *B. amyloliquefaciens* produce natural peptides *via* non-ribosomal peptide synthetases. Among these molecules, the lipopeptides which exhibit inhibitory activity against plant pathogens have received high attention. Lipopeptides can be grouped in three different families: surfactins, iturins and fengycins (Peypoux *et al*., 1999; Borriss, 2013; Lee *et al*., 2007; Zhu *et al*., 2012; Ongena and Jacques, 2008; Besson *et al.*, 1978). The higher accumulation of some lipopeptides more than others in the rhizosphere such as more surfactin accumulated in the tomato rhizosphere than bacillomycin and fengycin (Nihorimbere *et al*., 2011) may indicate to a role of surfactin in the colonisation.

It is interesting to mention that the results presented by Deravel (2011) experiment (figure 1) explain that a difference in the population of *Bacillus* species at the end of the experiment and they clearly have shown that the *B. amyloliquefaciens* strains had a higher biomass in the rhizosphere than *B. subtilis* strains. Furthermore, for the same species the surfactin producer strains are slightly better colonizers than the non-producer. But, the surfactin does not seem to be the major component to explain the good colonisation of rhizosphere: *B. amyloliquefaciens* CH1 (a non surfactin producer) is a better colonizer than *B. subtilis* BBG 131 and RFB 104 (surfactin producers). Thus, the role of surfactin on the colonisation was not clear and, in this work, we decided to study, among others, more in details the role of surfactin.

Until the moment this work was performed, there was not a detailed study conducted on the colonisation of the tomato rhizosphere and the factors by which it is influenced.

Different approaches were used: 1) based on Deravel's study (2011), the bacterial species have an effect on rhizosphere colonisation and the role of surfactin was not obvious. Therefore, this work studied the kinetic of bacterial population in the rhizosphere during 21 days taking into account that the optimal growth was obtained after 13 days (Nihorimbere *et al*., 2011). 2) The kinetic of surfactin production during 21 days was also studied in order to check its relationship with colonisation, 3) The tomato root exudates and their composition represent an important carbon sources for the microorganisms in the rhizosphere (Bais *et al*., 2006; Brigham *et al.*, 1999; Fray, 2002; Ryu *et al*., 2013**;** Huang and Sumner, 2011; Teplitski *et al*., 2000; Knee *et al*., 2001; Hooper, 2015; Choudhary, 2009). Their influence on bacterial growth and surfactin production was thus analyzed. 4) The last factor studied in this current work is the biofilm

formation which probably strongly influences the rhizosphere colonisation (Christensen, 1989; Sutherland, 2001; Donelli, 2014).

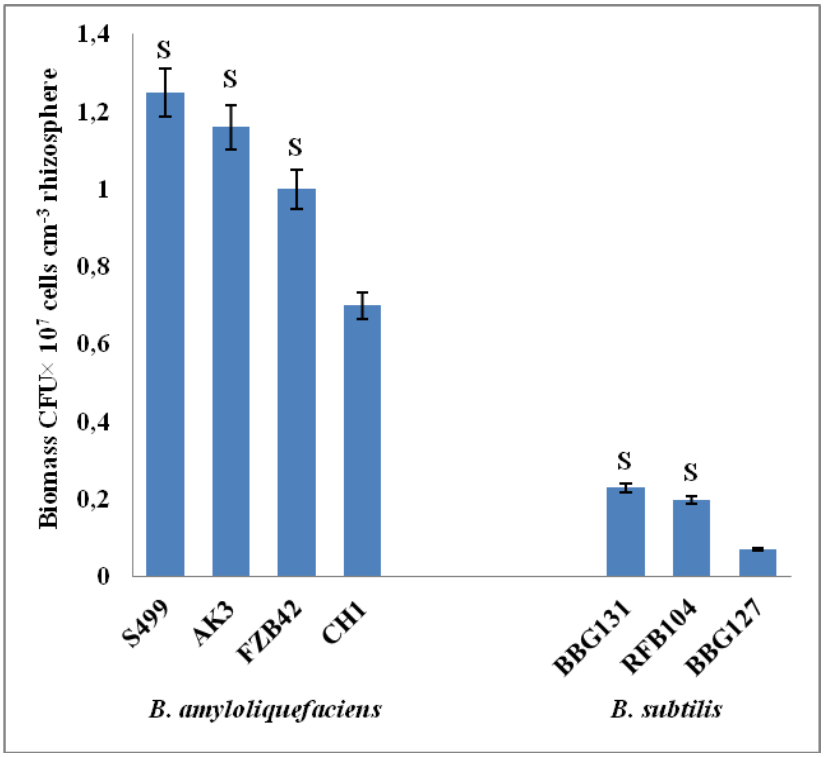


Figure 1 The population of bacteria in the tomato rhizosphere after 17 days of inoculation tomato seeds by *B. amyloliquefaciens* and *B. subtilis* species. S: strain produces surfactin. Adapted from (Deravel, 2011).

B. amyloliquefaciens FZB42 and *B. subtilis* BBG131 were chosen to perform a comparative study of their behaviour. The first one is a wild strain producing three lipopeptides: surfactin, fengycin and bacillomycin. The second one is a derived strain from *B. subtilis* 168 and it was genetically engineered in ProBioGem to overproduce surfactin. These two strains were chosen, based on their lipopeptide production and their differentiation in the final population after 17 days in the tomato rhizosphere which were reported in a previous study (Deravel, 2011). In addition, *B. amyloliquefaciens* FZB42 was widely studied as PGPR and a biocontrol agent (Butcher and Helmann, 2006; Chen *et al.*, 2009; Idris *et al.*, 2004; Koumoutsi *et al.*, 2004, 2007; Moldenhauer *et al.*, 2007; Schneider *et al.*, 2007; Krober *et al.*, 2014). Hence, we were interested to perform a comparative study between the two strains to answer the question; what are the important factors which influence the rhizosphere colonisation and by which factors a PGPR is distinguished to another non-PGPR strain?

Chapter II

Review of literature

II. Literature review

II.1. Population and food challenge

During the past 30 years, both the size of the world population and the production of crops to feed these people have increased considerably (Barker and Pilbeam, 2015). Between 1987 and 2012, the world population increased from 5 billions to about 7 billions, and the rate of population growth is such that it is estimated that by 2030 the number of people requiring food will exceed 8 billions (UN Department of Economic and Social Affairs, Population Division, 2013). More people and higher average incomes will result in greater food consumption. The increase in the global population and their income is normally followed by increasing food production and that requires greater inputs of land, water or energy, or a combination of these inputs (To and Grafton, 2015; UNEP, 2014).

Overlaying the food supply challenge is the issue of environmental sustainability due to land, biodiversity and water degradation, especially soil and fertility loss (Rickson *et al.,* 2015) and it is remarkable that of a total of approximately 400 000 species of flowering plants, less than 200 have been domesticated as food and feed plants and only 12 species provide 75% of the food eaten (Heslop-Harrison and Schwarzacher, 2012). According to the data of the Food and Agriculture Organization (FAO) of the United Nations, the production of the common crop groups (including cereals, oilseeds, fruits and vegetables) increased by 47% between 1985 and 2005. In this time period, the area of cropland only increased by 2.4%. This means that average crop yields per unit land area increased by 20% (Foley *et al.,* 2011). These yield increases were brought about by advances in crop production techniques, including in the use and application of fertilizers.

II.2. Agriculture and crop production

Land quality and crops production improvement, is essential to respond to the increase demands for food and other agricultural goods. Use of chemical fertilizers can improve soil quality and increasing crops yield, but these are costly. Inorganic fertilizers supply only nutrients

and they don't provide the beneficial effects on the soil physical properties; furthermore, the excessive, continuous and unbalance use of inorganic fertilizers causes eutrophization of water and it is considered to be the main cause for stagnating or declining crop productivity (Gebremedhin and Tesfay, 2015).

II.3. Ways of agricultural development

II.3.1. Use of organic matter

Organic fertilizers can provide nutrients to the soil; enrich humus content and also improve soil chemical and physical characteristics (Serranti *et al.,* 2015; Kasper, 2015). Use of raw dung as fertilizer is not advisable because it attracts white ants, which ultimately eat away the roots of vegetation growing in the area. The compost of different organic materials such as dung, dry leaves and agricultural residues is better as organic fertilizer than the raw material. Use of compost provides the nutrients as available form (Makinde, 2015; Cavagnaro, 2015).

Certain species of plants have the ability to fix nitrogen from the atmosphere and add it to the soil, and among these plants; the leguminous occupies an important role to improve soil nitrogen availability when they grow in a poor soil (Li *et al.,* 2015).

II.3.2. Crop rotation and mixed cropping

Crop rotation is one of the oldest strategies used to conserve soil productivity and it means alternately planting of different crops in the same field planned in order to compensate the nutrients removed by crops. These crops often belong to different families. The planned rotation may vary from 2 or 3 years or longer period (Blackshaw *et al*., 2015). It is very effective to use two crops in the same area; one derives the nutrients from the soil (cereals crops) and the other one adds the nutrients (leguminous crops) and this way of crops rotation is called mixed cropping. Additionally, the mixtures of cereals crops with legumes can directly reduce the infestation by insect pests (Paulsen *et al.,* 2006).

II.3.3. Irrigation and soil damage

Although only 20% of the world's cropland is irrigated, this land produces 40% of the global food. A sufficient supply of water is the limiting factor for food production in many parts of the world. Thus, supplying water to fields from underground aquifers, reservoirs, and diverted rivers has been considered to increase overall yield and the amount of land that can be farmed (FAO, 2011, Gliessman, 2015).

In addition to the likelihood that fertilizers will be leached from fields into local streams and rivers, irrigation can greatly increase the rate of erosion. The non-controlled irrigation leads to degradation in land quality. Excessive irrigation very often leads to sanitation or alkalinisation of soil. The irrigation water evaporates leaving the dissolved salts behind. Once, the soils become salt-affected, they are almost useless for agriculture, as crops do not grow there successfully. These areas are generally left fallow. But these soils can be reclaimed mainly by growing special types of plants' (halophytes) which are tolerant to salinity or alkalinity. When these plants grow there, the soil condition becomes better and finally other species can also grow. They provide cover to infertile soils because they survive better, improve the soil faster and also provide hay for the livestock (Guillou and Gérard, 2014; Gliessman, 2015)**.**

II.3.4. Aerial Seeding and carbon stocks

Aerial seeding is a technique of sowing seeds by spraying them through aerial mechanical means such as a plane or helicopter. Aerial seeding has also been widely used around the world (Schoonmaker *et al.,* 2014; Xiao *et al.,* 2015).

About of 86% of global vegetation carbon comes from the forest carbon. The change of forest carbon can lead to a huge impact on greenhouse gas emissions, which has an important role in climate changes (Buchholz *et al.,* 2014; Carvalhais *et al.,* 2014; Xiao *et al.,* 2015). Therefore, it is critical to evaluate the effectiveness of vegetation restoration on carbon stock. So these management strategies can be carefully implemented to maximize carbon sequestration capacity for mitigating the impacts of climate change, particularly in tropic and sub-tropic forests (Xiao *et al.,* 2015).

The use of aerial seeding plays a very important role for improving the degraded land and reducing the pressure on natural forests, grasslands, and grazing grounds. The aerial seeding was

the only restoration option in the areas with complex terrains after a forest fire. Gradually, the degraded land is transformed into a productive area and thus the carbon stocks are restored and a good ecosystem is maintained (Xiao *et al.,* 2015; Bassett *et al.,* 2015).

II.3.5. Industrial agriculture

The harness industry in agriculture for obtaining the maximum production is widely used in the recent decades. Among these processes; intensive tillage, monoculture, irrigation, application of inorganic fertilizer, chemical pest control, genetic manipulation of domesticated plants are a basic practices used in industrial agriculture for crop production. Industrial agriculture has been based on the practice of cultivating the soil completely (Gliessman, 2015).

II.3.6.Tillage and agriculture

Generally the tillage can be divided into two types: primary tillage is a deep tillage that fractures, sifts, or mixes the top six inches to two feet of soil. Primary tillage is applied to soils in order to eliminate soil pans, mix organic matter and other soil amendments, incorporate cover crops and crop residues, and aerate soils. Secondary tillage is a shallow and fine tillage.

Secondary tillage produces a fine seed or transplant bed by a series of operations that reduce the surface soil particle size. Tools and techniques are applied to the top 3 to 6 inches of soil and used to form fine, level, firm planting beds following primary cultivation (Miles and Martha, 2015). Du *et al.,* (2015)**,** pointed out that, the changes in tillage systems significantly influenced soil organic matter concentration in different soil fractions. No-tillage with residue and rotary tillage with residue, had higher soil organic matter than other type of tillage used. Carbon dioxide emission form soil and total carbon input from aboveground crop residue are significantly increased by tillage (Al-Kaisi and Xinhua, 2005).

II.3.7. Monoculture

Monoculture is the agricultural practice of producing or growing a single crop or plant species in a field at a time and it is one the different ways used in industrial agriculture. The monoculture system needs large quantities of chemical fertilizers in addition to pesticides. Growing the same crop in the same area year after year depletes the nutrients from the soil. The

monoculture fields are a highly attractive to weeds and insect pests therefore they need more pesticides than other systems (Reganold *et al.,* 1990, Gliessman, 2015)**.** The crops under monoculture system are more vulnerable to disease and insect pests. For instance, 73% of studies reported that disease suppression is widely found with intercropping system than with monoculture system (Boudreau, 2013; Brooker *et al.,* 2015; Horrigan *et al.,* 2002).

II.3.8. Use of fertilizers

The annual rate of world demand for total fertilizers was calculated as 1.9% between 2012 and 2016 (FAO, 2012). Gliessman, (2015), indicated that the use of chemical fertilizers at the last half of twentieth century was due to a high increase in yields. High quantity and unscientific use of chemical fertilizers lead to different environmental disadvantages. Li and Wu (2008), pointed out that the use of some chemical fertilizers can induce the accumulation of toxic by-components in soil resulting the worsening in soil ecological environment and result in the agriculture products to contain these by-components. The applied fertilizers led to an accumulation of heavy metals in the soil and changed the speciations and bioavailability (Tu *et al.,* 2000; Bi and Zhang, 2015). Besides the accumulation of heavy metals in soil, runoff and leaching of nutrients in chemical fertilizers and livestock manure can become a big problem in water quality and environment (Abler, 2015). Some of the plant nutrients added to the soil are not absorbed by the plant, thus they accumulate in the soil and other quantity discharges to the water bodies (Giang, 2015).

In addition to the influence of the chemical fertilizers on heavy metal accumulation in soil, fertilizer compounds also affect some soil physical properties (Bi and Zhang, 2015). Annaheim *et al.,* (2015), compared the influence of organic amendment treatments and chemical fertilization treatments, they found that the soil treated with chemical fertilizers treatments contained more and larger hard colds and the compacted topsoil was thicker due to higher soil dispersibility than soil treated with the organic amendments.

II.3.9. Herbicides and pesticides in agriculture

Pesticides are a necessary part in agriculture to keep plants saved from the pest and unwanted plants. Pesticides are chemicals that kill or manage the population of pests. There are

many different types of pesticides on the market today, but the most common are herbicides and insecticides, which kill or manage unwanted plants and insects. The increasing amounts of pesticides caused by the agricultural pests' detriment were represented as a global problem. The extensive use of pesticides can provide several benefits including, increase food production as a result to the prevention of diseases caused by the pests and combat the pests which consume a large portion of agricultural crops and thus, the economic situation will be improved (Aktar *et al*., 2009; Bresin *et al*., 2015; Cizmas *et al*., 2015).

Although there are benefits to the use of pesticides, there have also been many problems: the residues of pesticides used are mobile in the environment and come in contact with other organisms causing different disadvantages. According to the national pesticide information center (NPIC, 2015), the pesticides maybe kill the non-pest organisms and thus the ecosystem equilibrium will be disturbed. The use of pesticides also exposes human health to diseases. Around 20,000 to 40,000 people die of cancer each year because of poisoning by the pesticides (Miller and Scott, 2011).

II.4. Pollution of the Environment

The most important source of water pollution is agriculture. Agricultural pollutants come from pesticides, herbicides, fertilizers, salts and animal wastes (Gliessman, 2015). Some of the pesticides are known to remain for many decades such as DDT and today they are replaced by other less persistent, but often much more toxic pesticides. In addition to the environmental impact of pesticides, they represent a considerable human health hazard (Hellawell, 2012; Schafer, 2007). The centers for disease control (CDC) reported that all of the 9,282 people tested they had pesticides and their breakdown products in their bodies, and the average person had detectable quantities of 13 different pesticides (Schafer *et al.*, 2004). Similar incidences of exposure and detection were reported in the CDC's 2013 report (CDC, 2013). Pesticides enter our bodies through our food and our drinking water. Gilliom and Hamilton (2006), indicated that pesticide contamination was detected in 97% of streams tested in agricultural and urban areas, in 94% of streams tested in areas with mixed land use, and in 65% of streams tested in undeveloped areas. Pesticides were found in 61% of groundwater samples in agricultural areas and 55% of samples in urban areas.

The reduction of chemical compounds used in agriculture like chemical fertilizers and chemical pesticides is one of the most important challenges facing agriculture. Excessive use of these chemical compounds in agriculture processes leads to the pollution of soil which in turn is reflected in the plant, animal and human health. The pollution of soil is mostly a result of industrial activity and of the use of fertilizers and pesticides. The soil pollution can destabilize the life in the soil ecosystem. Thus many attempts have been occurred to reduce the use of these harmful compounds in agriculture. Among these attempts, the use of microorganisms as biofertilizers and biopesticides as alternative of chemical compounds is attracting a lot of attention.

It is very important to understand the relation between plant and microbe in the complex environment: the rhizosphere. This environment composed of different factors could affect the plant production such as the availability of nutrients, the quantity of water, and the composition of organic material etc... In the following part, we will detail the rhizosphere and its composition especially the root exudates as an important factor influencing plant growth promoting bacteria PGPR.

II.5. Rhizosphere as an environment for bacterial growth

II.5.1. Rhizosphere definition and its composition

The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root. This zone is about 1 mm wide, but has no well-defined boundary. Rather, it is an area of extreme biological and chemical reactions influenced by compounds exuded by the root, plant residues and by microorganisms feeding on the compounds (Tinker and Nye, 2000; Pinton *et al*., 2007; Ehrenfeld *et al*., 2005; Bais *et al*., 2006; Dessaux *et al.*, 2009).

Rhizosphere is generally divided into the following three zones (figure 2) (Pinton *et al*., 2007; Prasher *et al*., 2014).

1. Endorhizosphere that consists of the root tissue including the endodermis and cortical layers.
2. Rhizoplane is the root surface where soil particles and microbes adhere. It consists of epidermis, cortex and mucilaginous polysaccharide layer.
3. Ectorhizosphere which consists of soil immediately adjacent to the root.

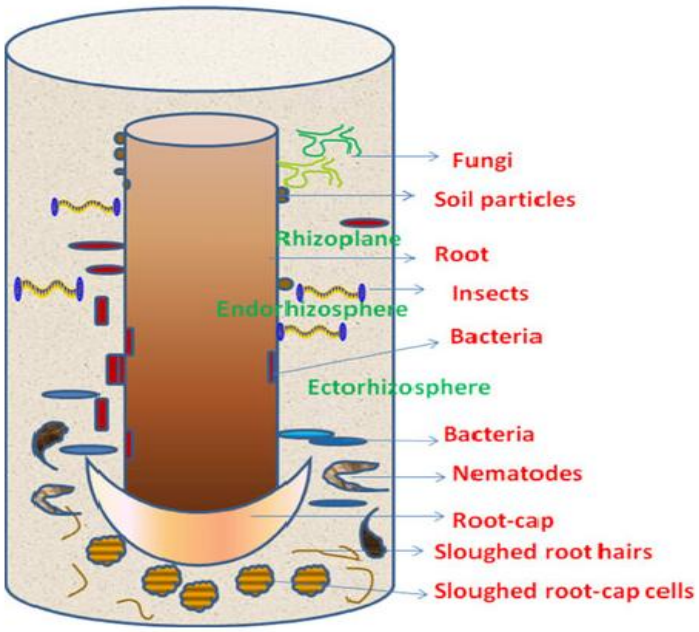


Figure 2 A simplistic diagram of the rhizosphere (Prashar *et al.*, 2014)

II.5.2.The influence zone

Many factors determine the rhizosphere characteristics, among these, are chemical and physical properties of the soil, plant types, photosynthesis activity and compound secretion. Some of the chemical soil activities happening in the rhizosphere are extending far beyond the rhizosphere such as mobility of some nutrients (Na, Cl, K, NO_3… etc) while phosphorus mobility is often less than a millimeter from the soil adhering to the root. In addition to these differentiations, the microbial activity plays an important role in rhizosphere distinguished in soil body. Based on these reasons, soil scientists broadly differentiated the first 20 centimeters of soil which have the most biological process activity between root and microorganisms into two regions:

1- A few millimeters of soil adhering around to the roots as the form of (sleeve rhizosphere) this region is accurately defined as rhizosphere.
2- The part of soil which is not directly adhering to the roots and it is followed (sleeve rhizosphere) is defined as bulk soil. This region has less biological activity than rhizosphere. (Kulmatiski and Beard, 2011; Lugtenberg, 2015).

II.5.3. Rhizosphere organic compositions and microorganisms

Root excretes various minerals and organic substances in parallel with water and mineral salt diffusion (Hinsinger, 1998; Hinsinger *et al.*, 2006; Dessaux *et al.*, 2009). Organic compounds are a carbon substrate by which the microflora contributes to the mobilization of elements in its metabolism. This microflora is induced by the changes in the rhizosphere such as root excretions covariance, and a feedback of microorganisms in the properties of soil and root. The complex interaction between the root and the soil are influenced directly or indirectly by the roots and their activity variation on the physical, chemical and biological properties of soil (Lemanceau *et al*., 1988; Violante and Caporale, 2015; Dessaux *et al.*, 2009).

II.5.4.The rhizodeposition

The "rhizodeposition" are all the organic compounds released by the roots. It includes actively secreted compounds (exudates, secretions, mucilages), and detached cells of the root. The importance of these excretions is organic carbon which represents 17% of photosynthesized material. The proportion of carbon released from roots has been estimated to as much as 50% in the young plants. The rhizodeposition might contribute to increase the availability of N and C in the soil for the crops (Pinton, 2007; Nguyen, 2003; Paterson *et al*., 2007; Zang *et al*., 2015).

II.5.5. The root exudates

The main part of rhizodeposition is the root exudates which are rapidly mobilized by the microorganisms. These are small organic molecules, water-soluble or volatile: carbohydrates, organic acids, amino acids, fatty acids, sterols, vitamins, enzymes, nucleotides, etc. Organic acids, sugars and a small quantity of amino acids represent the major three families found in root exudates (Curl *et al.*, 1986; Jones, 1998; Dakora *et al.*, 2002; Bacilio-Jiménez *et al.*, 2003). The composition and concentration of root exudates are extremely changeable. This variation in composition and concentration is depending on the type of plant, growth stage, root geometry, the seasons and the physical and chemical environment characterizations. Some of these molecules react specifically with some elements for example: phosphates on phosphate compounds, siderophores on iron. Others are more adaptable as oxalate and citrate which create a strong complex with metal cations (Szmigielska *et al.*, 1997; Jones, 1998; Strobel *et al.*, 1999).

Root compounds are discharged at least by two potential mechanisms, and the rates of exudation differ widely among species and different environmental conditions (Kochian *et al.*, 2005). Root exudates are transported across the cellular membrane and secreted into the surrounding rhizosphere. In addition to root excretion, plant products are also released from roots, such as border cell and root border like cells which separate from border as they grow (Bais *et al*., 2006). However it is important to note that it is very difficult to identify root exudates with respect to the chemical composition and the concentration in the soil because of methodological difficulties (Paul, 2014; Pinton *et al*., 2007). When the plant excreted the organic compounds via the roots, these compounds are directly attacked by the microorganisms in the root zone and thus, there is not enough time of their accumulation to enrich the environment. Additionally to root exudates variation by the time, it is difficult to obtain these exudates in natural conditions. Then, sterile hydroponic conditions are necessary to study their nature and quantity (Pinton *et al*., 2007; Bacilio-Jiménez *et al.*, 2003).

To quantify the root exudates, the production of CO_2 in the rhizosphere labeled of C^{14} has been adopted. It has been estimated that 12-40% from total carbohydrates amount produced by photosynthesis, are excreted into the soil surrounding roots. Root exudates are mainly composed of water soluble sugars, organic acids and amino acids, but also contain hormones, vitamins, amino compounds, phenolics and sugar phosphates esters (Pinton *et al*., 2007). These low molecular weight compounds are released through the plasma membrane using the passive process along the sheer concentration gradients which habitually subsist in the cytoplasm of root cells (millimolar range). Passive or direct diffusion happens through the lipid bilayer of the plasma membrane for transferring these compounds. The permeability of membrane estimates the compound diffusion and depends on the physiological state of the root cell and on the polarity of the compounds which facilitates the permeation of lipophilic exudates (Rudrappan *et al.*, 2007). Several factors like extreme temperature, nutrients deficiency or exudation stress that affects the membrane integrity may enhance the exudation efficiency (Ratanyake *et al.*, 1978). Qualitative and quantitative root exudates are affected by a variety of environmental factors including soil type, soil temperature, light intensity, nutrient availability pH, oxygen status, and the presence of microorganisms. These factors have a higher impact on root exudates than the effect due to the plant species (Singh and Mukerji, 2006).

The root exudates are characterized to be changeable according to the plant growth stage. For example, at the stage of six leafs the quantity of carbohydrates and root mucilage is higher than at earlier stage. Other compounds are also of substantial importance such as the rhizodeposition of nitrogen which are estimated as 20% of the total plant nitrogen at the maturity (Boulter *et al.,* 1966; Jensen, 1996; Wacquant *et al.,* 1989).

Low nutrient availability can constraint plant growth in many environments of the world especially the tropics where soils are extremely deficient in these oligoelements nutrient and thus, it influences the biological processes which in turn affect the root exudates (Pinton *et al.,* 2007). Some species typically exude organic acids anions in response to P and Fe deficiency or phytosiderophores due to Fe and Zn deficiency (Hynes, 1990).

As previously mentioned, the rhizosphere is the region which is very close to the roots of plant where intense bacterial activity is found because this zone is extremely rich in carbon sources and nutrient needed by the microorganisms (Nihorimbere *et al.,* 2011; Shukla *et al.,* 2011; Dessaux *et al.,* 2009). The signal between the plant and microbial community in the rhizosphere is highly influenced by the root secretion which represents important resources for microorganisms growth. These chemical compounds help the microorganisms to attract the rhizosphere and then permits the colonisation of the root zone especially by the rhizospheric bacteria by the mean of inducing chemotactic responses (Bcilio *et al.,* 2003; Dessaux *et al.,* 2009).

Among all these chemical compounds which are secreted from root, organic acids are important because they supply the substrates for microbial metabolism which affects the biogeochemical processes in the soil (Hinsinger, 2006; Bacilio-Jiménez *et al.,* 2003).

Root excretion has positive and negative effects in the rhizosphere. The positive effect mainly represents the symbiotic association with beneficial microbes such as mycorrhiza, rhizobia and plant growth promoting rhizobacteria while the negative effect represents the association with parasitic plant, pathogen microbes and invertebrate herbivores. The main role of microorganism, especially bacteria, is the degradation of the residue in the soil while the plant provides nutrients for the bacteria. This process has major importance in the environment because it contributes to reduce the pollutant and finally colonize the rhizosphere (Walker *et al*., 2003; Bais *et al*., 2008; Pereg and McMillan, 2015; Ahemad and Kibret, 2104).

The sugars such as glucose, fructose, sucrose, galactose, pentose, arabinose, xylose raffinose, ribose and mannitol have been found in root exudates and glucose is the dominant source among them (Jones and Darrah, 1995; Lugtenberg, 1999; Toal, 2000). The amino acids such as aminobutyric acid, mugineic acid, homoserine, l-hydroxypriline, and all twenty amino acids for proteinogenic acids have been detected and the root exudates also include organic acids such as malic, l-glutamic, succinic, acetic, chorismic, p-hydrobenzoic, shilimic, l-aspartic, salicylic, sinapic isocitric and caffeic acids (Seal *et al.,* 2004; Vivanco and Baluška, 2012).

The study of Kravchenko *et al.,* (2003) showed that the extract of swollen seeds incubated for two days were dominated by pyruvic, oxalic and ketoglutaric acids while the citric and oxalic were dominant in extract of 4-days old tomato seedlings and in the extract of 14-days old, citric and malic were dominant. This study, showed that in general the concentration of total organic acids was higher with 14-days old than with 2 and 4-days old extracts. On the other hand, the dominant sugars were: fructose and glucose for swollen seeds, maltose for 4-days old plants incubated and the total amount of sugars increased 9 times for 14-days old plants.

The study of Kamilova *et al.,* (2006) was performed for three important crops: tomato, cucumber and sweet pepper. The results showed that the total amount of organic acids per seeds and per plant increased with plant growth: seedling and root exudates have 2 and 26 times more organic acids than the seed exudate. Citric acid was found in all plant growth stages, whereas succinic acid strongly increased when the seedling became a plant. The major sugars in root exudates are glucose, fructose and xylose and the amount of sugars in seeds and plants increased with plant growth. It is 2.7 and 5.3 higher in seedling and root exudates than in seed exudates. On the other hand, in grass the analyzed root exudates showed that citric, malic, and succinic acids were major organic acids and fructose, glucose and xylose were in minor quantities (Kuiper *et al.,* 2001; Kamilova *et al.,* 2006).

II.5.6. Organic acids

Organic acids are abundant compounds in the rhizosphere. The root exudation is the main source of organic acids. The microorganisms produce and also consume and may litter decomposition in some environments (Jones *et al.,* 2003). Organic acid in soil and soil solution include well-defined aliphatic and aromatic carboxylic acids having a maximum molecular weight of about 300 Da (Strobel, 2001; Vranova *et al.,* 2013).

Aliphatic organic acid in soil solution include mono, di and tricarboxylic acids with compounds containing unsaturated carbon atoms and hydroxyl groups. Most di-and tricarboxylic acids found in the soil solution are metabolites of the citric acid cycle (citric, acotinic, succinic, fumaric and malic). The major portion of the aromatic organic acid soil solution are substituted benzoic acids and cinnamic acids with hydroxyl and methoxy groups as the most common substituents, with some mentions of phthalic acid and catechol. In most soil solution (neutral pH) at least one carboxyl group is dissociated by the compound (Strobel, 2001; Vranova *et al.*, 2013; Tan, 2010; Sposito, 2008).

The dissociation of carboxylic groups from organic acids leads these organic acids to be negatively charged. These negative charges play a role in the movement of anion and complex formation with cations in the soil environment.

Many processes happen in the soil by dint of the presence of organic acids (figure 3) such as detoxification of heavy metals by plants and microbial grown in the rhizosphere, mobilization of macro and microelements by plants and microorganisms and soil minerals dissolution, which lead to pedogenesis. Depending on the negative charges of potential carboxyl groups, the solid phase of the soil quickly and simply adsorb the organic acids like other groups with negative charges (nitrate, phosphates etc.). Different and important implications on the mobility of these ligands and on their ability to complex formation and biodegradation happen by adsorption but usually receive not a lot of attention (Jones, 1998; Cataldo *et al.*, 1987; Banks *et al.*, 1994; Burckhard *et al.*, 1995; Harvey *et al*, 1997; Roussel- Debet *et al*., 2000).

The diffusion rates of degradation and adsorption of organic acids and their complex on the solid phase clearly contend the heterogeneity in the reaction field. Distances of compounds adsorbant to the solid phase like monocarboxylic acids (acetate) can reach or exceed 5 mm, while this distance is much lower with di-and tricarboxylic acids whose field of influence in the efficient root zone is estimated between 0.2 and 1.0 mm depending on the soil type, the type of organic acid and time (Darrah, 1991; Jones *et al.,* 1996, 2003). The value of concentration ranges are 0-5 µM for di- and tricarboxylic acids and 0-1 µM for mono acids, this experimental estimation do not show the heterogeneity; on the other hand, a high concentration (up to 5mM) of citrate dissolved in the vicinity of white lupin roots (*Lupinus albus* L.) is found (Baziramakenga *et al.,* 1995; Strobel, 2001; Ullman *et al.,* 2002; Dinkelaker *et al.,* 1989; Jones, 1998). The study of (Jones *et al.,* 2003) indicates that the buffering capacity of the soil,

sampling, and microbial control affect the heterogeneity and it is difficult to estimate the actual concentration and gradients techniques in various soil microsites. Nevertheless, root exudates symbolize only small quantities of all organic compounds in a lot of the surface soil layer. Strobel et al., (1999) explained that the carboxylic acids represent about 10% of dissolved soil organic carbon, the remainder contain carbohydrates and amino acids but mostly fulvic acids. Van Hees *et al.*, (1999) analyzed the soil solutions of five horizons of a podzolized soil. The results showed that low molecular weight; organic acids compounds made up 1–3% of the dissolved organic carbon and 0–14% of the acidity and all of the organic molecules in the soil are highly variable and unknown because of the large diversity of the complex processes in the soil environment.

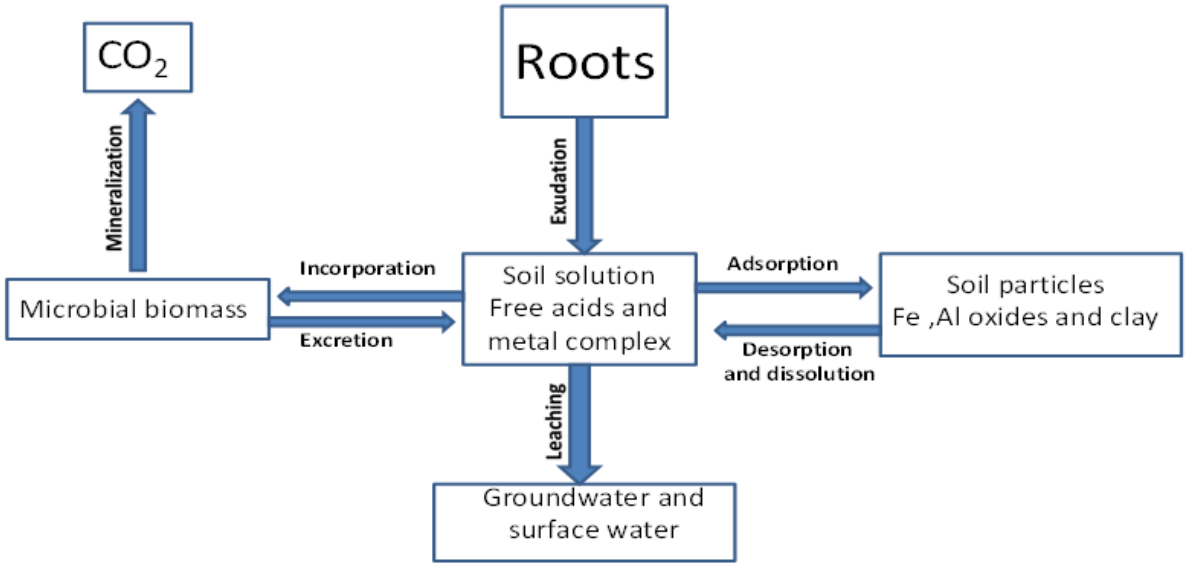


Figure 3 Organic acid flux in the rhizosphere (Jones, 1998)

II.5.7. Sugars

The sugars present in the soil are in a simple form (mono or di-saccharides) or complex form (polysaccharides). Cellulose as a polysaccharide represents more than 15% of the soil organic matter (Gobat *et al.*, 2010). These sugars are essential for the composition of exopolymers which is secreted by microorganisms in the environment (Chenu *et al.*, 1996). The major source of simple sugars in the root exudates is the degradation of complex

polysaccharides. Some sugars such as fructose and glucose are exhausted and rapidly metabolized by the microorganisms. In the soil, the numerous sugars are extremely diffused because they have no charges, so they are not or only a little adsorbed on the solid phase (Darrah, 1991).

II.5.8. Humin, humic and fulvic acids

Organic matter is exposed in the soil to many and different chemical and biochemical processes, among these is humification process, by which humin, humic and fulvic acids are produced. (González-Pérez *et al.,* 2010; Bot and Benites, 2005; Tadini *et al.,* 2015).

Humic and fulvic acids are heterogeneous mixtures of organic matter; fulvic acids have lots of aliphatic side chains or natural peptides with aromatic nucleus; they are derived from humic acids by polycondensation and they have shorter side chains and a larger aromatic nucleus than humic acids (Gobat *et al*., 2004). The humin and humic acids are not soluble in water while fulvic acids have a high solubility in water and all pH values. The huge solubility of fulvic acids is due to their richness in carboxylic acid, phenolic and ketonic groups which are provided by their electronegativity, a good ability to complexation of divalent and trivalent cations and a high tendency for adsorption (Stumm *et al.,* 1996; Gobat *et al.,* 2004). Fulvic acids adsorb cations associated with the surface and reduce the number of cations accessible to the surface, "coating" the surface of the mineral and reducing their dissolution. Humic acids contain more carbon than fulvic acids, in particular aromatic carbon. In addition, humic acids molecules are generally larger, with molecular weights up to approximately 100,000 Da compared with 1000 to 10,000 Da for fulvic acids (Sidstedt *et al*., 2015; Strobel, 2001; Krepelova, 2007; Eljack, *et al*., 2015).

II.5.9. Rhizosphere and microorganisms communities

The rhizosphere is the region of soil where there is a mutual influence between the roots and microorganisms. The plant is a principal partner in the biocoenotic system and all the changes which happen in the physiological properties of this system through vegetation life are reflected in the aspect of coexisting microorganisms. In the rhizosphere, the system of plant microorganisms cannot be separated and all of them are exposed to the long and short period of variation which relies on the plant growth stage in addition to the agro-ecological conditions.

The rhizosphere is a better environment to provide beneficial microorganisms for their study and isolation than the bulk soil (Das and Dkhar, 2011; Das *et al*., 2010; Pinton, 2007; Dessaux *et al.,* 2009).

Challenging the permanent variations in the surrounding environment is the main reason for the survival of plants which depends on the plant ability to respond to these changes by adaptation to these local fluctuations. The growth and development of neighboring plant species and microorganisms within the rhizosphere can be enumerated as local changes (Walker *et al*., 2003).

The roots naturally secrete several molecules and proteins as a response to meet the challenges (Badri *et al.,* 2012). The beneficial microorganisms and pathogens in the rhizosphere are induced by the root exudates and thus a role of symbiosis or defense is played as a plant ultimately involved in these microorganisms depends on the other elements in the local environment (Choudhary, 2009; Arora, 2013). Contrary to the wide advancement in studying the relationship between plant and plant, insect or microbe which occurs in the aerial plant organs, few investigations have concentrated on the root interaction with root, microbe and insect in the rhizosphere. A number of researches have indicated that root exudates play an important role in root microbe interaction, including the presence of flavonoid in the legumes root exudates which contribute to the nodulation process by activating the *Rhizobium meliloti* genes (Peters *et al*., 1986; Hooper, 2015).

Some studies have elucidated that flavonoid compounds may induce root colonisation by vesicular-arbuscular mycorrhiza, as the data obtained on olive trees showed that the amount of phenolics compounds is modified by the tree colonized with AM fungi (Larose *et al*., 2002; Mechri *et al*., 2015). In contrast to foliar plant infections, relatively a little is known about the nature of root defenses versus pathogens and in reaction to this underground hostility, root cells secrete secondary molecules such as phytoalexins and defense proteins in addition to other compounds and yet unknown chemicals (Flores *et al.,* 1999; De Coninck *et al*., 2014).

Many researches have been conducted to study the root exudates reliant on the chemodiversity identification. For example, Bais *et al*., (2004b) identified the presence of multifunctional caffeic acid ester (Rosmarinic acid) in the root exudates of hairy root cultures of sweet basil (*Ocimum basilicum*) which is elicited by fungal cell wall of *Phytophthora cinnamon*. Rosmarinic acid is excreted by roots *in situ* challenge with *Pythium ultimum* while there is no

Rosmarinic acid detected in the untreated plant with fungi. Rosmarinic acid demonstrated potent antimicrobial activity against an array of soil-borne microorganisms including *Pseudomonas aeruginosa* (Bais *et al*., 2004a). A similar study by Brigham *et al.*, (1999) reported that the production of Pigmented naphthoquinone derivatives of shikonin is observed in the rhizosphere of *Lithospermum erythrorhizon* as a challenge versus the pathogenic fungi *Rhizoctonia solani*, *Pythium aphanidermatum*, and *Nectria hematococca* and these compounds showed microbial activity; these observations strictly recommend an important role of root exudates in the rhizosphere defense against pathogens.

Furthermore, the above-mentioned studies complement other researches focused on the production and regulation of these small signaling molecules by supplying worthy insights into the biological importance of these secondary molecules (Fray, 2002; Ryu *et al*., 2013**;** Huang and Sumner, 2011). For instance, Teplitski *et al*., (2000) and Knee *et al*., (2001) reported that seedling root exudates of pea (*Pisum sativum*) contain several bioactive components that mimicked Acyl-Homoserine Lactone (AHL) signals in well-characterized bacterial reporter strains, stimulating AHL-regulated behaviours in some strains while inhibiting such behaviours in others. The chemical nature of such active mimic secondary metabolites is currently unknown and some reports illustrate the important role of AHLs produced in the rhizosphere as a crucial factor in plant pathology. However, it was also reported that crude aqueous extracts from several plant species exhibit AHL inhibitory activity. Thus, it is possible that roots may develop defense strategies by secreting compounds into the rhizosphere that interfere with bacterial quorum sensing responses such as signal mimics, signal blockers, and/or signal-degrading enzymes (Schikora *et al.*, 2011; Fuqua and Greenberg, 2002; Witzany, 2011).

The rhizosphere has a positive or negative impact on the plant by attracting the pathogen and plant growth promoting bacteria. The root exudates have an important role to the growth of these bacteria and thus there is a competition between them to the sources of carbon and energy close to the roots. The root exudates of plants considerably influence their symbiosis with bacteria found in the rhizosphere; PGPR are very important in the ecology system via gradually reducing the pesticides by these bacteria which are able to rapidly colonize the rhizosphere and suppress the growth of phytopathogens (Kravchenko *et al*., 2003; Paungfoo *et al.*, 2015; Ahmad *et al*., 2011; Arora, 2013; Dessaux *et al.*, 2009). Besides the positive effect of root exudates, the composition of organic acids extracted from watermelon have increased the population of

bacteria in the natural conditions which attribute an important role to the root exudates on rhizosphere colonisation (Ling *et al*., 2011; Dessaux *et al.,* 2009). Other works confirmed the previous results, like the impact of organic acids secreted by roots on the chemotaxis and swarming activation and thereafter increasing the bacterial population in the rhizosphere and also contribute to biofilm formation and hence protect plant against pathogens (De Weert *et al.,* 2004; Pandey and Jain, 2002; Donelli,2014).

Apparently, bacteria possess a processing mechanism by which they move towards chemical compounds in the environment; bacteria restore to this demeanor in search for the optimal conditions in order to survive. It is the first step for bacterial colonisation of the plant roots, and thereafter they provide the protection of plant from pathogens by root colonisation (Adler and Tso, 1974; Caetano-Anollés *et al*., 1988; Tan *et al.,* 2013). Equally important, many studies have confirmed that organic acids secreted from plant root were evaluated for their effect on the chemotaxis, biofilm formation, swarming ability and growth (Bais *et al*., 2006; Bais *et al*., 2004a; Tan *et al*., 2013; Compant *et al.,* 2005).

Besides, the root exudates provide the nutritive and energetic sources for bacterial development, root secretions have the ability to stimulate certain genera of bacteria in order to remove organochlorine pesticides as they have a huge potential for bioremediating and showed a high power to reduce pollution in the rhizosphere (Alvarez *et al*., 2012; Shelton *et al*., 1996; Lal *et al*., 2010; Kidd *et al*., 2008).

II.5. 10.Mechanism of root excretion

Different mechanisms that root cells use to secrete different compounds are described below (figure 4):

II.5.10.1. Diffusion

Diffusion is the net movement of a substance (e.g., an atom, ion or molecule) from a side of high concentration to a side of low concentration. That means the movement down towards a gradient substance. By this process, the low molecular weight organic compounds such as sugars, amino acids, carboxylic acids and phenolic compounds involve steep concentration gradients between the cytoplasm of intact root cells and the soil or the solution external to the root; these molecules can be diffused directly through the lipid bilayer of the plasmalemma. This

process depends on the physiological state of the root cell and on the polarity of the compounds to be exuded. Permeation of lipophilic exudates is generally facilitated by this method (Guern *et al.*, 1987; Leigh and Sanders, 1997; Wink, 1997). This process is influenced by certain factors including deficiency of nutrient like potassium, phosphorus and zinc, extreme temperatures, or oxidative stress (Cakmak and Marschner, 1988; Jones and Darrah, 1995; Jones *et al.*, 1994; Ratnayake *et al.*, 1978; Rovira, 1969; Marschner, 1995; Lambers, 2008; Ryan and Delhaize, 2001).

II.5.10.2. Ion channel

Some molecules cannot be transported through cell membrane by using diffusion process; these specific carboxylic compounds such as citrate, malate, oxalate, etc… are typically excreted in high concentration. In that case, the anion channels control the discharge of these products by the roots. Together with the responsibility of anion channel for specific carboxylic molecules, many studies showed that using anion channel antagonists indicated the participation of those channels due to their repressive effects on transport in general (Neumann *et al*., 1999; Ryan *et al.*, 1995; Sakaguchi *et al.*, 1999; Zheng *et al.*, 1998; Ryan and Delhaize, 2001).

II.5.10.3. Vesicle transport

Transport of high-molecular- weight compounds generally involves vesicular transport (Battey and Blackbourn, 1993). Transport of mucilage polysaccharides across the root cap is mediated by Golgi vesicles, while secretory proteins like ectoenzymes (e.g., phytase, acid phosphatase, peroxidase, phenoloxidase) which are synthesized by membrane-bond polysomes, enter the lumen of the endoplasmic reticulum using vectorial segregation (Neumann and Romheld, 2007). Proteins are detached from Golgi apparatus to vacuolar compartmentation which transports them to the plasmalemma by transfer vesicles (Chrispeels, 1991; Beevers and Raikhel, 1998). Extracellular and intracellular calcium levels influence processes that involve exocytosis, phenolic compounds and phytosiderophores (Marschner, 1995; Gagnon *et al*., 1992; Rougier, 1981; Mori and Nishizawa, 1987; Eshel and Beeckman, 2013; Marschener, 1998), as well as high-molecular-weight compounds are stored and released using vesicles, but the exact mechanisms utilized remain unknown. Root exudates occasionally release chemical compounds in large quantities and these are generally exposed to the physical (sorption), chemical (metal oxidation) and biological (microbial degradation) processes in the rhizosphere (Cheng, 1999;

Chen, 1995; De kroon, 2003; Walker *et al.*, 2003; Hinsinger *et al.*, 2006; Bertin *et al.*, 2003; Gregory, 2006; Hinsinger, 1998).

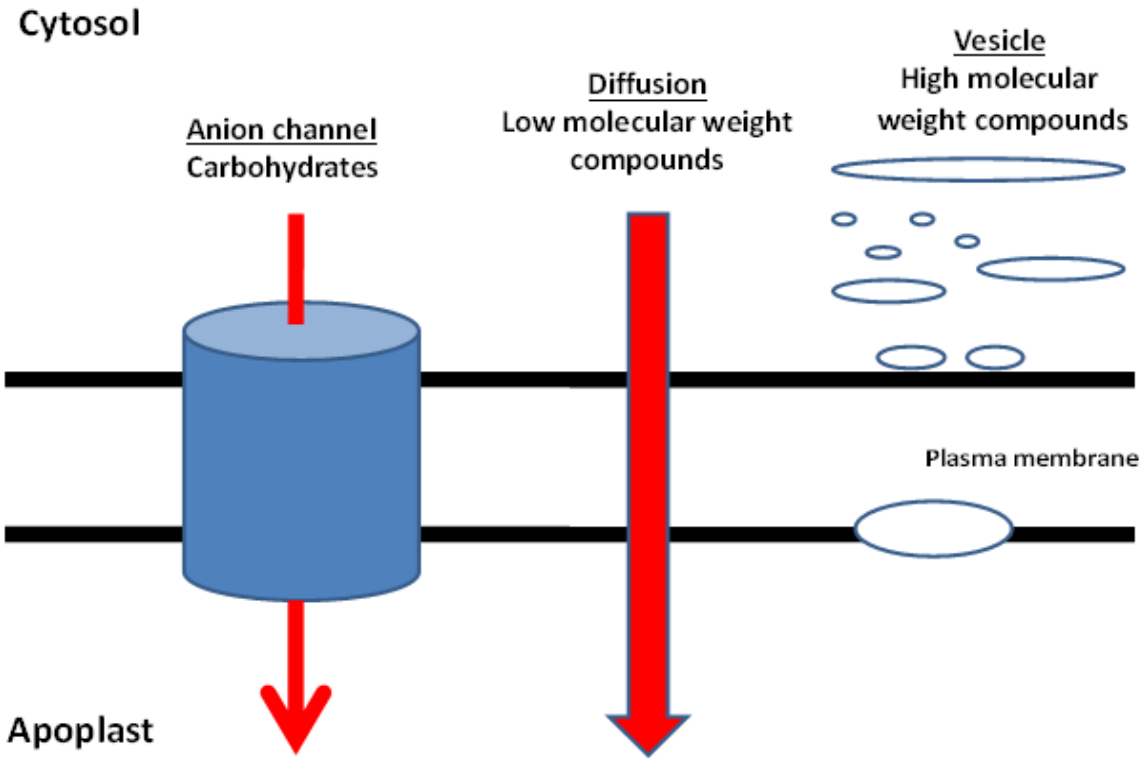


Figure 4 Proposed mechanisms of root exudation through the plant cell membrane. Sugars, amino acids carboxylic acids and phenolic acids transport through diffusion mechanism, phytase, acid phosphatase, peroxidase and phenoloxidase transport through vesicle transport mechanism and carbohydrate, citrate, malate oxalate transport using anion channel mechanism (adapted from Bertin *et al.*, 2003).

II.5.11. Role of root exudates to enhance beneficial bacteria

Root exudates compounds play a remarkable and positive role in the rhizosphere and this is clearly observed in root-soil beneficial microorganisms' interaction. Among these favorable microorganisms, PGPR are given a lot of attention in the agriculture practices (Walker *et al*., 2003; Bais *et al*., 2008; Pereg and McMillan, 2015, Pinton *et al*., 2007; Ahmad *et al*., 2011). Organic acid compounds secreted from root exudates are well studied as a booster to enhance the PGPR growth. Liu *et al.*, (2014), reported that plant growth-promoting rhizobacterium, *Bacillus amyloliquefaciens* SQR9, enhance chemotaxis and biofilm formation in cucumber plant root exudates, likewise, the root secretion of fumaric and citric acids is enhanced by *Fusarium oxysporum* which infects cucumber plant. The results propose that root exudates improve

cucumber root colonisation by *B. amyloliquefaciens* SQR9. Moreover, four organic acids identified in tomato root exudates (malic acid, citric acid, succinic acid and fumaric acid) showed a considerably induction in the chemotaxis response and swarming motility by *B. amyloliquefaciens* T-5 in addition to increasing bacterial population and promoting root colonisation under natural conditions (Tan *et al.*, 2013).

II.6. Plant growth promoting bacteria

Plant growth-promoting bacteria can be defined as the absolutely necessary part of rhizosphere biota in stimulating the growth of the host plant in association with bacteria (Bhattacharyya and Jha, 2012). Among plentiful species of bacteria which grow in the rhizosphere, PGPR can be restricted to the bacterial strains that gift at least two of three criteria such as forceful colonisation, plant growth stimulation and biocontrol (Weller *et al.*, 2002; Vessey, 2003; Van Loon, 2007; Ahemad and Kibret, 2104). The interaction which occurs between bacteria and plant root is classified as a harmful, beneficial or neutral interaction. Rhizobacteria that have an inhibitory role in plant growth have been designated as deleterious rhizobacteria (DRB). On the other hand, the rhizobacteria that have a beneficial role in plant growth have been called plant growth promoting rhizobacteria (PGPR) (Van Loon and Bakker, 2003; Van Loon, 2007; Ahemad and Kibret, 2104).

PGPR have a direct and indirect way to promote rhizobacteria growth (figure 5). Direct effect includes several processes such as facilitating resource acquisition (nitrogen, phosphorus and essential minerals) like atmospheric nitrogen fixation, minerals solubilization (phosphorus and trace elements), siderophores production which solubilize and sequester iron, or modulate plant hormone levels to improve plant growth at different stages. Indirect effect on plant growth can occur by improving growth restricting conditions such as producing antagonism substances or inducing resistance against pathogens in addition to decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents (Glick, 2012; Persello-Cartieaux *et al.*, 2003; Ahemad and Kibret, 2104).

The production of phytohormones such as cytokinins, gibberellins and auxins, production of siderophores and enzymes, lowering of ethylene levels and system resistance inducing represent the essential mechanisms to plant growth promoting directly enhanced by PGPR

(Bhattacharyya and Jha, 2012). PGPR indirectly benefit the plant growth by the biocontrol of deleterious microorganisms or root pathogens that inhibit plant growth, including antibiotic production, parasitism, competition for nutrients and niches within the rhizosphere, synthesis of extracellular enzymes to hydrolyze the fungal cell wall, decreasing pollutant toxicity (Bhattacharyya and Jha, 2012; Zahir *et al.,* 2003). Both of monocots and dicots plants are colonized by PGPR and by which the architecture of the root system is modified depending on the phytohormones production and other signals. Hence, this leads regularly to enhance and develop the root hairs and lateral root branching (Vacheron *et al.,* 2013).

Numerous bacterial species from PGPR have the ability to produce the auxin phytohormone indole-3-acetic acid (IAA). Different biosynthesis pathways have been identified and redundancy for IAA biosynthesis and is widespread among plant-associated bacteria. Interactions between IAA-producing bacteria and plants lead to diverse outcomes on the plant side, varying from pathogenesis to phytostimulation (Spaepen *et al.,* 2007).

Because of the importance of PGPR in agricultural development, many experimental and practical studies are performed for this purpose. Talboys *et al.,* (2014) reported that *B. amyloliquefaciens* FZB42 increases wheat root production at a low availability of inorganic phosphorus and on the contrary, it significantly represses root inorganic phosphorus uptake. Equally important, they indicate that applied exogenous auxin causes an increase in root carbon exudation and, at high external concentrations of inorganic phosphorus, root production is promoted by *B. amyloliquefaciens* FZB42, while inorganic phosphorus uptake is unchanged.

PGPR can be divided according to their action with plant in the rhizosphere, into three categories. First is biopesticides with microorganisms which have the ability to control plant phytopathogens and thereupon, the growth of plant is promoted. The second is biofertilizers with microorganisms which have the ability to increase the availability of essential elements for the plant such as phosphorus and nitrogen. The third is phytosimulator with microorganisms which have the ability to produce phytohormones (Bhattacharyya and Jha, 2012).

Generally speaking, the use of PGPR as a biofertilizers have been well reported, many researchers have shown that the inoculation plants with PGPR stimulate plant growth by increasing phosphorus and potassium availability, rising nitrogen and other elements uptake, expanding plant yield as a result of enhancing the growth parameters (Gupta *et al*., 2014; Kumar and Dangar, 2013; Rani *et al*., 2014). Phosphorus solubilizing bacteria and *Rhizobium* are

important PGPR in plant nutrition. These microbes play a significant role in crop biofertilization, under those circumstances of lowering the pH value by secreting spectrum of carboxylic acids in the rhizosphere and thus, increasing phosphorus solubilization. They can also produce some substances that will increase phosphorus availability, like phytases, phosphatases and phosphonate hydrolysis (Mardad *et al*., 2014; Hunter *et al.,* 2014). Dashti *et al*., (2014), elucidated in their study on soybean plant that the ability of PGPR to stimulate soybean nodulation is related to the capacity of soybean root colonisation and it is influenced by the variability of rhizosphere temperatures. Additionally, they articulated that addition of genistein (plant-to-bacteria signal molecule) motivates soybean nitrogen fixation and the growth was greater than adding PGPR alone, but only at 25 and 17.5°C, and not at 15°C Ahamd *et al.,* (2014); Shahverdi *et al.,* (2014), brought to light that *Rhizobium* and *Pseudomonas* contain ACC-deaminase providing the ability to reduce the damages caused by the salinity stress on physiology and quality parameters in plant when they inoculate mung bean together or separately. But co-inoculation with two strains was most effective to reduce the inhibitory effect of salinity on photosynthetic rate, chlorophyll content, stomatal conductance and CO_2 assimilation rate in addition to the advance of increasing phosphorus and protein concentration in grain and improvement nutrient balance.

Coupled with the promotion of plant growth by enhancing the availability of different essential nutritive elements such as nitrogen, phosphorus, potassium, iron, etc..., PGPR have the capability of decreasing the deleterious effect of pollution and thus plant growth is promoted. For instance, Hassan *et al.,* (2014) found that PGPR significantly decrease the deleterious effects of Pb pollution and increase maize growth under all Pb concentrations soil *via* chelation in the soil, and ultimately influence its release and uptake. On the other hand, PGPR which are able to produce ACC-deaminase and fix atmospheric nitrogen are more effective and resistant against Pb pollution than PGPR which have only one of them. Indeed, PGPR that produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, promote plant growth by segregating and cleaving plant-produced ACC, so the level of ethylene in the plant decreases, which would allow the plant to be more resistant to a wide variety of environmental stresses (Glick, 2005). Furthermore, another way used to promote plant growth has been showed by Rasouli *et al.,* (2014) who identified three strains: *Pseudomonas putida*, *P. fluorescens,* and *P. aeruginosa* in

the wheat rhizosphere which produces siderophore in big quantities. These bacterial siderophores have significantly and differently affected the uptake of ferrous ion by wheat genotypes.

One of the effective means to avoid plant diseases is the use of PGPR as biocontrol agents. Likewise, in addition to their ability to enhance crop yield through nutrient uptake and plant growth regulators, they also play an important role as biocontrol agents by keeping plant from the deleterious factors, triggering induced local systemic resistance, production of antibiotics, or effects of xenobiotics by degradation and act as rhizo-remediators. It is very useful for sustainable agriculture to introduce bacteria to act at the same time as biopesticides and as biofertilizers and in this manner PGPR could represent a group of bacteria with great significance (Srivastava, and Sharma, 2014).

Induced systemic resistance (ISR) and system acquired resistance (SAR) are a widespread phenotype in the beneficial rhizosphere microbiome. ISR is similar to SAR and it is effective against a wide category of pathogens. ISR is diagnosed by the presence of ethylene and jasmonic acid as the principal regulator in the ISR pathways, while the plant defence hormone salicylic acid represents an important key for SAR (Pieterse *et al.,* 2015; Bakker *et al.,* 2013).

Rhizobacteria can be used as a biopesticide by inducing resistance through the salicylic acid-dependent SAR pathway, or requiring jasmonic acid and ethylene produced in the plant for ISR. *Pseudomonas* and *Bacillus* are rhizobacteria which are well known for their ability to activate ISR (Kloepper *et al*., 2004). This ability to induce resistance against phytopathogens could be valuable for formulating new biopesticides which improve plant production (Beneduzi *et al.,* 2012).

Similarly to the above-mentioned studies, Muzammil *et al.,* (2014) pointed that *Saccharothrix algeriensis* induces ISR against *Botrytis cinerea* and requires salicylic acid and to some extent NADH oxidase in addition to the jasmonic acid and ethylene. Equally important, Annapurna *et al*., (2013), observed that inducing resistance in plant is elicited by different bacterial strains from PGPR such as *Pseudomonas putida, Serratia marcescens, Flavomonas oryzihabitans, Bacillus pumulus,* etc. This elicitation depends on several bacterial determinants like siderophore, pyoverdine, salicylic acid, fucose, rhamnose and flagellins which play a notable role in plant resistance. On the other hand, they indicated that NPR1 (Natriuretic peptide receptor) regulates the transcription of PR (protein resistance) genes that are activated during SAR and it also requires ethylene and jasmonic acid which mediates in the defence response

through ISR activation. The same study also mentioned different enzymes associated with ISR, including chitinase, β-1, 3-glucanase, peroxidase (PO), lipoxygenase (LOX), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and proteinase inhibitors.

ISR and induced systemic tolerance (IST) in plants can be also elucidated by volatile organic compounds which are secreted from rhizobacteria and this contributes to shed light to new insights in the biological and ecological approach of volatile organic compounds produced by rhizobacteria for modulating abiotic and biotic stress tolerance in modern agriculture (Ryu, 2015; Bakker *et al.*, 2013).

Another key point, Yi, (2013), evaluated the potential of an endophytic PGPR, *Bacillus pumilus* INR7, to induce systemic resistance against bacterial spot caused by *Xanthomonas axonopodis* pv. *vesicatoria* in pepper, it can be increased by combining PGPR with benzothiadiazole under field conditions. Whereas, Jetiyanon and Kloepper, (2002), conducted a greenhouse experiment to explain the effect of the combination and individual PGPR on inducing resistance activity. The results obtained, showed that four mixtures of PGPR and one individual strain treatment significantly reduced the severity of all diseases compared in this study: 18 mixtures reduced anthracnose of long cayenne pepper, 16 mixtures reduced bacterial wilt of tomato, 11 mixtures reduced cucumber mosaic virus and 7 mixtures reduced damping off of green kuangfutsoi. Furthermore, most mixtures of PGPR provided greater disease suppression than individual PGPR strains. The outcomes suggest that the mixtures of PGPR can elicit the induced systemic resistance to fungal, bacterial, and viral diseases.

II.7. *Bacillus subtilis* and *Bacillus amyloliquefaciens*: Colonisation of the rhizosphere and suppression of the pathogens

Some beneficial microbes have been cultured and formulated and are being sold as commercial products and applied as biopesticides, plant protection products or biofertilizers.

Various species of both Gram-positive and Gram-negative bacteria are widely used as beneficial bacteria, but in the recent years, *Bacillus* and *Pseudomonas* species have occupied a wide area of related research (Borriss, 2015). Bacilli are quite the largest part of bacteria used on biopesticides market in North America (Maheshwari, 2011), and among the first biocontrol

agents used against pathogens and insects, the members of the genus *Bacillus* were skillfully accomplished (Powell and Jutsum, 1993).

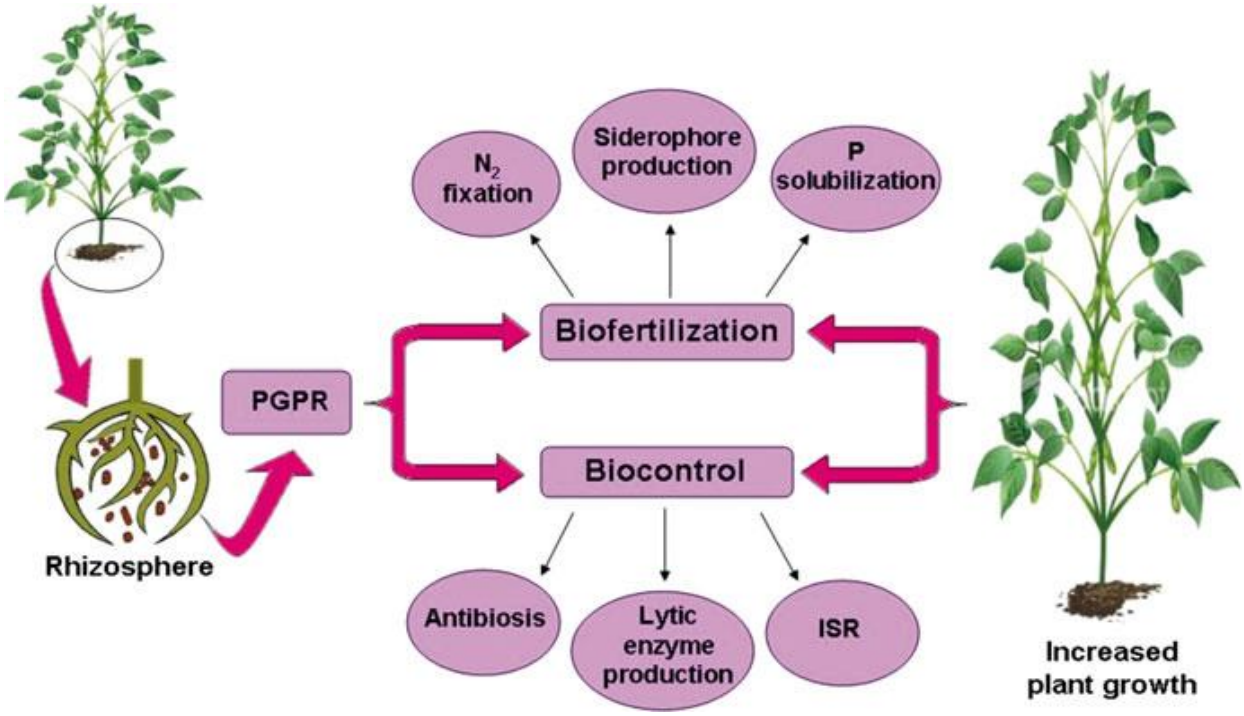


Figure 5 Schematic illustration of important mechanisms known for plant growth promotion by PGPR. Different mechanisms can be broadly studied under (1) Biofertilization, and (2) Biocontrol of pathogens. Biofertilization encompasses: (a) N2 Fixation, (b) Siderophore production, (c) P inorganic solubilization by rhizobacteria. Biocontrol involves: (a) Antibiosis, (b) Secretion of lytic enzymes, and (c) Induction of Systemic Resistance (ISR) of host plant by PGPR (Kumar *et al.*, 2011).

The wild strain of *Bacillus amyloliquefaciens* FZB42 was firstly isolated from soil infested with plant pathogens (Krebs *et al.,* 1998). It is well known as a Gram-positive, aerobic, endospore-forming bacteria with plant-growth promoting activity, and is investigated more profoundly for its potential to suppress plant-pathogenic microflora and to stimulate plant growth. The beneficial action of FZB42 and its closely related "cousin" FZB24 with respect to that of PGP and biocontrol is well notarized and the genome of FZB42 was the first Gram-positive PGPR that has been completely sequenced (Bochow, 1992; Krebs et al., 1998; Dolej and Bochow 1996; Kilian et al. 2000; Schmiedeknecht *et al.,* 1998, 2001; Grosch *et al.,* 1999; Bochow *et al.,* 2001; Yao *et al.,* 2006; Burkett-Cadena et al. 2008; Chen et al. 2007; Maheshwari, 2011).

Bacillus amyloliquefaciens FZB42 and other representatives of the *B. amyloliquefaciens plantarum* subspecies secrete different molecules surrounding plant root which are very

important for stimulating ISR, which represent the main mechanism in the effectiveness of biocontrol in the presence of Gram-positive endospore-forming bacteria (Borriss, 2015).

Coupled with *B. amyloliquefaciens*, the beneficial rhizobacterium *Bacillus subtilis* can promote plant growth and protect against a fungal pathogen attack. It is very abundant in the rhizosphere, which gives it an active role in organic matter decomposition in soil (Utkhede and Smith, 1992; Asaka and Shoda, 1996; Emmert and Handelsman, 1999). The two species *B. amyloliquefaciens* and *B. subtilis* are successfully commercialized as biofertilizer by ABiTEP GmbH (http://www.abitep.de/) and as biocontrol agents for fungal diseases of crops (Emmert and Handelsman, 1999; Narayanasamy, 2013). Moreover, many studies describe that Gram-positive *Bacillus subtilis* are the best biocontrol agents against *Fusarium oxysporum* and are widely marketed as biofertilizers, biopesticides and soil amendments (Baysal *et al.*, 2008; Cazorla *et al.*, 2007; Chung *et al.*, 2008; Hervás *et al.* 1998). The commercial biofungicide, Serenade, which contains a *B. amyloliquefaciens* strain, is reported to be effective against a variety of pathogenic bacteria, including *Pseudomonas*, *Xanthomonas* and *Erwina* strains (http://www.agraquest.com). Both *B. subtilis* and *B. amyloliquefaciens* produce a vast array of secondary metabolites of antibacterial and antifungal compounds including lipopeptides such as surfactin, bacillomycin and fengycin and polyketides like bacillaene, difficidin and macrolactin, hence these compounds could explain their inhibitory effect (Peypoux *et al.*, 1999; Chen *et al.*, 2009 ; Borriss, 2013; Besson *et al.*, 1978).

The root-microorganisms communication in the rhizosphere is a considerable precondition of the biocontrol agents applied to soil for effective biological control (Bais *et al.*, 2004a; Ping and Boland, 2004). Additionally, the mechanisms of competition, growth promotion and induction of systemic resistance and antibiosis are principal requirements for pathogens suppression by *B. subtilis* (Raupach and Kloepper, 1998; Romero *et al.*, 2007).

Other recent studies have reported the usefulness of *B. subtilis* and *B. amyloliquefaciens* in the rhizosphere for plant production. For instance, Cao *et al.*, (2011) indicated that *Bacillus subtilis* SQR 9 can colonize cucumber root rapidly and efficiently after rhizosphere inoculation. The results obtained by fluorescence microscopy showed that bacterial cells often colonized the surfaces of the primary roots, the zone of differentiation and elongation and the lateral root junctions. This strain also suppressed *Fusarium oxysporum* in the rhizosphere of cucumber. Shen *et al.*, (2015) showed that the application of *B. amyloliquefaciens* for two years was more

effective in controlling *Fusarium* wilt disease and improved fruit yields under field conditions, whereas, Babu *et al.,* (2015) reported that the treatment of tomato plant with PGPR resulted in a significant increase in seed germination as well as in tomato growth and weight, and they attributed these results to the ability of PGPRs to produce IAA and enhance nutrient uptake.

Field experiments of *B. amyloliquefaciens* FZB42 applied to lettuce plants showed a high capability to colonize the lettuce rhizosphere. The disease severity caused by *Rizoctonia solani* was significantly reduced in the presence of *B. amyloliquefaciens* FZB42. The rhizosphere communities were affected by the presence of pathogen while *B. amyloliquefaciens* FZB42 successfully established in the rhizosphere without having durable effects on other rhizosphere communities (Chowdhury *et al.,* 2013).

In addition to the high potential of the use of *B. amyloliquefaciens* FZB42 as a biocontrol agent, *B. amyloliquefaciens* showed an important role in improving root production of *Triticum aestivum* at low conditional phosphorus concentrations and on the contrary, inorganic phosphorus uptake by root showed a significant reduction. These simultaneously occurred with an expression of the inorganic phosphorus transporters by an-auxin-mediated reduction (Talboys *et al.,* 2014).

A study was carried out to investigate the role of maize root exudates to induce certain genes of *B. amyloliquefaciens* FZB42. The results indicated that 8.2% of a total 302 genes studied, showed high levels of expression in the presence of root exudates. Among these genes were the genes responsible for: metabolic pathways relating to nutrient utilization, bacterial chemotaxis motility, and non-ribosomal synthesis of antimicrobial peptides and polyketides (Fan *et al.,* 2012).

II.8. *Bacillus* and lipopeptides

It is commonly known, that the synthesis of the lipopeptides by *B. subtilis* and *B. amyloliquefaciens via* non-ribosomally multi-enzymes, can be grouped in three different families, i.e. the surfactins, iturins and fengycins (Lee *et al*., 2007; Zhu *et al*., 2012; Caldwell *et al.,* 2005; Ongena and Jacques, 2008).

The lipopeptides molecules have an away of activities: antibacterial (Thimon *et al.,* 1992; Toure *et al.,* 2004; Stein 2005), antiviral (Kracht *et al.,*1999), antifungal (Thimon *et al.,* 1992), antimycoplasma properties (Vollenbroich *et al.,*1997) and antitumour activity against Ehlich's ascites carcinoma cells (Cameotra *et al.,* 2004) , in addition to inhibition of the fibrin clot formation and hemolysis (Arima *et al.,* 1968; Cameotra *et al.,* 2004), inhibition of the cyclic adenosine-3, 5-monophosphate phosphodiesterase (Hosono *et al.,* 1983) and formation of ion channels in lipids bilayer membranes (Sheppard *et al.,* 1997).

The chain of lipophilic hydrocarbon in the lipopeptide compounds interacts with the plasma lipid moiety whereas the peptidic part of lipopeptides, which contains polar amino acid, interacts with the polar phosphatidyl moiety (Mikkola, 2006). The interaction between the two parts of lipopeptides with the cell membrane aims to penetrate it (Makovitzki, 2006; Heerklotz, 2004).

The three families of the lipopeptides produced by *Bacillus* have the amphiphilic character but this criterion is not the sole feature influencing the biological activity. It seems that several homologues within each family are more active than others (Fickers *et al*., 2009; Bonmatin *et al*., 2003). Moreover, the peptide moiety is very important to show a specific function (Ongena and Jacques, 2008; Bonmatin *et al.*, 2003).

Owing to their structure, the lipopeptides are resistant to enzymatic cleavage by proteases and peptidases in addition to the conservation of their stability (Carmona-Ribeiro and Carrasco, 2014; Mandal *et al.,* 2013).

II.8.1. Surfactin family

The first molecule with biosurfactant properties isolated from *B. subtilis* was discovered in 1968 (Arima *et al*., 1968). The lipopeptides pertaining surfactin family are β-hydroxy hepta cyclic depsipeptides with possibilities of Ala, Val, Leu or Ile amino acid variations at positions 2, 4, and 7 in cyclic depsipeptide moiety and C13 to C16 variation in β-hydroxy fatty acid chains (figure 6) (Peypoux *et al*., 1994; Kowall *et al*., 1998; Hue *et al*., 2001).

Surfactin is one of the biosurfactants which is defined as microbially produced surface-active compounds (Jennings and Tanner, 2000). The biosurfactants are distinguished by containing both hydrophilic and hydrophobic parts causing them to aggregate at the interfaces between fluids with different polarities such as water and hydrocarbon (Banat, 1995; Fiechter,

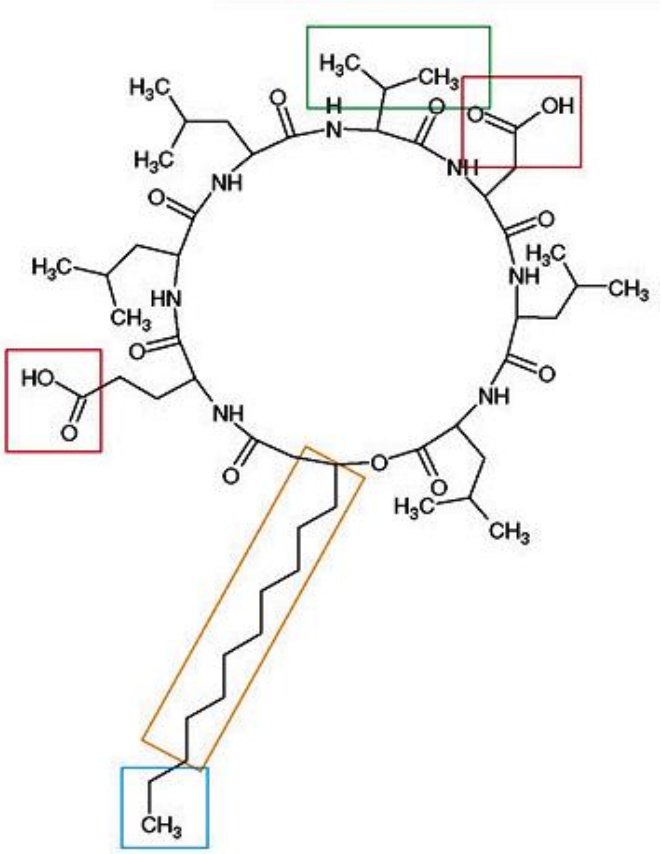
1992; Georgiou, 1992; Kosaric, 1993; Karanth *et al.*, 1999). These molecules are well known for being able to enhance the nutrient transport across membranes. They act in diverse host-microbe interactions and provide biocidal and fungicidal protection for the producing organisms (Lin, 1996; Banat, 1995a; Banat, 1995b).

Biosurfactants are used as potential replacements for synthetic surfactants in several industrial processes, such as lubrication, wetting, softening, fixing dyes, making emulsions, stabilizing dispersions, foaming, preventing foaming, as well as in food, biomedical and pharmaceutical industry, and bioremediation of organic- or inorganic-contaminated sites (Reis *et al*., 2013). Among these molecules, surfactin represents 12% of 255 biosurfactants and bioemulsifiers patents issued worldwide (Shete *et al.*, 2006).

About twenty diverse lipopeptides belong to the surfactin family (Bonmatin *et al.*, 2003). Many reports indicate that the surfactins can willingly link and strongly attach into the lipid layers, induce the plant systemic resistance and promote plant root colonisation (Bonmatin *et al.*, 2003; Jourdan *et al.*, 2009; Ongena and Jacques 2008). Moreover, surfactin has shown exceptional emulsifying and foaming properties causing the ability to reduce water surface tension from 72 mN m^{-1} to values in range of 25-30 mN m^{-1} (Bonmatin *et al.*, 2003). The critical micelle concentration (CMC) of surfactin is 10 mg L^{-1} depending on the carbon chain length; CMC value of surfactin C-13 and C-14 is more important than the CMC value of surfactin C-16 (Ishigami *et al*., 1995; Li *et al*., 2009).

Surfactin has also proved to carry out antiviral, antitumor, anti-inflammatory and insecticidal activities (Ongena and Jacques, 2008, Vollenbroich *et al*. 1997; Kracht *et al*. 1999; Kim *et al*., 2010; Kim *et al*., 1998 Geetha *et al*., 2010). Moreover, biofilm formed by *Salmonella* species and other infectious microorganisms was reduced after treatment with surfactin (Mireles *et al.*, 2001) and surfactin has also shown the ability to reduce colonisation of pathogenic bacteria like; *Enterobacter sakazakii* and *Salmonella enteritidis*, when it is applied to solid surfaces prior to infection (Nitschke *et al*., 2009; Korenblum *et al*., 2012).

Surfactin family:



Name of molecule	Amino acids in surfactin groups	Length of cycle chain
Esperin[**]	L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu-COOH	
Lychenysin[***]	L-XL_2-LXL_2-D-Leu-L-XL_2-L-Asp-D-Leu-L-XL_7	*i*-c_{13}, *ai*-c_{13}, *n*-c_{14}, *i*-c_{15}, *ai*-c_{15}
Pumilacidin	L-Glu-L-Leu-D-Leu-L-Leu-L-Asp-D-Leu-L-XP_7	
Surfactin	L-Glu-L-XS2-D-Leu-L-XS4-L-Asp-D-Leu-L-XS_7	*i*-c_{14}, *n*-c_{14}, *i*-c_{15}, *ai*-c_{15}

[**] β-carboxyl of Asp_5 is engaged in the lactone
[***] β-carboxyl of Asp is engaged in the halobacillin
XL_1 = Gln or Glu; XL_2 = Leu or Ile; XL_4 and XL_7 = Val or Ile;
XP_7 = Val or Ile; XS2: Val, Leu or Ile; XS4 = Ala, Val, Leu or Ile;
XS_7 = Val, Leu or Ile

n, linear
i, iso
ai, anteiso

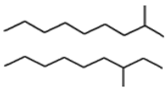


Figure 6 Structure of representative members within surfactin family. Boxed blue, type of branching (linear, iso, anteiso); boxed orange, acyl chain length; boxed red, ionisable or polar groups; boxed green, hydrophobicity of residue in position 4; boxed yellow, L-Asx(1)-D-Tyr(2)-D-Asn(3) sequence (Ongena and Jacques, 2008).

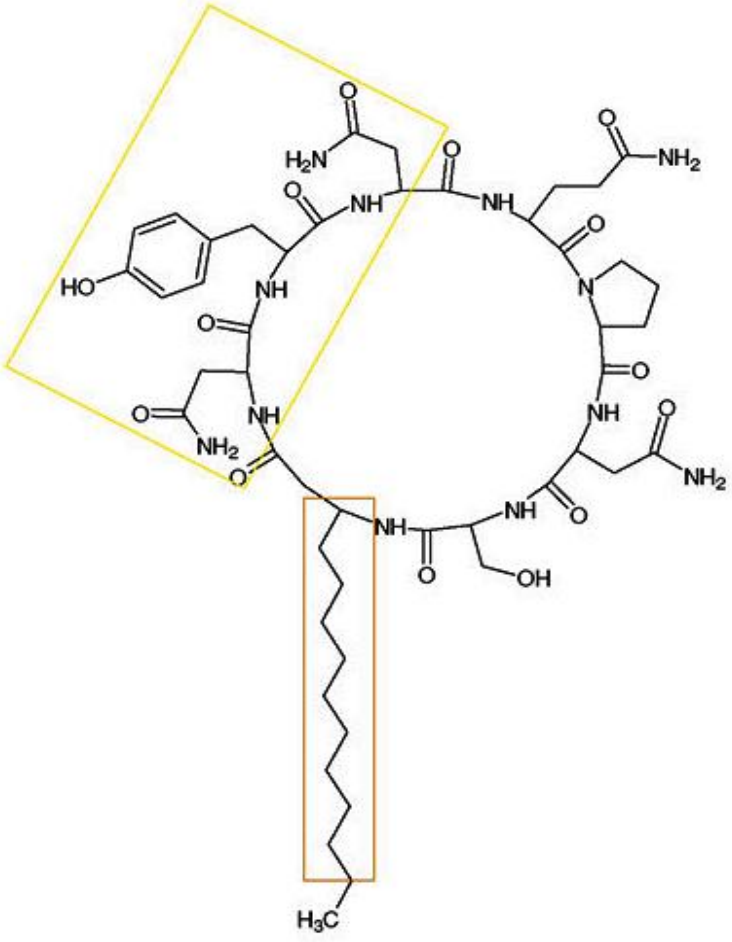
II.8.2. Iturin family

Iturin is an antibiotic, showing a strong antifungal activity. It was extracted the first time from a culture of a *B. subtilis* (Delcambe and Vignat, 1957; Delcambe, 1965). The iturin family is composed of an acyclic heptapeptide as the constant chiral form LDDLLDL interlinked with β-amino fatty acids (14-17 carbon atoms) by the acylation process (figure 7) (Maget-Dana, 1994; Isogai *et al.*, 1982; Chen, 2009; Besson *et al.*, 1978). The critical micelle concentration (CMC) of iturin is about 40 mg mL^{-1} and it has the ability to reduce water surface tension to 54 mN ml^{-1} (Maget-Dana, 1994).

The three antibiotics belonging to the iturin family (iturinA, mycosubtilin and bacillomycin) exhibit an antifungal activity (Besson *et al*., 1984; 1978) against a broad range of fungi and this gives them a high potential for use in agriculture by replacing the chemical pesticides (Hsieh *et al*., 2008).

They also exhibit strong antifungal activities against a wide variety of pathogenic yeasts and fungi but their antibacterial activities are restricted to some bacteria such as *Micrococcus luteus*. The antifungal activity is related to the interaction of the iturin lipopeptides with the cytoplasmic membrane of target cells, the K^+ permeability of which is greatly increased (Maget-Dana, 1994). The iturin compounds capability to increase the membrane cell permeability is due to the formation of ion-conducting pores and it is attributed to their ability to form aggregates (lipopeptide aggregates or lipopeptide/phospholipid complex aggregates) in the phospholipid membrane. The lipid tail, peptide ring and the residue of D-Tyr play an important role in the peptide backbone which needs to have a free hydroxyl group for optimal interaction with the target cells and in forming pores (Maget-Dana, 1994; Bonmatin *et al*., 2003).

Iturin family



Name of molecule	Amino acids in surfactin groups	Length of cycle chain
Bacillomycin D	L-Asn-D-Tyr-D-Asn-L-Pro-L-Glu-D-Ser-L-Thr	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Bacillomycin F	L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Thr	*n*-C_{16}, *i*-C_{17}, *ai*-C_{17}
Bacillomycin L	L-Asp-D-Tyr-D-Asn-L-Ser-L-Gln-D-Ser-L-Thr	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Bacillomycin LC*	L-Asn-D-Tyr-D-Asn-L-Ser-L-Gln-D-Ser-L-Thr	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}, *i*-C_{16}
Iturin A	L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Iturin A_L	L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	*n*-C_{16}, *i*-C_{16}
Iturin C	L-Asp-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Mycosubtilin	L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Ser-L-Asn	*n*-C_{16}, *i*-C_{16}, *ai*-C_{17}

*or bacillopeptin
n, linear
i, iso
ai, anteiso

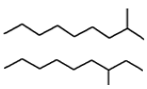


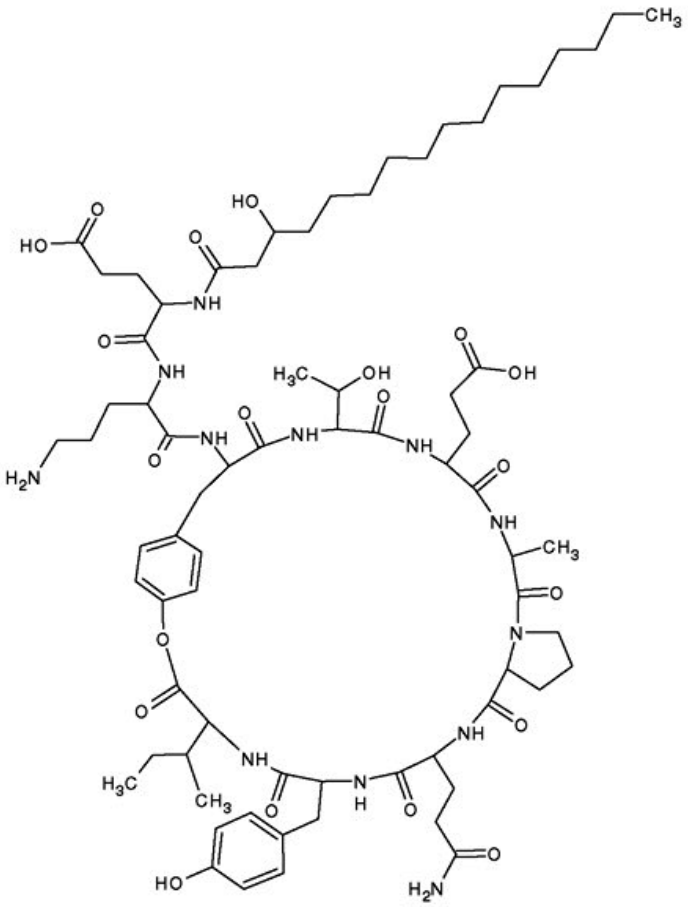
Figure 7 Representative members within iturin family. Boxed orange, acyl chain length; boxed yellow, L-Asx (1)-D-Tyr (2)-D-Asn (3) sequence (Ongena and Jacques, 2008).

II.8.3. Fengycin family

The fengycin or plipastatin is the third family of lipopeptides which was first isolated from *B. subtilis* in 1986 by Vanittanakom and Loeffler**.** Fengycin inhibits filamentous fungi but is ineffective against yeast and bacteria. The inhibition is antagonized by sterols, phospholipids and oleic acid, whereas two other unsaturated fatty acids increase the antifungal effect. Fengycin consists of two main components differing by one amino acid exchange. Fengycin A is composed of I D-Ala, 1 L-Ile, 1 L-Pro, 1 D-allo-Thr, 3 L-Glx, 1 D-Tyr, 1 L-Tyr, I D-Orn, whereas in fengycin B the D-Ala is replaced by D-Val. The lipid moiety of both analogs is more variable, as fatty acids have been identified as anteiso-pentadecanoic acid (ai-C15), iso-hexadecanoic acid (i-C16), n-hexadecanoic acid (n-C16), and there is evidence for further saturated and unsaturated residues up to C19 (figure 8) (Vanittanakom and Loeffler, 1986). Additionally to antifungal activity, fengycin and plipastatins reduce the acute hypersensitivity, inflammation and blood platelet aggregation by the inhibition of the phospholipase A2 enzyme (Volpon *et al*., 2000).

Fengycins are deca-peptides with an internal lactone ring in the peptidic moiety and also with a β-hydroxy fatty acid chain (C14- C18). Fengycins are found to have a strong antifungal activity but are less hemolytic than iturins and surfactins. They have the ability to interact with lipid layers and thus they have a certain extent of capability to change cell membrane structure and permeability based on dose way (Hathout, 2000; Vanittanakom *et al*., 1986).

Fengycin family



Name of molecule	Amino acids in surfactin groups	Length of cycle chain
Fengycin A**	L-Glu-D-Orm-D-Tyr-D-aThr-LGlu-D-Ala-L-Pro-L-Gln-L-Tyr-L-Ile	*ai*-C_{15}, *i*-C_{16}, *n*-C_{16}
Fengycin B	L-Glu-D-Orm-D-Tyr-D-aThr-LGlu-D-Val-L-Pro-L-Gln-L-Tyr-L-Ile	*ai*-C_{15}, *i*-C_{16}, *n*-$C_{16,}$ C_{17}
Plipastatin A	L-Glu-D-Orm-L-Tyr-D-aThr-LGlu-D-Ala-L-Pro-L-Gln-D-Tyr-L-Ile	*n*-C_{16}, *ai*-C_{17}
Plipastatin B	L-Glu-D-Orm-L-Tyr-D-aThr-LGlu-D-Val-L-Pro-L-Gln-D-Tyr-L-Ile	*n*-C_{16}, *ai*-C_{17}

** Double bond between carbons 2-3, 3-4 or 13-14 were reported for some acyl chains

n, linear
i, iso
ai, anteiso

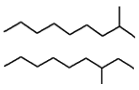


Figure 8 Structure of representative members within fengycin family (Ongena and Jacques, 2008).

II.8.4. Lipopeptide synthesis

Lipopeptides (LPs) are biosurfactants produced by a variety of bacterial genera, including *Bacillus* (Ongena and Jacques, 2008) in addition to *Actinomycetes*, filamentous fungi and marine microorganisms (Finking and Marahiel, 2004). Scientists realized in 1960s that lipopeptides are

not synthesized by means of the ribosomal machinery (Finking and Marahiel, 2004) and for the first time, it was presented that the biosynthesis of gramicidin (a peptide antibiotic) is possible in the presence of an inhibitor of ribosomal machinery (Gevers *et al.*, 1968).

Nonribosomal peptide synthesis is carried out by the so-called nonribosomal peptide synthetases (NRPSs) or hybrid polyketide synthetases (PKSs) and NRPSs. These NRPSs and PKSs are organized as multi-enzymes with a notable size (Schwarzer *et al.*, 2002). The synthesis of these molecules is modularly organized, every module represents a part of polypeptide chain in NRPSs and is responsible for the incorporation of one amino acid into the final product (Schwarzer *et al.*, 2002; De Bruijn *et al.*, 2008; Sieber and Marahiel, 2005, Jacques, 2011). Figure 9 shows an overview of these modules in of all these multifunctional proteins in *Bacillus spp*.

Each module of peptide synthetases are subdivided into domains which correspond to the enzymatic units that catalyze the individual steps of nonribosomal peptide synthesis (figure 10) (Schwarzer *et al.*, 2002; Bruijn *et al.*, 2008; Ansary *et al.*, 2004).

The biosynthesis of the peptides backbone in NRPS machinery depends on four domains for substrate recognition (figure 10) (Stachelhaus and Marahiel, 1995; Mootz and Marahiel, 1997; May *et al.*, 2001), activation and transport to the respective catalytic centers (Ehmann *et al.*, 2000; Stachelhaus *et al.*, 1996) as well as the formation of the peptide bond (Stachelhaus *et al.*, 1998; Bergendahl *et al.*, 2002) and release of the peptide and is also frequently involved in the formation of a macrocyclic product (lactones and lactams) or the oligomerisation of peptide units (Kopp and Marahiel, 2007).

Domains of equal function share a number of highly conserved sequence motifs. These "core-motifs" allow the identification of individual domains on the protein level (Schwarzer *et al.*, 2003).

The domains in each module are divided into main domains and secondary domains:

- **The main domains:**

In general, the NRPS biosynthesis involves four main domains (figure 10): adenylation (A), thiolation (T), condensation (C) and thioesterase (TE) domains (Jacques, 2011). The adenylation domain is responsible for amino acids selection and activation as amino acyl adenylates and this process is stimulated with the expense of ATP (figure 10) (Dieckmann *et al.*, 2001; Stachelhaus and Marahiel, 1995; Mootz and Marahiel, 1997; May *et al.*, 2001). As an

example of this domain, recently, the crystal structure of the A domain from the gramicidin synthetase (GrsA) with L-phenylalanine and adenosine monophosphate bound from *B. brevis* (Conti et al., 1997; Challis *et al.,* 1999) and the 2,3-dihydroxybenzoate (2,3-DHB) activating A-domain DhbE from *B. subtilis* (May *et al.,* 2002) has been determined. These structures represent the important role of ten amino acid residues in the coordination of the substrate (Jacques, 2011). The so-called non-ribosomal code can be used as rules to rationally alter the specificity of adenylation domains and to predict from the primary sequence the specificity of biochemically uncharacterized adenylation domains (Stachelhaus *et al.,* 1999).

After amino acid activation in the A-domain, it is transferred to the peptidyl-carrier protein (PCP) or T-domain which represents the transport unit that accepts the activated amino acid that is covalently tethered to its 4'phosphopantheteinic (4-PP) cofactor as thioester (Ehmann *et al.,* 2000; Weber *et al.,* 1994; Jacques, 2011). This cofactor is post-translationally transferred to a serine of the PCP. This reaction is catalyzed by a phosphopantetheinyl transferase which is essential to transform apoform of NRPS in its holoform (Mofid *et al.,* 2004). PCPs are part of the CP superfamily, which comprises a number of different members. These members have been named according to the substrates they carry (Finking and Marahiel, 2004).

The formation of the peptide bond between amino acyl substrate bonds to PCPs of neighboring modules is involved in condensation (C) domain which represents the central entity of nonribosomal peptide synthesis (Finking and Marahiel, 2004; Jacques, 2011). The enzyme catalyzes the nucleophilic attack of the amino (or imino, hydroxyl) group of the activated amino acid bound to the downstream (with respect to the C-domain) module onto the acyl group of the amino acid tethered to the upstream module, as shown in figure 11 (Finking and Marahiel, 2004). According to the multiple-carrier thiotemplate model (Stein *et al.,* 1996), the C-domain possesses a site for the nucleophile (acceptor site) and a position for the electrophile (donor site) as shown in Figure 10 (Doekel and Marahiel, 2000). Finally, the termination of synthesis is catalyzed by the terminal enzyme of the last module. In most cases and for lipopeptide synthesis, this reaction is performed by a thioesterase domain (TE). This allows the release of the peptide and is also frequently involved in the formation of a macrocyclic product (lactones and lactams) or the oligomerisation of peptide units (Kopp and Marahiel, 2007). Other alternative release mechanisms can be achieved by the reduction of the peptidyl-S-PCP final product to generate a linear aldehyde or alcohol (Jacques, 2011).

- **Secondary or additional domains:**

Additional domains can be involved in the biosynthesis of the peptide to modify the structure of the monomer contributing in the primary structure or to add some external compounds to the peptide. Among these are tailoring domains, cyclisation (Cy), methylation (Me), oxidation (Ox), glycosylation, epimerisation (E) and addition of fatty acid chain. The final two domains are engaged in lipopeptide biosynthesis in *Bacillus spp*., the E-domain catalyses the epimerisation of the PCP-bound L-amino acid of the growing polypeptide chain. The addition of the fatty acid chain to the first amino acid of the peptide moiety is catalysed by a first specific condensation domain (figure 12). The added fatty acid chain can itself be partially synthesized by another main group of modular enzymes, called the polyketide synthetases (PKS). In this last case, a hybrid PKS/NRPS is required for the synthesis of the biomolecules (Du *et al.*, 2000).

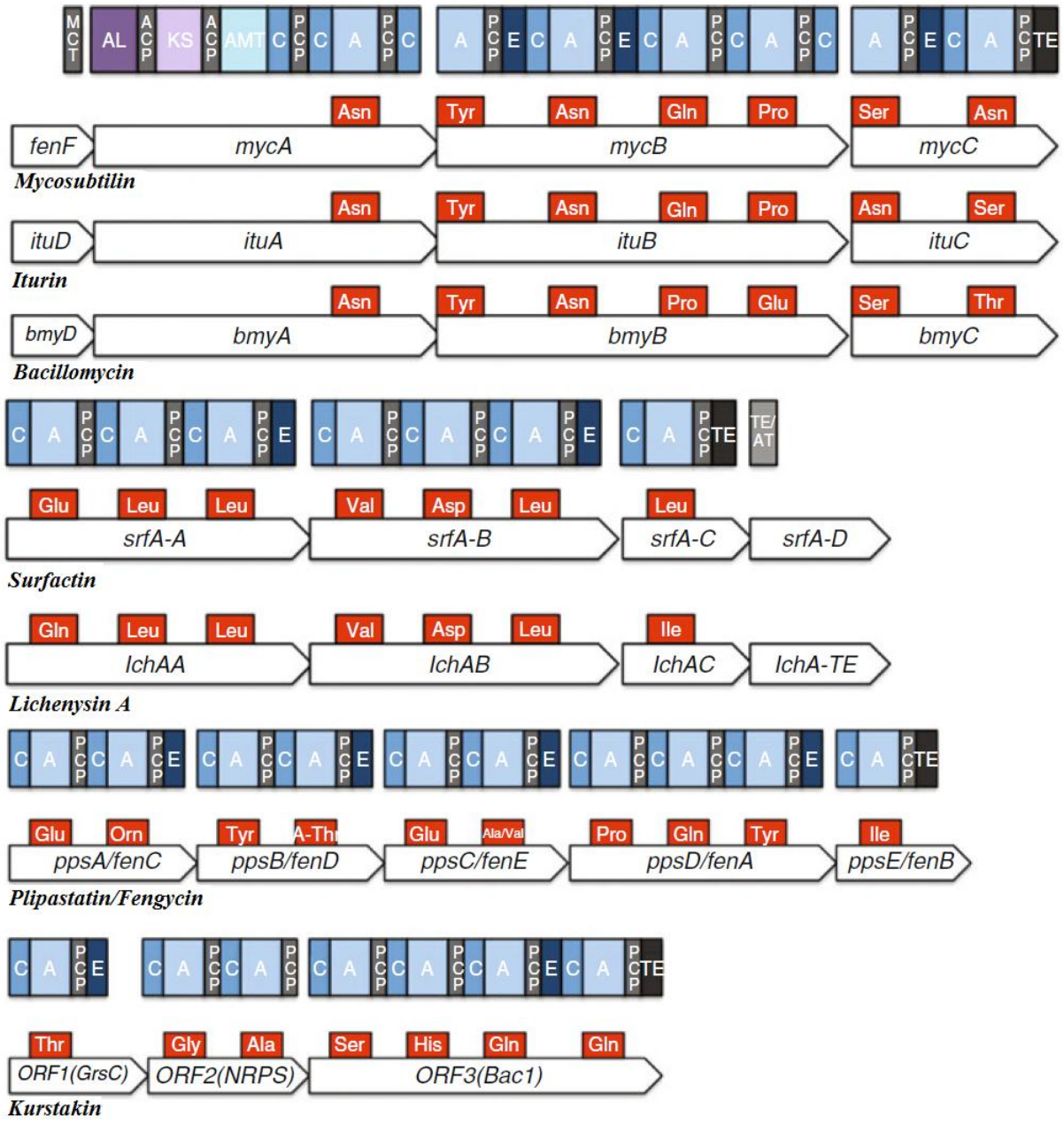


Figure 9 Operons responsible for lipopeptide biosynthesis in *Bacillus spp*. (Jacques, 2011).

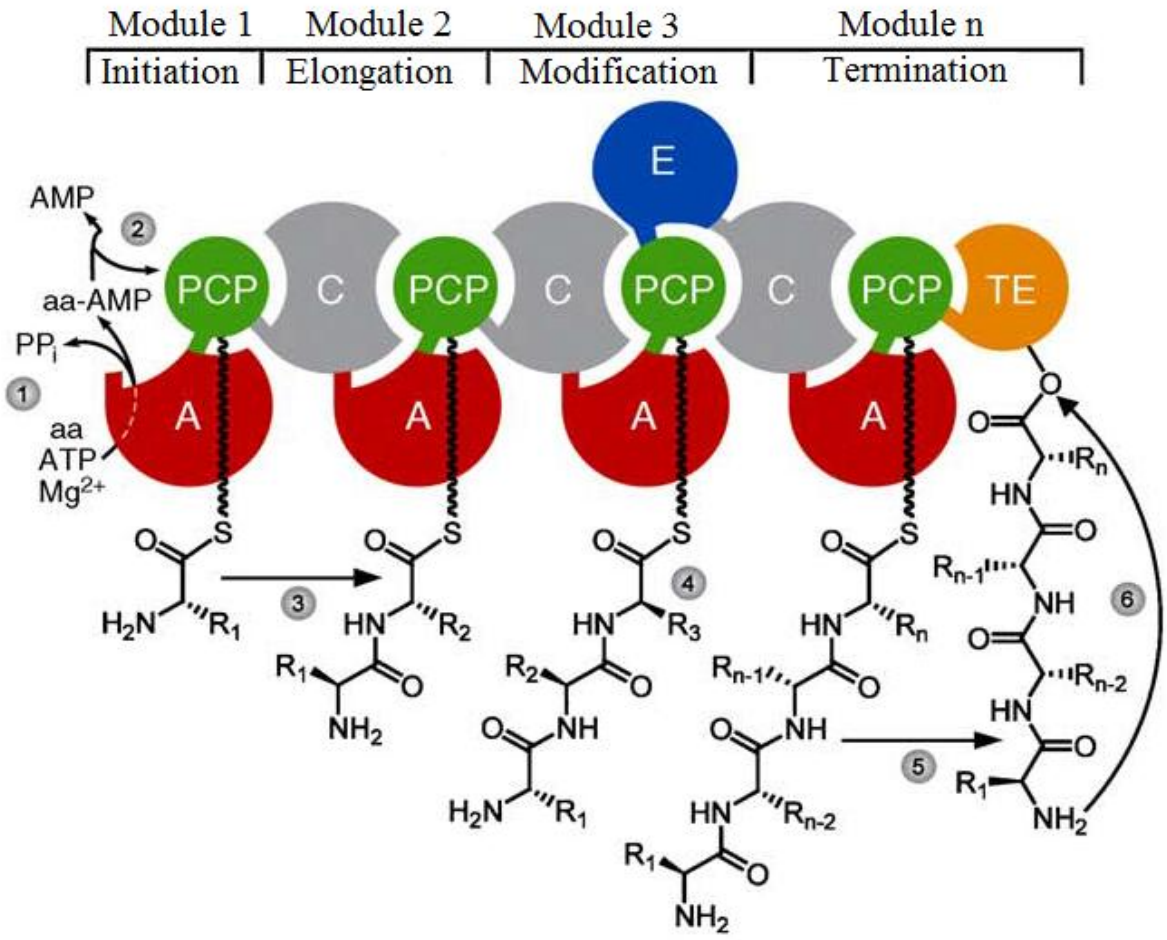
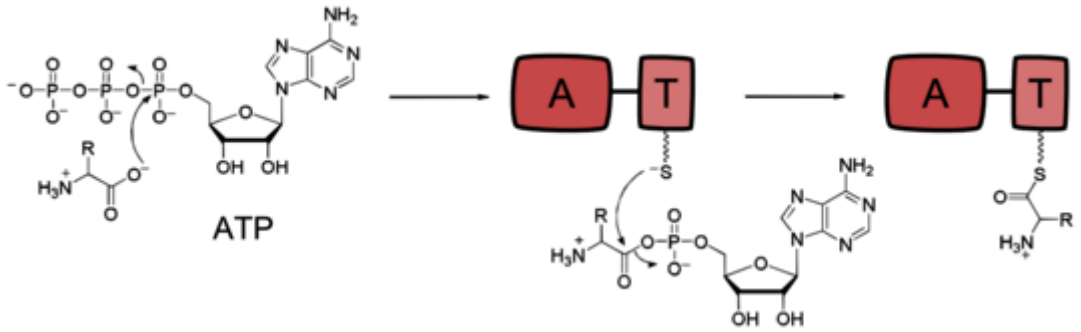
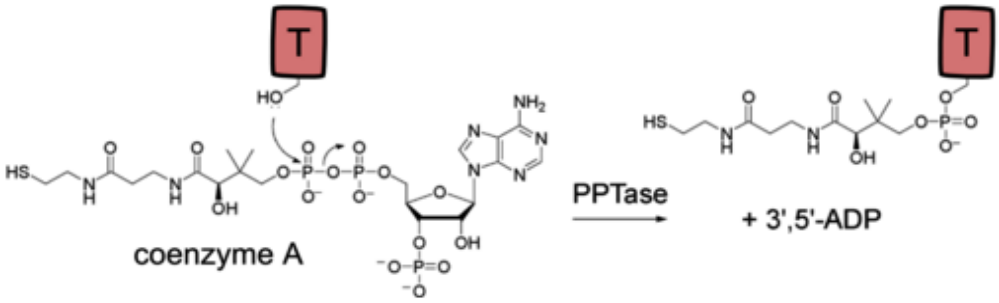


Figure 10 Simplified mechanism of nonribosomal peptide (NRP) synthesis. (1) The amino acid is activated as aminoacyl-AMP by the adenylation domain. (2) Transfer of the amino acid onto the PCP domain. (3) Condensation of PCP-bound amino acids. (4) Possibility of amino acid modifications, for example by epimerization domains. (5) Transesterification of the peptide chain from the terminal PCP onto the TE domain. (6) TE catalyzed product release by either hydrolysis or macrocyclization (Strieker *et al.*, 2010).

(A) A-Domain



(B) T-Domain



(C) C-Domain

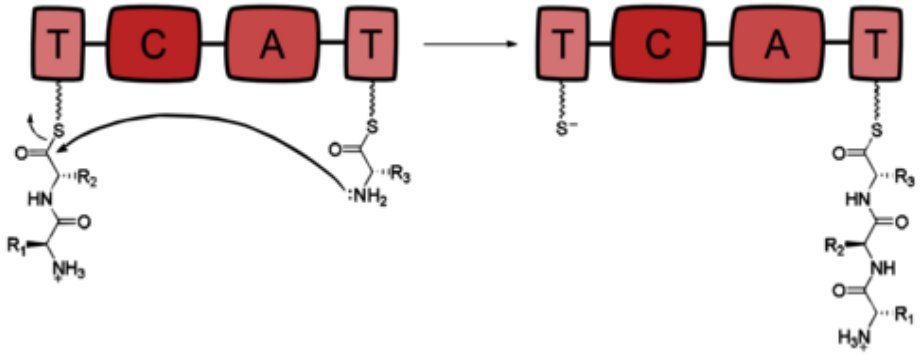


Figure 11 Reactions catalysed by the three basic NRPS domains. A) The adenylation (A) domain selects the amino acids and covalently binds it to the T domain, B) Thiolation (T) domains are the carrier domains of the monomers and the growing peptide chain. The transfer of phosphopantetheine from coenzyme A to a conserved serine in the T-domain is essential for non-ribosomal peptide synthesis as the thiole residue is necessary for monomer binding. It catalyzes two reactions: First, the activation of the monomer by ATP-binding and second, the acylation to the downstream T domain. c) Chain elongation is catalyzed by condensation (C) domains. The C domain enables peptide bond formation between the monomer (acceptor) and the growing peptide chain (donor), resulting in a translocation of the peptide chain to the acceptor T domain (Fischbach and Walsh, 2006).

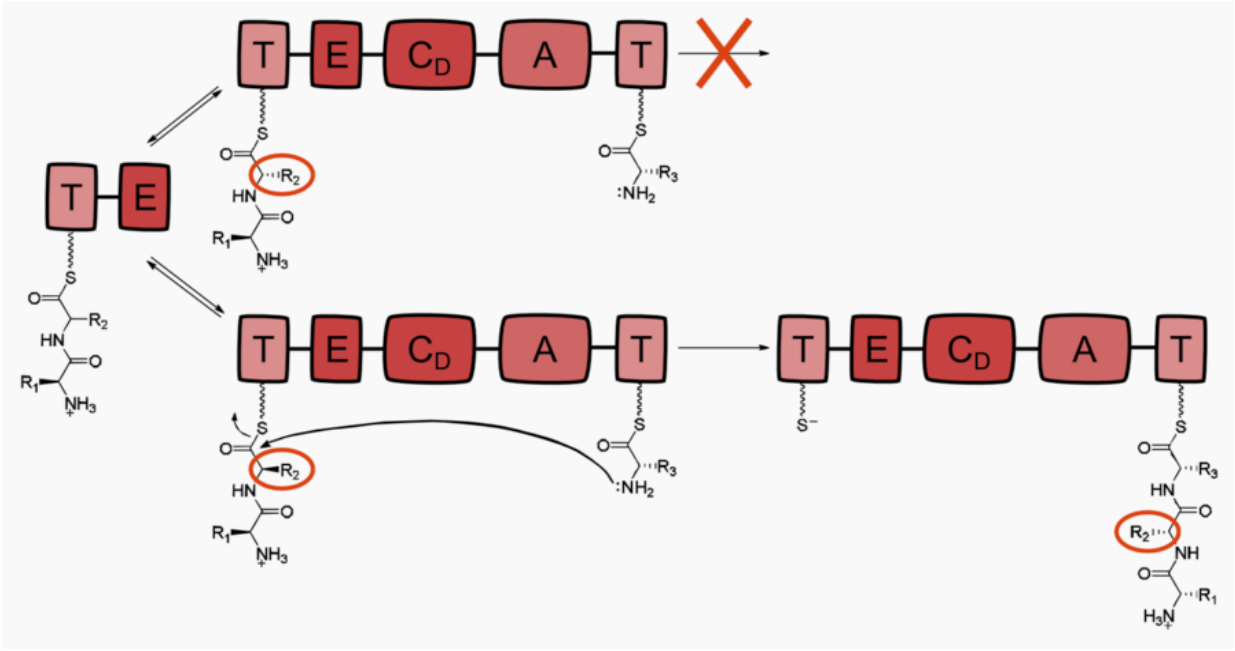


Figure 12 A combination of epimerisation (E) and C domains allow for the incorporation of L-amino acids into NRPs. First, the E domain racemises the donor amino acid. Subsequently, a C domain specific for D-amino acids catalyzes the condesation reaction (lower reaction). For the L-stereo conformation, no peptide bond can be formed (Zhu *et al.*, 1998).

II.8.5. Lipopeptide application

The various and interesting physiological and biological properties of lipopeptides have led to use them in wide array of applications. They are used in the biomedical applications owing to their ability to disturb cell membranes integrity and permeability and, thus, to destabilize cell membranes (Ortiz *et al.*, 2009; Zaragoza *et al.*, 2009; Sánchez *et al.*, 2010). These molecules are nontoxic or less toxic than their synthetic counterparts and that encourages their use in the biomedical and pharmaceutical fields (Muthusamy *et al.*, 2008; Cochis *et al.*, 2012; Ayed *et al.*, 2015; Meena and Kanwar, 2015).

Different lipopeptides produced from different bacterial species isolated from soil have demonstrated an antimicrobial activity. Among them, Polymyxin A produced by *Bacillus polymyxa* was discovered first (Jones, 1949). Equally important, surfactin, fengycin, iturin, bacillomycin and mycosubtilin are produced by *B. subtilis* and *B. amyloliquefaciens* (Ongena and Jacques, 2008; Vater *et al.*, 2002). Whereas lichenysin and pumilacidin are produced by *B. licheniformis*, *Bacillus pumilus* respectively (Naruse *et al.*, 1990; Yakimov *et al.*, 1995; Grangemard *et al.*, 2001; Landman *et al.*, 2008). In addition, *Streptomyces roseosporus* produces

a cyclic lipopeptide; daptomycin and *Pseudomonas* produces viscosin which are known as antimicrobial lipopeptides (Saini *et al.*, 2008; Baltz *et al.*, 2005).

In addition to their antimicrobial activity, lipopeptides can be used to prevent microbial infections as described by many researchers (Deleu *et al.*, 2004; Cameron *et al.*, 2005; Hill *et al.*, 2008), and they also have an activity to prevent skin and mucosa diseases (Groux *et al.*, 2005).

Besides their potential use in the medical fields, they could be also applied in the food industry. In certain conditions of food industry, microorganisms can multiply and form biofilm from nutrient residues and microorganisms cells and the elimination of these harmful conditions represents a major challenge in this industry (Bagge-Ravn *et al.*, 2003; Kim *et al.*, 2006; Hood and Zottola, 1995; Irie *et al.*, 2005; Walia and Cameotra, 2015). Among these microorganisms, *Pseudomonas aeruginosa*, *Pseudomonas fragi*, *Pseudomonas fluorescens*, *Micrococcus* sp., *Enterococcus faecium*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, and *Bacillus cereus* are found to form biofilms/adhering to food and food contact surfaces, which represents a real problem in food industry (Hood and Zottola, 1995; Maukonen *et al.*, 2003). Lipopeptides which present biosurfactant properties could be one of the solutions to reduce the biofilm formation, thereby preventing microorganism adhesion on the surfaces of foods or equipment in which foods are manufactured (Rodrigues *et al.*, 2007; Quinn *et al.*, 2013). For instance, the synergetic activity of lipopeptide produced by *B. subtilis* V9T14 increases the activity against biofilm formed by *Escherichia coli* CFT073 (Rivardo *et al.*, 2011). On the other hand, the amount of biofilm formed *by S. typhimurium, Salmonella enterica, E. coli and Proteus mirabilis* in PVC plates and vinyl urethral catheters is inhibited by surfactin treatment (Mireles *et al.*, 2001).

The pre-treatment of silicone rubber with *Streptococcus thermophilus* surfactant inhibits 85% of the adhesion of *Candida albicans* (Busscher *et al.*, 1997). Irie *et al*., (2005) also reported the important role of rhamnolipids in interrupting the biofilm formed by *Bordetella bronchiseptica* and reducing the adhesion rates of *Streptococcus salivarius* and *Candida tropicalis* in silicone rubber. In addition, Velrade *et al.*, (1996) indicated that the surfactants produced by *Lactobacillus fermentum* and *Lactobacillus acidophilus* adsorbed by glass reduces the number of adhering uropathogenic cells of *Enterococcus faecalis.*

Depending on the properties of some lipopeptides which are classified as biosurfactants, having emulsification, dispersion, surface, detergency activity, and enhanced solubility led to use

them in agriculture for the remediation of soil polluted with hydrocarbons, heavy metals, and pesticides (Kosaric, 2014). Mulligan *et al.,* (2001) demonstrated the ability of lipopeptides; surfactin from *B. subtilis*, rhamnolipids from *P. aeruginosa* and sophorolipids from *Torulopsis bombicola* to remove metals from soil. Biosurfactant significantly reduced soil permeability, and adding this compound to the soil allowed to remove about 96% Zn and Cu and to reduce Pb and Cd concentrations in groundwater (Rufino *et al.,* 2011, 2012). Equally important, it has been reported that the biosurfactants are widely used in the hydrocarbon degradation and bioremediation in soil (Thavasi *et al.,* 2009; Liao *et al.,* 2015).

Among the numerous applications of lipopeptides, their use in agriculture attracted a high attention. Lipopeptides produced by different species of bacteria are well known for their ability to suppress different phytopathogens through direct way by inhibiting directly pathogens or indirect way by inducing resistance in plant (Jacques *et al.,* 2008) (figure 13) Velho et al., (2011) reported that lipopeptides produced by a *Bacillus* strain inhibited the growth of different phytopathogenic fungi such as *Fusarium spp., Aspergillus spp.,* and *Biopolaris sorokiniana*. Romero *et al.,* (2007) reported that lipopeptides produced by the two strains of *B. subtilis* were able to reduce cucurbit powdery mildew caused by *Podosphaera fusca* by inhibiting conidial germination. Whereas, the surfactin produced by *Brevibacillus brevis* HOB1 presented strong antibacterial and antifungal properties (Haddad, 2008) and the cyclic surfactin from *Bacillus mojavensis* RRC 101 showed high activity against *Fusarium verticillioides* (Snook *et al.,* 2009).

Besides their activity in different fields, lipopeptides can play an essential role in the relationship between plant and microorganisms (negative or positive interaction) such as motility, signaling, and biofilm formation (Sachdev and Cameotra, 2013; Ongena and Jacques, 2008).

The role of lipopeptides in the interaction between plant and microorganisms can be explained as follows (figure 13):

1. Expediting microbial mobility on plant root surface (Deziel *et al.,* 2003).
2. Creating microcolonies by adhesion and dispersion of biofilms on plant surfaces (Davey *et al.,* 2003).
3. Improving plant nutrients uptake by increasing soil moisture content and thus supporting better soil fertility (Sachdev and Cameotra, 2013).

4. Protecting from toxic hydrocarbons and heavy metals compounds (Chrzanowski *et al.*, 2009).

Altogether, the lipopeptides occupy an important place in different fields and as far as we are concerned here is the main role in the replacement of chemical compounds both in the field of biological control and in the field of soil fertility by increasing the readiness of nutrients for plants (Kosaric, 2014).

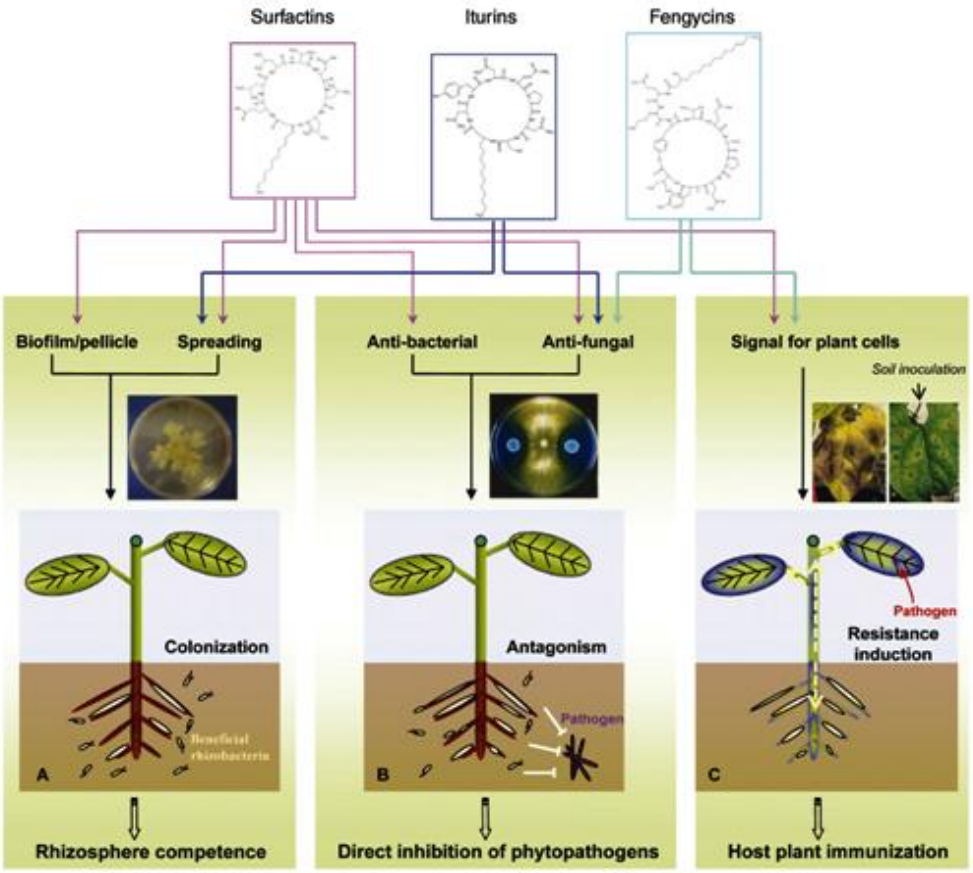


Figure 13 Overview of *Bacillus* lipopeptide interactions in the context of biological control of plant diseases. From left to right, the three photographs show bacterial spreading, fungal growth inhibition through the production of fungitoxic compounds by blue bacterial cells and leaf disease reduction following inoculation of the beneficial bacterium on roots. They illustrate how to get experimental indications about the potential involvement of one particular strain in the three phenomena schematically represented in (A), (B) and (C). Establishment in biofilm and/or microcolonies of the rhizobacterium is represented in (A), (B) represents direct antibiosis that can be exerted by the established biocontrol strain toward pathogens sharing the same microenvironment. In (C), the arrows illustrate the emission of a signal following perception of the rhizobacterium at the root level (Ongena and Jacques, 2008).

II.8.6. Role of lipopeptides and biofilm in colonisation

Until current work, there have been no indications of direct effect of lipopeptides especially the surfactants on the root or rhizosphere colonisation. On the other hand, Ongena and Jacques, (2008) reported the importance of lipopeptides and the variety of their modes of action in the biocontrol activity of numerous strains of *Bacillus* species by killing the phytopathogen, stimulating resistance in plant and maybe also facilitating root colonisation. The detection of only surfactin among other lipopeptides and polyketides in the extracts of Lemna plantlets colonized by *B. amyloliquefaciens* FZB42 under laboratory conditions (Fan *et al.,* 2011) could refer to a contribution of surfactin in root colonisation. Furthermore, Bais *et al.,* (2004) indicated that the extensively form of biofilm and secreted surfactin by *B. subtilis* 6051 may act together to protect plant against pathogenic bacteria.

In natural environment, microorganisms' cells associate together by forming a complex of aggregate cells and extracellular product. This complex is cemented by a mixture of polysaccharides (Christensen, 1989; Sutherland, 2001). Plant root or rhizosphere is one of the natural environments in which different PGPR could physically interact with the plant in various ways. All these interactions commonly appear as colonisation of root and rhizosphere. The bacteria adhere to the external part of plant tissues as individual and aggregate cells and these aggregated cells are defined as biofilms and they display a range of dimensions, locations and compositions (Nongkhlaw *et al*., 2014). The plant microenvironment has different characteristics such as saturation levels, nutrient availabilities and surface chemistries, which strongly influence the form and activity of biofilms (Ramey *et al*., 2004).

Some studies have indicated a probable role of biofilm in root colonisation. For instance, Watnick and Kolter (1999) elucidated in their study a high correlation between root colonisation and forceful biofilm formation by *B. amyloliquefaciens* FZB42. Equally, biofilm formation was detected on the primary root tips of *Arabidopsis* thaliana colonized by *B. subtilis* and *P. polymyxa* (Bais *et al*., 2004; Timmusk *et al*., 2005) and a similar observation was shown in the colonisation of *Arabidopsis* seeds by *B. amyloliquefaciens* (Reva *et al*., 2004).

Most microorganisms tend to form a biofilm by associating a complex of microorganisms cells. In this mechanism, extracellular products play an important role by attaching bacterial cells together as a biofilm (Christensen, 1989). The complex of cells and products forming the biofilm

is aggregated and cemented by the mixture of extracellular polymeric substances which are secreted by the cells (Sutherland, 2001; Lembere *et al*., 2012).

The extracellular polymeric substances synthesis ability is prevalent among the microorganisms. These compounds are almost similar and their amount synthesized within the biofilm relies on the carbon compounds availability and on the equilibrium between carbon and other nutrients (Fang and Ong, 2009; Davey and O'toole, 2000; Sutherland, 2001; Sutherland, 2008).

The extracellular polymeric substances contribute directly to the properties of the biofilms and mostly consist of polysaccharides, proteins, nucleic acids and lipids, which supply mechanical stability of the biofilms, mediate their adhesion to surfaces and form a cohesion (Mayer *et al., 1999;* Flemming *et al.,* 2007; Flemming and Wingender, 2010; Donelli, 2014).

Numerous studies clearly indicate that exopolysaccharide compounds (EPS) have an important role in biofilm formation and the mutants which are incapable of synthesizing the EPS compounds are unable to form biofilms, even though they may still form micro-colonies and attach to the surfaces in limited scope (Allison and Sutherland, 1987; Watnick and Kolter, 1999; Sutherland, 2001; Beauregard, 2012).

Chapter III

Objectives

III. The objectives:

III.1. Background of the current study

In a previous work, Jovana Deravel had initiated a study about the correlation between surfactin production and tomato rhizosphere colonisation. Several strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* producing or not surfactin have been tested for their ability to colonize tomato roots. The results obtained are summarized in figure 1. These results showed that the rhizosphere colonisation was mainly species dependent (strains of *B. amyloliquefaciens* were clearly better colonizers than strains of *B. subtilis*) and surfactin could enhance colonisation but was not the main factor. These findings, in addition to others from literature concerning this subject led us to develop this current study which aims to determine the important criterion for biocontrol agent rhizosphere colonisation in more detail.

III.2. Objectives

Based on what has been noted above, one model strain of each species was chosen: *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131. They were used:

- To study the role of surfactin on the tomato rhizosphere colonisation by performing kinetic experiments to determine the biomass and the amount of surfactin produced by both strains during 21 days in tomato rhizosphere.
- To study the role of tomato root exudates and their different components on rhizosphere colonisation and lipopeptide production.
- To study the role of biofilm formation in rhizosphere colonisation. This part of the study was divided into two assays.
    - First, the biofilm formed by *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 was evaluated *in vitro* in the presence of different carbon sources found in the tomato root exudates.
    - Then, biofilm formation was also tested *in vivo*. For this assay a mutant strain of *B. amyloliquefaciens* FZB42 with a knock-out of the gene *eps* encoding the exopolysaccharide production needed for biofilm formation was obtained. Its behaviour was tested in comparison with the wild type strain.

Chapter IV

Materials and methods

IV. Materials and methods

IV.1. Culture media and buffers

IV.1.1. LB medium

Lysogeny Broth or Luria-Bertani medium is used for bacterial growth. It contains 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl. pH is adjusted to 7.2 by addition of NaOH. 17 g L^{-1} agar is added for solid medium. This media is sterilized by autoclaving at 121°C for 20 min.

IV.1.2. Tryptone salt

Tryptone salt broth is recommended for preparation of specimens, stock suspensions and decimal dilutions for the purposes of microbiological tests. It contains 8.5 g L^{-1} NaCl and 1 g L^{-1} tryptone. These compounds are dissolved in 1L of distilled water. The medium is sterilized by autoclaving at 121°C for 15 min.

IV.1.3. PDA medium

This medium is used for the fungi growth. It is composed of 4 g L^{-1} potato extract, 20 g L^{-1} dextrose and 15 g L^{-1} agar. These components are dissolved by heating with slowly stirring. pH is adjusted to 7.0 and the medium is sterilized by autoclaving at 121°C for 20 min.

IV.1.4. Phosphate buffer

This buffer is used for washing cells. It is composed of 35 g L^{-1} K_2HPO_4 and 15 g L^{-1} KH_2PO_4. These compounds are dissolved in 1L of distilled water. pH is adjusted to 7.0 and the solution is sterilized by autoclaving at 121°C for 20 min.

IV.1.5. Landy medium

Landy medium (Landy *et al*., 1948) is used for lipopeptides production. It contains as follows: 20 g L^{-1} glucose, 5 g L^{-1} glutamic acid, 1 g L^{-1} yeast extract, 1 g L^{-1} K_2HPO_4, 0.5 g L^{-1} $MgSO_4$, 0.5 g L^{-1} KCl, 1.6 g L^{-1} $CuSO_4$, 1.2 g L^{-1} $Fe_2(SO_4)_3$, 0.4 g L^{-1} $MnSO_4$ and 1M of 3-[N-

morpholino]-propane sulfonic acid (MOPS) is added as a buffer. The pH is adjusted to 7 with the addition of 3M KOH and the medium is sterilized by autoclaving at 110°C for 30 min.

IV.1.6. Stock solutions of Landy medium

IV.1.6.1. Glucose solution (20X)

It is composed of 400 g L^{-1} glucose dissolved in 1L distilled water and sterilized by autoclaving at 110°C for 20 min.

IV.1.6.2. Glutamic acid solution (20X)

The solution is composed of 100 g L^{-1} glutamic acid dissolved in 1L distilled water. pH is adjusted to 8.0 by adding 5 M KOH solution and it is sterilized by filtration through a 0.2 µm filter.

IV.1.6.3. Solution A (20X)

This solution contains 20 g L^{-1} yeast extract and 10 g L^{-1} $MgSO_4$ dissolved in 1L distilled water and sterilized by autoclaving at 121°C for 20 min.

IV.1.6.4. Solution B (20X)

This solution is composed of 20 g L^{-1} K_2HPO_4 and 10 g L^{-1} KCl dissolved in 1L distilled water and sterilized by filtration through a 0.2 µm filter.

IV.1.6.5. Solution C (20X)

This solution is composed of 32 mg L^{-1} $CuSO_4$, 24 mg L^{-1} $MnSO_4$ and 8 mg L^{-1} $FeSO_4$ dissolved in 1L distilled water. This solution is acidified to pH 1.8 with concentrated H_2SO_4 and sterilized by filtration through a 0.2 µm filter.

IV.1.6.6. MOPS solution (20X)

It is prepared by dissolving 420 g L^{-1} of MOPS in 1Ldistilled water and it is sterilized by filtration through a 0.2 µm filter.

IV.1.7. Recombination of Landy medium

For preparation of 1L of Landy medium, 50 ml of each solution (glucose, glutamic acid, A, B, C and MOPS solution) are added to 700 mL of distilled and sterilized water. pH is adjusted to 7.0 by adding 3 M KOH which is already sterilized by autoclaving at 121°C during 20 min.

IV.1.8. SOB medium

This medium is composed of 20 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 0.5 g L^{-1} NaCl and 18.6 g L^{-1} KCl. These compounds are dissolved in 1L distilled water. pH is adjusted to 7.2. This medium is sterilized by autoclaving at 121°C for 20 min.

IV.1.9. SOC medium

This medium is prepared by adding 0.5 mL of 2 M $MgCl_2$ (sterilized by autoclaving at 121°C during 20 min) and 2 mL of 1M glucose (sterilized by autoclaving at 110°C during 20 min) to 100 ml of SOB medium.

IV.1.10. NCM medium

This medium is used to prepare competent cells for electro-transformation and consists of: 17.4 g K_2HPO_4, 11.6 g NaCl, 5 g glucose, 5 g tryptone, 1g yeast extract, 0.3 g trisodium citrate, 0.05 g $MgSO_4.7H_2O$ and 91.1 g sorbitol. These components are dissolved in 1L distilled water and pH is adjusted to 7.2 (Ito and Makoto, 2001). This medium is sterilized by autoclaving at 121°C for 15 min.

IV.1.11.TBE – Buffer solution (10X)

This buffer is used for gel electrophoresis and it is composed of 40 mM [2-Amino-2-hydroxymethyl-propane-1,3-diol] (Tris), 890 mM boric acid and 20 mM [2-({2-[Bis(carboxymethyl)amino] ethyl} (carboxymethyl) amino) acetic acid] (EDTA). These compounds are dissolved in 1L distilled water. pH is adjusted to 8.3. This medium is sterilized by autoclaving at 121°C for 15 min.

IV.1.12. DNA agarose gel

This solution is prepared for gel electrophoresis migration and contains 7.2 g agarose dissolved in 1L TBE buffer dissolved by heating in the microwave for 2.5 min.

IV.1.13. Transformation buffer solution

It is composed of 1X SMM solution, 1mM [ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid] (EGTA), 5 g L^{-1} glucose and 20 mM $MgCl_2$. All these components are dissolved in 1L distilled water. This medium is sterilized by autoclaving at 121°C for 15 min.

IV.1.14. MD buffer solution

This buffer is composed of 1X PC solution, 1g L^{-1} glucose, 50 mg L^{-1} L-tryptophan, 100 mg L^{-1} $FeCl_3$, 100 mg L^{-1} sodium citrate and 3 mM $MgSO_4$. These components are dissolved in 1L distilled water. This medium is sterilized by autoclaving at 121°C for 15 min.

IV.1.15. MDCH buffer solution

This buffer is composed of 1X PC solution, 1g L^{-1} glucose, 50 mg L^{-1} L-tryptophan, 100 mg L^{-1} $FeCl_3$, 100 mg L^{-1} sodium citrate, 3 mM $MgSO_4$, 1 g L^{-1} casein hydrolysate and 2.5 g L^{-1} sodium glutamate. This medium is sterilized by autoclaving at 121°C for 15 min.

IV.1.16. SMM solution

This solution contains 20 g L^{-1} $(NH4)_2SO_4$, 140 g L^{-1} K_2HPO_4, 60 g L^{-1} KH_2PO_4 and 10 g L^{-1} sodium citrate. All these components are dissolved in 1L distilled water and sterilized by autoclaving at 121°C for 15 min.

IV.1.17. PC solution (10X)

0.8 M K_2HPO_4, 0.45 M KH_2PO_4 and 0.35 M of sodium citrate are dissolved in 1L distilled water to prepare PC solution. pH is adjusted to 7.0. This solution is autoclaved at 121°C for 15 min.

IV.1.18. Antibiotic solutions

Different concentrations of antibiotic stock solutions are prepared. The concentrations are 20 mg mL^{-1} for ampicillin, 10 mg mL^{-1} for neomycin, 2 mg mL^{-1} for chloramphenicol, 2 mg mL^{-1} for tetracyclin, 10 mg mL^{-1} spectinomycin and 2 mg mL^{-1} for erythromycin. The antibiotics are dissolved in distilled water and they are sterilized by filtration through a 0.2 µm filter.

IV.1.19. M9 mineral medium

This medium is used as a minimal medium for bacterial growth. 1 liter of M9 is composed of: 33.7 mM $NaHPO_4$, 22.0 mM KH_2PO_4, 8.55 mM NaCl, 9.35 mM NH_4Cl, 1 mM $MgSO_4$, 0.3 mM $CaCl_2$, 1 µg biotin, 1 µg thiamin and 1X trace elements. This medium is prepared from the following stock solutions.

IV.1.19.1. M9 salt solution (10X)

$Na_2HPO_4.2H_2O$ 75.2 g L^{-1}, KH_2PO_4 30 g L^{-1}, NaCl 5 g L^{-1} and NH_4Cl 5 g L^{-1} are dissolved in 800 mL distilled water. pH is adjusted to 7.2. Water is added to a final volume of 1L and is autoclaved at 121°C for 15 min.

IV.1.19.2. $MgSO_4$ (1M)

For 100 mL stock solution, 24.65 g $MgSO_4.7H_2O$ is dissolved in 87 mL water. It is autoclaved at 121°C for 15 min.

IV.1.19.3. $CaCl_2$ (1M)

For 100 mL stock solution, 14.70 g $CaCl_2.H_2O$ is dissolved in 94.5 mL water. It is autoclaved at 121°C for 15 min.

IV.1.19.4. Thiamin 1mg mL^{-1}

For 50 ml stock solution, 50 mg thiamin-HCl is dissolved in 45 mL distilled water. Water is added to a final volume of 50 mL. The solution is sterilized by filtration through a 0.2 µm filter.

IV.1.19.5. Biotin 1mg mL^{-1}

For 50 mL stock solution, 50 mg biotin is dissolved in 45 mL distilled water. Small aliquots of 1M NaOH were added until the biotin was dissolved. Water is added to a final volume of 50 ml. The solution is sterilized by filtration through a 0.2 µm filter.

IV.1.19.6. Trace elements solution (100X)

This solution is composed of: EDTA 5 g L^{-1}, $FeCl_3.6H_2O$ 0.83 g L^{-1}, $ZnCl_2$ 84 mg L^{-1}, $CuCl_2.2H_2O$ 13 mg L^{-1}, $CoCl_2.2H_2O$ 10 mg L^{-1}, H_3BO_3 10 mg L^{-1} and $MnCl_2.4H_2O$ 1.6 mg L^{-1}. To prepare this solution, 5g EDTA is dissolved in 800 mL of distilled water and the pH is adjusted to 7.5 with NaOH. Then the components are added and the water is added to a final volume of 1L. The solution is sterilized by filtration through a 0.22 µm filter.

IV.1.20. Recombination M9 solution

1L of M9 solution is composed by mixing the volumes as follows: 100 mL of M9 salt solution, 1 mL $MgSO_4$, 0.3 mL $CaCl_2$, 1 mL of biotin and thiamin and 10 mL of trace elements are added to 867 mL distilled and sterilized water. For preparing minimal medium containing sole carbon source, 2g L^{-1} of each source: glucose, sucrose, fructose, maltose and xylose as sugars and glutamic, malic, succinic, fumaric and acetic as organic acids. They are separately added to 1L volume of M9 medium. pH is adjusted to 7.0.

IV.1.21. Hoagland solution

Hoagland solution is prepared for plant growth. It is composed of macro and micro elements. For 1L of Hoagland solution the following compounds are added:
1 mL of 1M $NH_4H_2PO_4$, 6 mL of 1M KNO_3, 4 ml of 1M $Ca(NO_3)_2$, 2 mL of 1M $MgSO_4$, 1 mL of 2.86 g L^{-1} H_3BO_3, 1 mL of 1.81 g L^{-1} $MnCl_2$ $.4H_2O$, 1mL of 0.22 g L^{-1} $ZnSO_4$ $.7H_2O$, 1 mL of 0.08 g L^{-1} $CuSO_4.5H_2O$, 1 mL of 0.02 g L^{-1} $H_2MoO_4.H_2O$ and 0.25 mL of iron stock solution and

water is added to a final volume of 1L. The solution is sterilized by filtration through a 0.22 µm filter.

IV.1.22. Iron stock solution

This solution is prepared by dissolving 26.1 g EDTA in 286 mL water containing 19 g KOH. Then 24.9 g $FeSO_4.7H_2O$ is dissolved in 500 mL distilled water. The iron sulfate solution is slowly added to the potassium EDTA solution and is aerated overnight with stirring. The pH rises to about 7.1 and the solution is wine red and very little precipitation occurs. The volume is completed to 1 liter and stored in a bottle covered with foil (dark).

IV.2. Instruments used

IV.2.1. High performance liquid chromatography (HPLC)

The components of HPLC system include: the solvent reservoir, a high-pressure pump, a column, an injector system and a detector (figure 14). HPLC is a form of column chromatography that pumps a sample mixture or analyte in a solvent (mobile phase) at high pressure through a column with chromatographic packing material (stationary phase). The sample is carried by a moving carrier gas stream of helium or nitrogen. HPLC has the ability to separate and identify compounds present in any sample that can be dissolved in a liquid. HPLC is used in a variety of industrial and scientific applications, such as pharmaceutical, environmental, forensics and chemical. Sample retention time will vary depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s). As the sample passes through the column, it interacts between the two phases at different rate, primarily due to different polarities in the analytes. Analytes that have the least amount of interactions, with the stationary phase or the most amount of interaction with the mobile phase will exit the column faster.

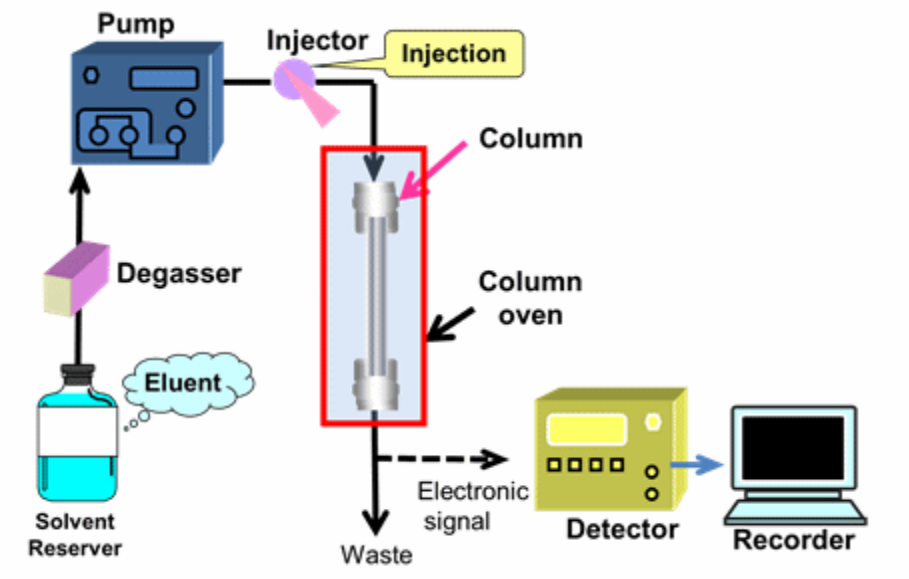


Figure 14 Components of HPLC

IV.2.2. MALDI-TOF mass spectrometry

This is an analytical technique to identify qualitatively and quantitatively the atomic and molecular composition of inorganic and organic materials, using mass-to-charge ratios and it recently emerged as powerful technique for identification of microorganisms (Piseth *et al*., 2010, Elena *et al*., 2014). MALDI-TOF MS can identify bacteria and fungi directly from colonies grown on culture plates in a few minutes and with simple procedures. Samples are prepared by mixing the analyte with a matrix made of small acid molecules that possesses a strong optical absorption in the range of the wavelength used by the laser device; DHB 2,5dihydroxybenzoic acid and CHCA α-cyano-4hydroxycinnamic acid are optimal matrices for the detection of lower mass ions. After co-crystallization of the sample and matrix, the latter absorbs energy from the laser, leading to the desorption and ionization of the analytes in the gas phase. Ions are then accelerated through an electrostatic field (created by a potential of about 20 kV) into the high vacuum flight tube until they reach the detector smaller ions travel faster than larger ones (figure 15). Thus, the time of flight (TOF) required to reach the detector is dependent on the mass and charge of the bioanalyte, resulting in an unique spectral profile for a given species, composed of peaks ranging usually from 2 to 20 kDa (Elena *et al*., 2014).

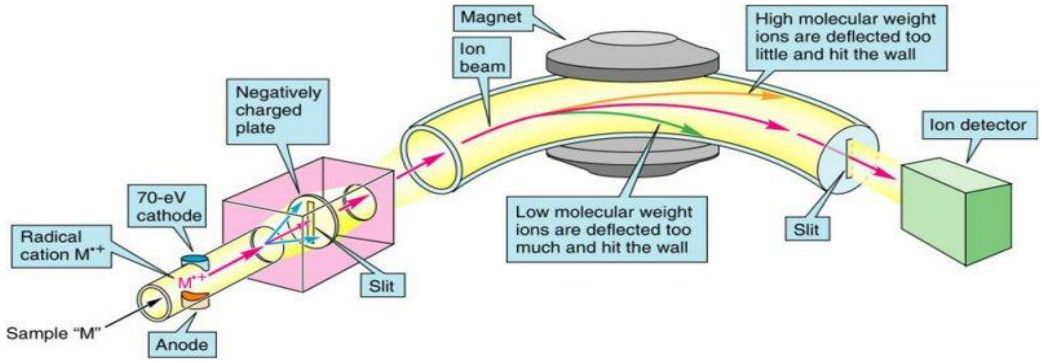


Figure 15 Principle of mass spectroscopy

IV.2.3. The polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was developed in 1983 by Kary Mullis (who earned his Bachelor of Science degree in chemistry from Georgia Tech in 1966) for which he earned the Nobel Prize in Chemistry in 1993. PCR (figure 16), uses an enzyme (polymerase) to replicate DNA regions of interest in a test tube by repeating the copying process. A small number of DNA molecules can be reliably increased up to billions within several hours. The resulting PCR products are then separated and detected. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications (Asadulla *et al*., 2015, Brown and Terry, 2006).

Figure 16 PCR instrument

IV.2.4. Gel electrophoresis

Gel electrophoresis is a technique by which the macromolecules, DNA, RNA and proteins are separated and analyzed base on their size and charge. It is used in clinical chemistry to separate proteins by charge and/or size (IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through a matrix of agarose (figure 17).

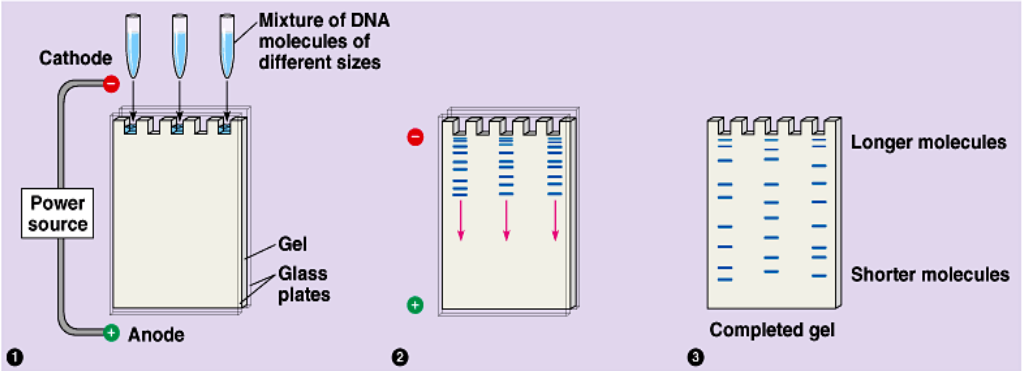


Figure 17 Schema explains gel electrophoresis

IV.2.5. Biolector system

Biolector is a microfermenter system providing different conditions of temperature, relative humidity, oxygen and shaking for the microorganism's growth (figure 18). With this system we studied the effect of root exudates and different carbon sources (sugars and organic acids) at a temperature of 21°C, a volume of 1200 µL and a shaking of 1200 rpm, using flower plate with 48 wells (figure 19). The results were analyzed using biolection program (http://www.m2p-labs.com).

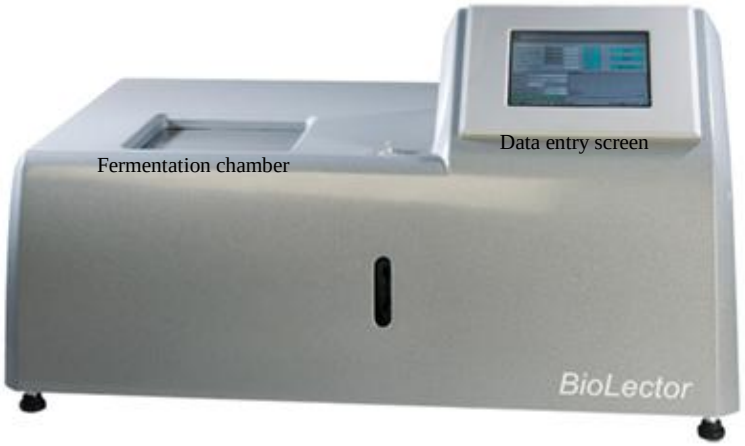


Figure 18 Biolector system

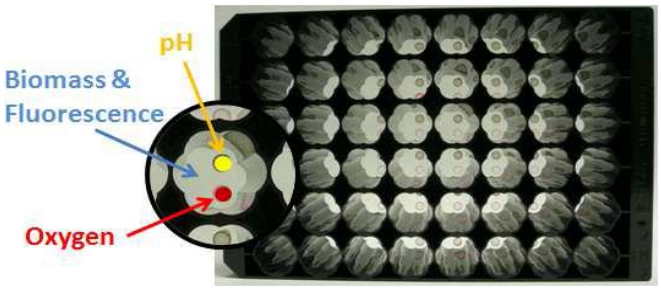


Figure 19 Flower Plate with different sensors (http://www.m2p-labs.com).

IV.3. Strains used in this study

Two strains were used in this comparative study. *Bacillus subtilis* BBG131 is derived from wild strain *Bacillus subtilis* 168 in the ProBioGem laboratory. This strain is a high producer of surfactin. The second strain is *Bacillus amyloliquefaciens* FZB42. It is a wild strain that produces three families of lipopeptides: bacillomycin, fengycin and surfactin but in small quantities. The inoculums are prepared from strains conserved at -80°C in 40% glycerol.

IV.4. Tomato seeds preparing for culture:

Tomato seeds *Solanum lycopersicum*, (Merveille des Marché cultivar) are used in this study for root exudates recovery and in the experiments of bacterial growth and lipopeptides production in the rhizosphere. At any experiment by which tomato seeds are used, the seeds are

surface sterilized by putting them in 70% ethanol for 2 min and in sodium hypochlorite for 15 min after ethanol removal and then the hypochlorite is removed and they are washed five times with distilled and sterilized water and then they can be directly used for the germination.

IV.5. Bacterial colonisation of tomato rhizosphere

Surface-sterilized and pregerminated tomato seeds are soaked for 10 min in bacterial cell suspensions of the strains in the concentration of 1×10^5 cells mL^{-1} at the exponential phase grown at 37°C in LB medium. After sterilization, the seeds are left for germination in petri dishes containing wet filter papers with Hoagland solution. The germinated seeds are placed into a sterilized glass tube containing 2 g of perlite, and 9 mL Hoagland solution. 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, 3 tubes are randomly chosen, aerial parts are removed and 10 mL of physiological solution is added to each tube. The tubes are vortexed at 2500 rpm for 5 min and then series of dilutions are prepared for bacterial plate count on LB agar. Results are expressed in total CFU per cm^3 of perlite (figure 20).

Figure 20 Tomato plants in rhizosphere (perlite).

IV.6. Kinetics of rhizosphere colonisation and surfactin production

Tomato seeds are prepared and grown as described earlier. Every three days, six samples of 3 treatments (sample without inoculum, sample inoculated with *B. amyloliquefaciens* FZB42 and sample inoculated with BBG131) were randomly taken. Three samples were used for plate count and three for surfactin quantification by HPLC.

IV.7. Extraction of surfactin from rhizosphere

For the study of surfactin production kinetics, every three days of the experiment, three tubes were randomly selected. The aerial parts of the plants were removed. Then, the surfactin was extracted from the perlite by adding 9 mL of acetonitrile/formic acid 0.1% and 2 g of glass beads to each tube. Tubes were vortexed at 2500 rpm for 5 min and then the tubes are incubated overnight at 30°C with agitation at 140 rpm min^{-1}. The tubes are centrifuged at 5000 g during 10 min. Surfactin is recovered by passing the supernatant through Solid-Phase Extraction Cartridges C18 (Altech Maxi-Clean) and the supernatants are vacuum dried (Speed Vac Plus, SC 110 A, Savant, GMI, Ramsey, USA). Dried residues are suspended in 200 µL of acetonitrile 80% formic acid 0.1% and HPLC is used for determination the lipopeptides after a filtration through 0.22 µm. The HPLC was a Waters (Online Degaser, 717 Autosample, 660S Controller, 626 Pump, 2996 PhotoDiode Array; Waters corporation, Milford, MA, USA). The column used was a C-18 (5 µm, 250 x 3 mm, VYDAC 218 TP53; Grace, Deerfield, Illinois, USA). The liquid phase was acetonitrile (0.1% trifluoroacetic acid) and double distilled water (0.1% trifluoroacetic acid), the volume of injection was 20 µL and the flow rate was 0.6 mL/min, table (1).

Table 1 Isocratic gradient for surfactin, fengycin and bacillomycin quantified by HPLC.

Time/min	Flow/min	ACN/TFA*	Water/TFA*
0	0.6	40	60
20	0.6	40	60
35	0.6	65	35
40	0.6	80	20
55	0.6	80	20
56	0.6	100	0
61	0.6	100	0
62	0.6	40	60
70	0.6	40	60

***ACN/TFA: Acetonitrile with 0.1% trifluoroacetic acid and Water/TFA water with 0.1% trifluoroacetic acid.**

IV.8. Sample preparation for microscope observation

The microscopic observation on tomato plant roots are done after 21 days of cultivation. The dye used is acridine orange at a concentration of 0.01% (w/v) prepared in 0.1 M acetate buffer, pH 4.0 (36 mL of 0.1 M sodium acetate mixed with 164 mL of 0.1 M acetic acid).

Several plants are randomly chosen. The aerial parts are removed and the roots are put in acridine orange for 5 minutes. The samples are protected from light during treatment with acridine orange. The roots are then fixed between slide and cover slip. The observation is done with a fluorescence microscope, Nikon EFD-3 with magnification X100. The images are obtained by using the Nikon DS-1 Fi camera connected to a computer.

IV.9. Root exudates collection

After germination, sterilized seeds are put in sterilized tubes containing Hoagland solution. The germinated seeds are left for growth at the condition of 8:16 (dark/light) hours of photoperiod and at room temperature (21°C). After 21 days, the root exudates are collected by

recovering all solutions from a hydroponic experiment. The solution was sterilized by filtration through a 0.22 µm filter and then concentrated and kept at -20°C until use (figure 21).

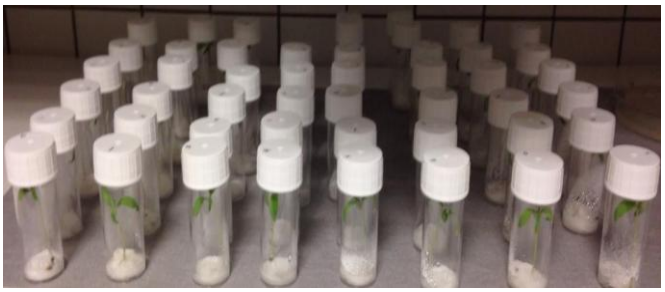


Figure 21 Sterilized culture tubes for root exudates collection.

IV.10. *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 growth on root exudates

The kinetic of bacterial growth in the presence of root exudates is studied by using the Biolector system, a micro system for fermentation, which provides different conditions for growth and measurement of different factors at the same time. 1200 microliters of root exudates were put in each well of the Biolector and the culture conditions were 21°C and 1200 rpm for 72 hours.

IV.11. Surfactin production with root exudates

Five mL of root exudates were inoculated with a bacterial suspension of *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 at 1×10^5 cells mL^{-1} at the exponential phase grown at 37°C and 160 rpm of agitation. After 72 h of incubation at 21°C and 160 rpm, series of dilutions were released for bacterial plate count. The growth in root exudates was compared with LB medium in the same conditions. Two mL of the 72h bacterial suspensions were taken and centrifuged at 10000 rpm during 30 min. The supernatants were recovered by passing through Solid-Phase Extraction Cartridges C18 (Altech Maxi-Clean) which were prepared by passing 20 mL of 100% methanol and then were washed with 8 mL of double distilled water. The supernatants were vacuum dried (Speed Vac Plus, SC 110 A, Savant, GMI, Ramsey, USA). Dried residues were suspended in 200 µL of 100% methanol and HPLC was used for determination of the surfactin after a filtration through a 0.22 µm filter as described earlier (section IV.7).

IV.12. Biofilm formation

To quantify *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 biofilm formation, the polyvinylchloride (PVC) microtiter plate assay with minor modifications was used, based on a procedure described previously (Hsueh et al., 2006). The strains were grown in LB medium until mid-log phase and the cells were collected by centrifugation and resuspended in minimal medium containing different sugars and organic acids. All carbon sources (2 g C L^{-1}) used were inoculated with the 1% of 10^5 of bacterial density. The microtiter plate was sealed with plastic wrap and incubated at 30°C without shaking for 48 h. The contents of each well were then removed and the well was washed five times with PBS 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 with 200 µL of 33% acetic acid and measured at $A_{590\ nm}$.

IV.13. *Bacillus amyloliquefaciens* FZB42 transformation

IV.13.1. Chemicals and standard procedures

All enzymes used for DNA manipulation such as restriction enzymes and DNA-ligase were purchased from Fermentas (WWW.fermentas.com).

For amplified PCR products, pGEM- T Easy vector was used from Promega (Madison, USA). The antibiotics used were sterilized by filtration through a 0.22 µm filter. The concentration of ampicillin sodium salt (Euromedex) was 50-100 µg mL^{-1} and of erythromycin (Sigma-Aldrich) was 20 µg mL^{-1}.

- Procedures used for DNA manipulations:

DNA digestions with restriction enzymes, preparing of recombinant plasmid DNA and cloning of DNA fragment were performed according to (Sambrook *et al*., 2003).

- Extraction of chromosomal DNA:

B. amyloliquefaciens FZB42 was grown overnight in LB medium at 37°C with agitation 160 rpm. Total genomic DNA was extracted of 1mL of fresh culture using Promega Kit « Wizard® Genomic DNA Purification Kit » (Promega Madison, USA) according to the procedure

supplied by the manufacturer and *Taq* polymerase "Arrow" from Qbiogene (Montreal, Canada) was used for PCR.

IV.13.2. Plasmid extraction

Five mL of an overnight fresh culture was used to extract the plasmid. The extraction was conducted according to the protocol supplied by the manufacturer of the «kit GeneJET Plasmid Miniprep ». After restriction enzyme digested, the fragments were purified in 0.72% agarose and then were extracted using «QIAquick Gel Extractin Kit». The purification was conducted according to the protocol supplied by the manufacturer.

IV.13.3. PCR reaction

The primers used for the amplification of the *epsA* gene were designed by the primer3 software on the basis of published genome of *B. amyloliquefaciens* FZB42 from NCBI site. The primers were synthesized by Eurogentec.

PCR product was amplified using the mixture of: PCR Master Mix (2X) 25µL, upstream primer (20 uM) 2.5 µL, downstream (20 µM) 2.5 µL, DNA template 5 µL and Nuclease-Free Water 15 µL.

PCR program was based on the Primer3 software results and consists of: predenaturation temperature 94°C for 3 min, 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 and then 5 min at 72°C. The PCR product was kept at 4°C.

IV.13.4. Cloning in plasmid

This technique is used for insertion of a DNA fragment into a vector. To obtain sufficient amount of the DNA fragment, it is first inserted into a commercial vector, pGEM- T Easy (figure 22) and this plasmid can replicate in *E. coli* JM109. After plasmid replication in *E. coli* JM109, it was extracted using «kit GeneJET Plasmid Miniprep ». The plasmid with fragment was digested using XmaIII and KpnI enzymes in order to insert in p-Mutin-GFP$^+$ plasmid (figure 23) and then it was replicated in *E. coli* JM109 before use in *B. amyloliquefaciens* FZB42 transformation.

The ratio 1/3 (vector/insert) was used for ligation and it is calculated as follows:

50 ng (vector) × (size of insert/size of vector) × 3 = ng of insert for a ratio of 1/3 (vector/insert). The volumes used for ligation were 2 µL ultra-pure water, 5 µL buffer (2x), 1 µL vector, 1 µL purified PCR fragment and 1µL ligase.

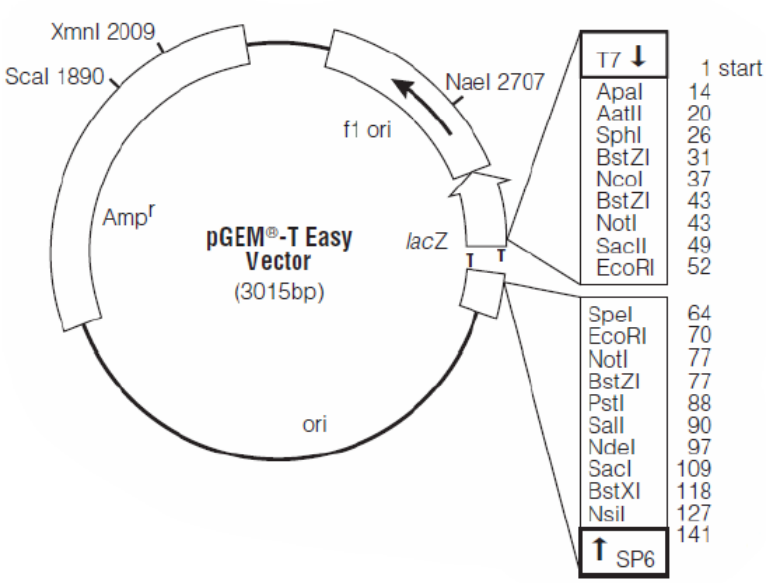


Figure 22 pGEM-T Easy vector circular map

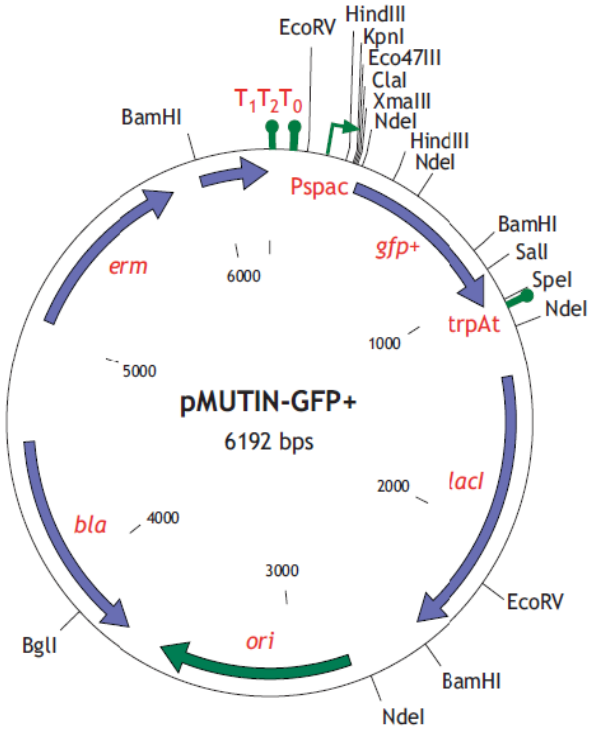


Figure 23 p-Mutin-GFP^{+} vector circular map

IV.13.5. Transformation of *E. coli*

To transfer plasmid into *E. coli* using thermal chock technique, tubes containing *E. coli* JM109 (competent cells stored at -80°C) are thawed on ice for 5 min. Then the ligation mixture is carefully added to 50 µL of the competent cells and gently flicks the tubes and incubated on ice for 20 min. They are exposed to 42°C for 90 sec in a water bath. Then they are immediately transferred on ice for 2 min. 950 µL of SOC medium was added and they were incubated at 37°C for 90 min with 150 rpm. The transformants were spread on LB medium containing the required antibiotics and they were incubated at 37°C for 24 h.

IV.13.6. *eps* fragment cloning in pGEM-T Easy and p-MUTIN- GFP$^+$ and transformation of *E. coli* JM109

A fragment from the *eps* operon (1390 bp) was amplified by PCR using the primers; forward: 5'GGTACCCTTTTCTTCTGCGG'3 and reverse: 5'CGGCCGGCTCGTTAAGAC'3 designed by both Primer3 (Version 4.0) and Amplifix programs, using chromosomal DNA from *B. amyloliquefaciens* 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 (1390 bp) was ligated to p-MUTIN-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 p-MUTIN-GFP$^+$::*epsA-C* was used to transform *B. amyloliquefaciens* FZB42 as following.

IV.13.7. Preparation and transformation of competent cells of *B. amyloliquefaciens* FZB42

To obtain competent cells of *B. amyloliquefaciens* FZB42 and to transform them we used the following protocol (Zhang *et al.* 2011; Cao *et al.* 2011):

1- An overnight LB culture of the *B. amyloliquefaciens* FZB42 cells was diluted 100-fold to NCM fresh medium.

2- Cell walls were awaked when the optical density reached to 0.5, by adding 3.89% glycine and 1.06% DL-Thyronine.
3- After 1 hour of shaking, the cells were cooled on ice for 20 min.
4- Cells were collected by centrifugation at 4°C and 8000 g for 5 min.
5- Cells were washed four times with ice-cold ETM buffer (0.5 M sorbitol, 0.5 M manitol and 10% glycerol), containing KH_2PO_4, K_2HPO_4, and $MgCl_2$ at the concentration 0.25, 0.25 and 0.5 mM respectively. pH was adjusted to 7.
6- The electro-competent cells were resuspended in 1/100 volume of the original culture.
7- 100 µL of the electro-competent cells were mixed with 100 ng of column-purified p-Mutin-GFP^+ plasmid.
8- The mix was loaded into a prechilled 1-mm gap electroporation cuvette.
9- The cuvette containing the mixture was briefly incubated on ice and then it was shocked by a single 2.1 kV cm^{-1} pulse generated with resistance and capacitance set at 200 Ω and 25 µf, respectively.
10- The cells were immediately diluted into 1 mL of recovery medium (growth medium containing 0.38 M manitol and 0.5 sorbitol)
11- Cells were resuspended in the recovery medium, were heated in water bath at 46°C for 6 min. Then, the cells were gently shaken for 3 h at 37°C.
12- Aliquots were spread onto LB medium agar plate containing erythromycin antibiotic (20 µg mL^{-1}).

IV.13.8. Verification of *B. amyloliquefaciens* FZB42 transformation

The transformants were grown overnight in LB medium and samples were analysed by fluorescence microscopy and compared with the wild-type strain. To ensure that *epsA* was integrated within the corresponding chromosomal locus of FZB42, a fragment of 1,917 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. Then they were analyzed by agarose gel electrophoresis analysis and thus, the 1,917 bp amplicon was observed only in the Em-R

transformant, confirming the microscopic observation of integration the *eps-gfp* fusion in the chromosomal DNA of this mutant.

IV.13.9. Gel electrophoresis quantification

Gel electrophoresis in agarose gel is conducted to quantify extracted chromosomal DNA or plasmidic DNA, by using a size marker to estimate concentration of genetic material in a sample. 0.7% agarose gels were used for fragments greater than 1kb and 1-1.5 % agarose for fragments smaller than 1 kb. Migration was done at 110 V during 1 h in TBE buffer. A mix of 10 µL composed of 3µL loading buffer (50% sucrose, 50 mM EDTA, 0.01% bromophenol blue and 4 M Urea) and 7 µL of samples were placed in each well. Marker used was O'GeneRuler 10000 kb DNA Ladder (Fermentas). The results were determined by GelDoc from Bio-Rad. Analysis was conducted using Quantity One software (version 4.1.1).

Chapter V

Results and discussion

V. Results and discussion

V.1. Kinetics of bacterial growth and lipopeptide production in the rhizosphere

This study was conducted on the basis of previous preliminary results which showed a significant difference between two species of bacteria: *Bacillus amyloliquefaciens* strains presented more biomass in the tomato rhizosphere than *Bacillus subtilis* strains (Deravel, 2011). In our study, this phenomenon has been studied in more detail.

B. amyloliquefaciens FZB42 and *B. subtilis* BBG131 strains were chosen as model strains for this study. *B. amyloliquefaciens* FZB42 is a co-producer of the three families of lipopeptides and well-known as biocontrol agent. *B. subtilis* BBG131 is a derivate of *B. subtilis* 168, a reference strain, which overproduces surfactin.

Germinated tomato seed were inoculated with *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 strains and they were put in a series of perlite cultures to study the growth kinetic and surfactin production in the tomato rhizosphere for 21 days. As these two strains are surfactin producers, the role of surfactin on rhizosphere colonisation has been studied.

The two strains showed distinct kinetics to colonize the rhizosphere (figures 24 and 25). The bacterial population of *B. amyloliquefaciens* FZB42 in the rhizosphere continuously increased from an initial population of 1×10^5 CFU cm^{-3} to 2×10^8 CFU cm^{-3} at the end of the experiment (21 days). The bacterial populations of *B. subtilis* BBG131 increased only during the first 3 days of the experiment. The population varied from 1×10^5 CFU cm^{-3} to 8×10^6 CFU cm^{-3}. The final rhizosphere population of *B. amyloliquefaciens* FZB42 was 25 times more than for *B. subtilis* BBG131 population. For both strains, the surfactin production followed the bacterial growth. Final surfactin concentration was 30 µg cm^{-3} for *B. subtilis* BBG131 and 6 µg cm^{-3} for *B. amyloliquefaciens* FZB42.

As it has been noted in earlier reports, rhizosphere colonisation by plant growth promoting bacteria is the most important step for the biocontrol agents (Weller *et al*. 2002; Vessey 2003; Pii *et al*., 2015). The first observation which can be stated is that *B. amyloliquefaciens* FZB42 is a better colonizer than *B. subtilis* BBG131 and these results may

lead to that: *B. amyloliquefaciens* FZB42 is more adaptable in the rhizosphere than *B. subtilis* BBG131. The results are completely different for surfactin production. *B. subtilis* BBG131 produces 125 times more surfactin per cell than *B. amyloliquefaciens* FZB42. This result shows that there is no direct correlation between colonisation and surfactin production.

The *B. subtilis* BBG131 had the highest biomass value at the third day and then the biomass was constant until the end of the experiment. The growth seems thus limited by one unknown factor (nutrient limitation or presence of a toxic compound) which is not the case for *B. amyloliquefaciens* FZB42.

The different behaviour between *B. amyloliquefaciens* and *B. subtilis* species in the rhizosphere cannot be only explained by the role of surfactin on rhizosphere colonisation. Thus, other factors such as root exudates and their composition were studied; they may have an important influence on this criterion.

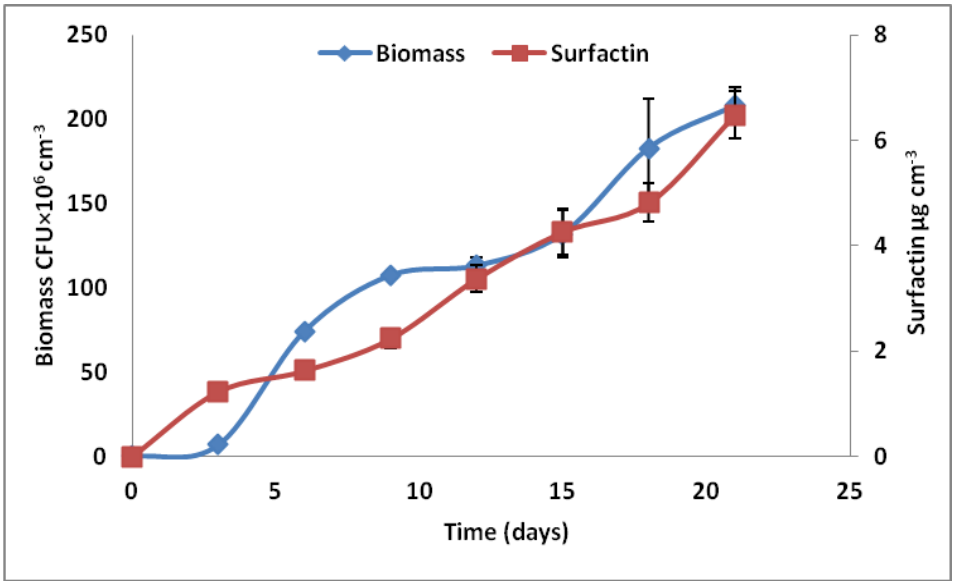


Figure 24 Kinetic of root colonisation and surfactin production by *B. amyloliquefaciens* FZB42 during 21 days in perlite tubes at room temperature.

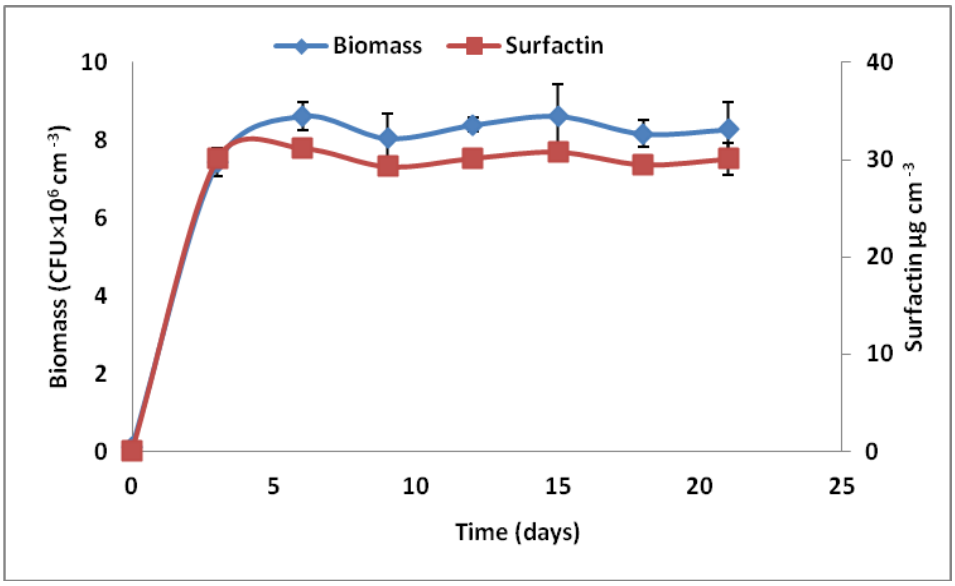


Figure 25 Kinetic of root colonisation and surfactin production by *B. subtilis* BBG131 during 21 days in perlite tubes at room temperature.

V.2. Microscope observation

Microscope observation of the colonisation process was performed. The images in figure 26 showed that, the *B. amyloliquefaciens* FZB42 strain (photo C) colonized almost all the tomato rhizoplane compared to the *B. subtilis* BBG131 (photo B). *B. subtilis* BBG131 strain colonized the rhizoplane by microcolonies and it appears as a longitudinal edge of the wall of root cells. The aggregates of colonies of *B. amyloliquefaciens* FZB42 and the separated cells of *B. subtilis* BBG131 confirm the results of differences of biomass population between the two strains in the rhizosphere (25:1).

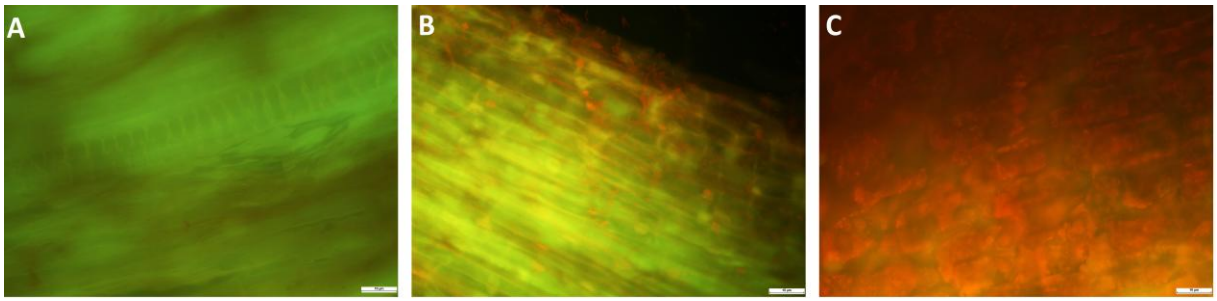


Figure 26 Example of tomato rhizoplaine colonisation by *B. amyloliquefaciens* FZB42 (C) and *B. subtilis* BBG131 (B) compared with control: non-inoculated sample (A). Bacterial cells were visualized under confocal laser scanning microscope using oily immersion with (100X) magnification (10 µm scale). The samples were treated with acridine orange dye.

V.3. Influence of root exudates

V.3.1. Effect of root exudates on growth of *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131

Plant root exudates are composed of a complex mixture of sugars, organic acid anions, nucleosides, phytosiderophores, vitamins, amino acids, purines, inorganic ions, volatile compounds, enzymes and root border cells which provide the main source of carbon and energy for microorganisms (Dakora and Phillipps, 2002). In this study, tomato root exudates were collected and used as a carbon source to stimulate the bacterial growth of *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131. The utilization of root exudates was evaluated in two experiments. The first one was performed to estimate bacterial growth using Biolector. The results obtained showed a big difference after three days between the two strains studied (figure 27). The optical density of *B. amyloliquefaciens* FZB42 reached approximately 1.4 after 72 h of incubation, while it was about 0.2 for *B. subtilis* BBG131. In the second experiment, the same root exudates were put in sterilized tubes, inoculated with the two strains and exposed to the same conditions used in the Biolector experiment. The population of bacterial cells was expressed as CFU per mL. The results confirm the results obtained in Biolector experiment. The CFU of *B. amyloliquefaciens* FZB42 was 2.5×10^{8} CFU mL^{-1} and 0.5×10^{8} CFU mL^{-1} for *B. subtilis* BBG131. The average proportion between the two strains was 5 times higher with *B. amyloliquefaciens* FZB42 than with *B. subtilis* BBG131.

This study represents the first comparative report on the use of tomato root exudates in the growth of *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 and it shows that the two strains were able to use the root exudates as a growth substrate. But *B. amyloliquefaciens* FZB42 growth is better than *B. subtilis* BBG131. The difference between the two strains may be due to the ability of *B. amyloliquefaciens* FZB42, more than *B. subtilis* BBG131, to consume the compounds in root exudates or it has less nutrient requirements than *B. subtilis* BBG131. These results are in agreement with those of Tan *et al*, (2013) who found that the root exudates from tomato plant support bacterial cell division and enhance the growth of *B. amyloliquefaciens*. In general, these results indicate the potential importance of tomato root exudates compounds to influence the bacterial growth and this in agreement with other studies (Baudoin *et al.,* 2003; Landi *et al.,* 2006; Paterson *et al.,* 2007; Henry *et al.,* 2008).

To put it another way, many biocontrol organisms grow very efficiently on root exudates (Lugtenberg *et al.*, 1999). In the present study, *B. amyloliquefaciens* FZB42 grew rapidly on root exudates collected from two tomato cultivars, which indicated that the root exudates provided growth factors as well as nutrient sources utilized by the bacteria. These results are consistent with the fact that some potential functional rhizospheric microbes can utilize the root exudates, such as *Pseudomonas putida* strain PCL1444 which was selected as an efficient consumer of the major exudates components for growth in *Barmultra* rhizosphere (Kuiper *et al*., 2002). Kamilova *et al*., (2005) also have reported that *Pseudomonas fluorescens* grew rapidly on tomato root exudates and controlled root rot in tomato plant. The ability to utilize root exudates has been identified as an essential quality for the *Pseudomonas* biocontrol bacteria to colonize the plant roots (Kravchenko *et al*., 2003; Lugtenberg *et al.*, 1999).

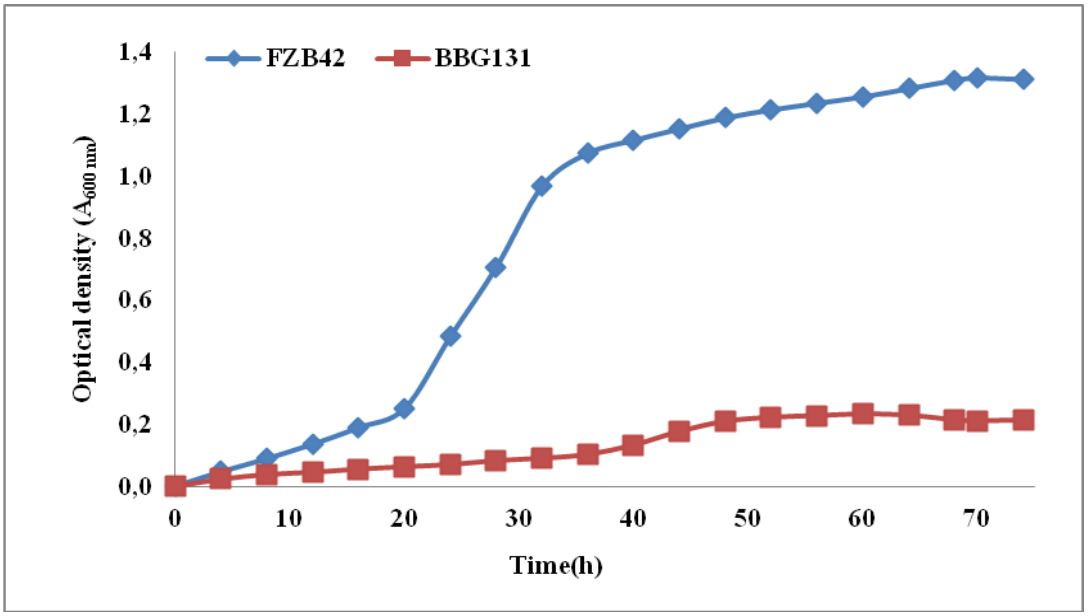


Figure 27 Effect of tomato root exudates on the bacterial growth of *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131. The culture was realized in concentrated tomato root exudates at 21°C and 1200 rpm during 72 h.

V.3.2. Effect of tomato root exudates on the production of lipopeptides

The production of lipopeptides by *B. amyloliquefaciens* strain FZB42 and *B. subtilis* strain BBG131 was tested in liquid culture with the tomato root exudates or LB medium or both.

The results of surfactin concentration produced by *B. subtilis* BBG131 (figure 28) were respectively 19.1, 289 and 338 mg L^{-1}, in the tomato root exudates, LB medium and LB medium with tomato root exudates. Whereas they were 10, 128 and 155 mg L^{-1} produced by *B. amyloliquefaciens* FZB42 in the same media (figure 30). *B. subtilis* BBG131 produces more surfactin than the wild strain *B. amyloliquefaciens* FZB42 in the three media (figure 29) *B. amyloliquefaciens* FZB42 produces also fengycin and bacillomycin in addition to surfactin (figures 30, 31, 32, 33, 34 and 35). The fengycin productions expressed as mg L^{-1} respectively were 3.11, 22.67 and 27.21 in the tomato root exudates, LB medium and LB medium with root exudates (figure 32), while the production of bacillomycin was: 1.6, 2.09 and 2.31 mg L^{-1} in the same media (figure 34). By the results of the two strains and all three lipopeptides produced, it seems that tomato root exudates have an important role as a carbon source inducing the lipopeptides production. It is also extremely clear that after adding the root exudates to the LB medium, the root exudates may have the elements responsible for inducing lipopeptides production.

More clearly, the results, showing the specific concentration of the three lipopeptides were 3.66, 38.49 and 44.20 µg 10^{-8} cells for surfactin produced by *B. subtilis* BBG131, respectively in the tomato root exudates, LB medium and LB medium with tomato root exudates (figure 29). With *B. amyloliquefaciens* FZB42, the concentrations were 0.39, 4.00 and 4.67 µg 10^{-8} for surfactin (figure 31), 0.12, 0.66 and 0.86 µg 10^{-8} cells for fengycin (figure 33) and 0.064, 0.065 and 0.069 µg 10^{-8} cells for bacillomycin (figure 35), respectively in the same media. As previously shown, the production of surfactin per cell is higher for *B. subtilis* BBG131 compared to *B. amyloliquefaciens* FZB42.

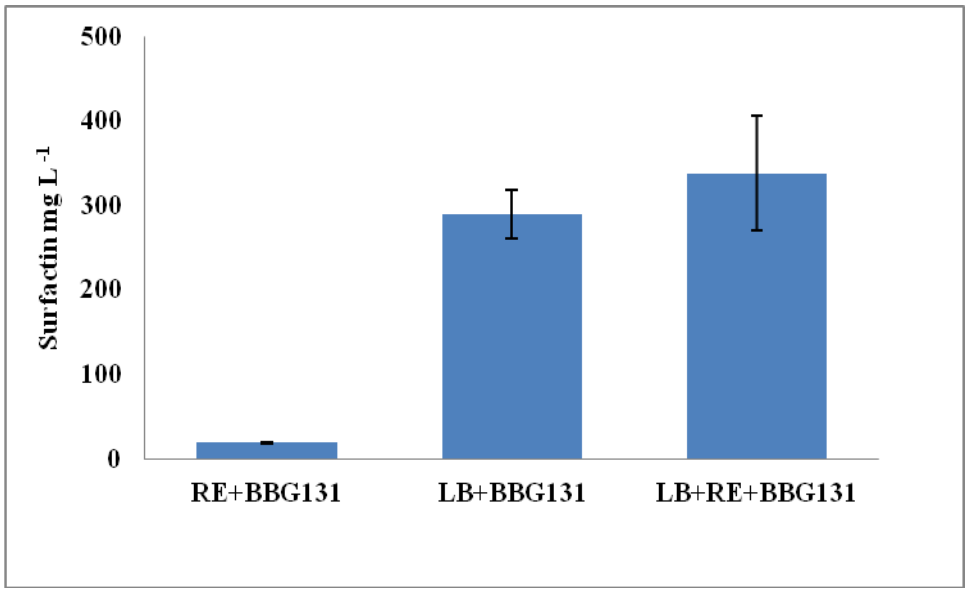


Figure 28 Effect of tomato root exudates on surfactin production by *B. subtilis* BBG131 as a compared with LB medium. Experimental conditions were: 21°C and 160 rpm for 72 h. LB; Luria Burtani medium, RE: tomato root exudates concentrated.

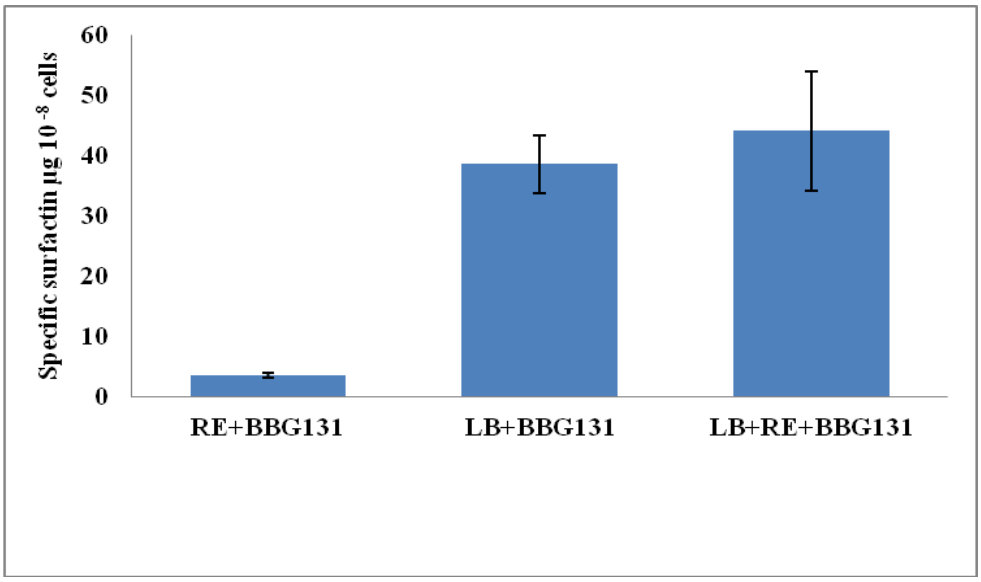


Figure 29 Specific surfactin calculated as µg 10^8 cells of *B. subtilis* BBG131. Experiment conditions were: 21°C and 160 rpm for 72 h. LB; Luria Burtani medium, RE: tomato root exudates concentrated.

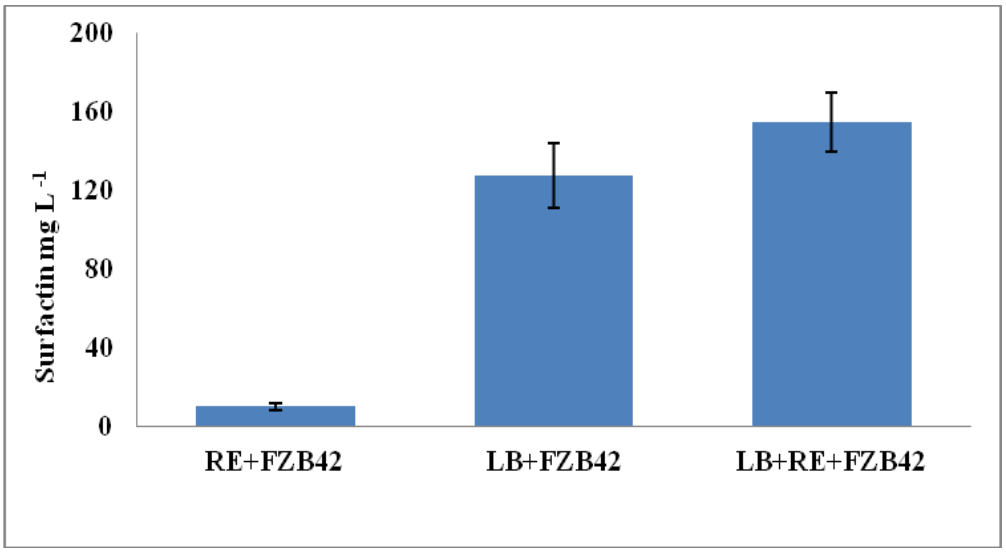


Figure 30 Effect of tomato root exudates on surfactin production by *B. amyloliquefaciens* FZB42 as a compared with LB medium. Experiment conditions were: 21°C and 160 rpm for 72 h. LB; Luria Burtani medium, RE: tomato root exudates concentrated.

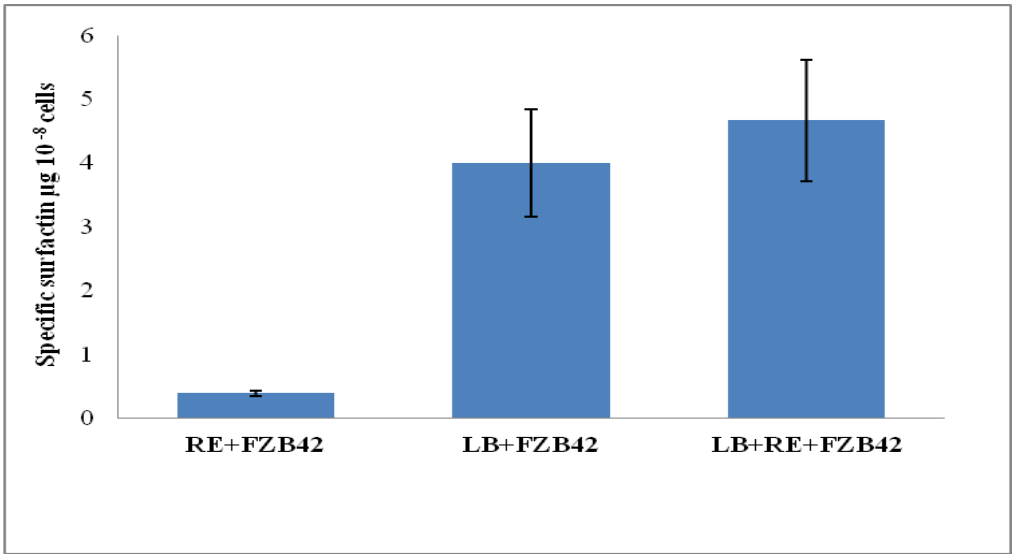


Figure 31 Specific surfactin calculated as µg 10^8 cells of *B. amyloliquefaciens* FZB42. Experiment conditions were: 21°C and 160 rpm for 72 h. LB; Luria Burtani, RE: tomato root exudates concentrated.

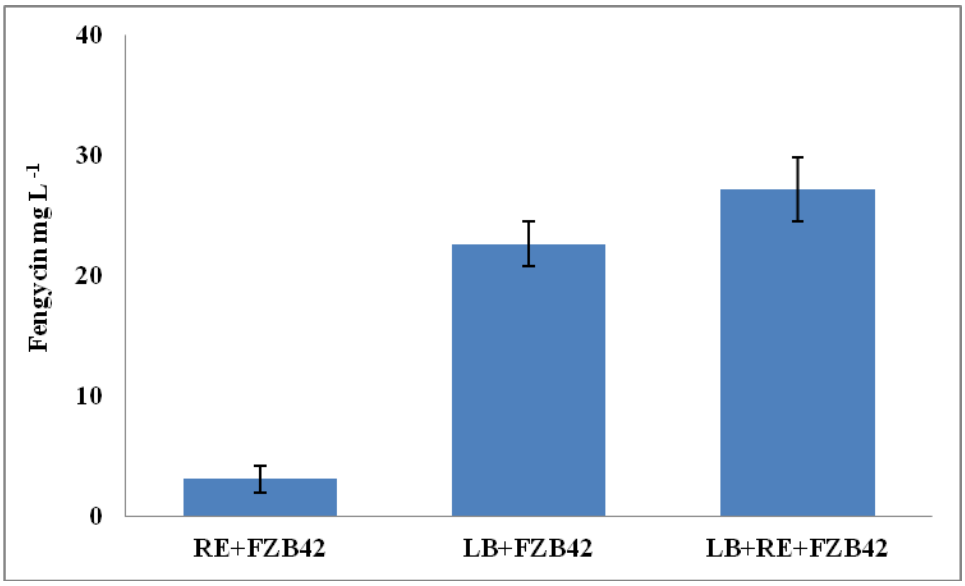


Figure 32 Effect of tomato root exudates on fengycin production by *B. amyloliquefaciens* FZB42 as a compared with LB medium. Experiment conditions were: 21°C and 160 rpm for 72 h. LB; Luria Burtani medium, RE: tomato root exudates concentrated.

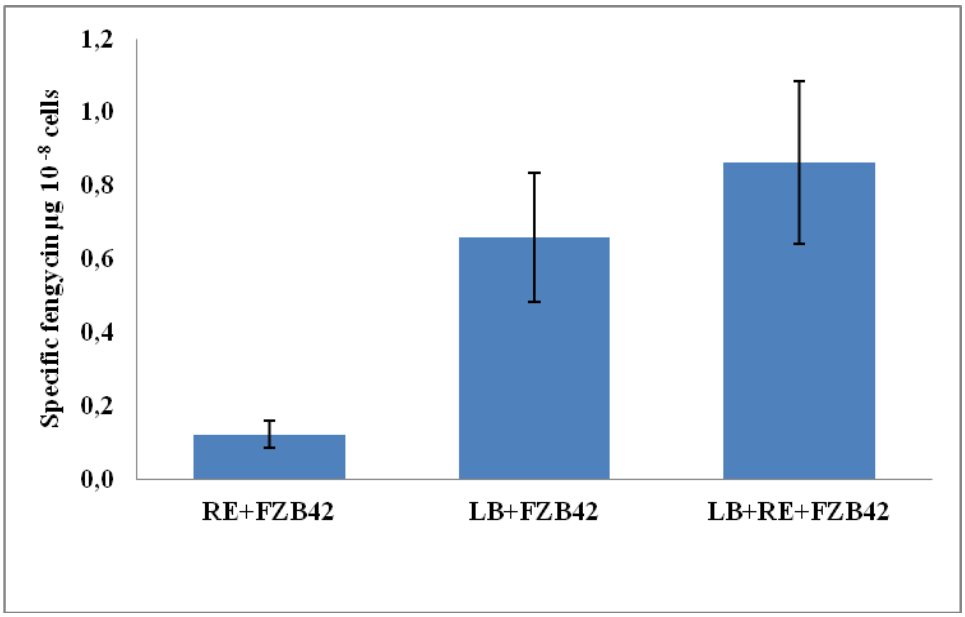


Figure 33 Specific fengycin calculated as µg 10^8 cells of *B. amyloliquefaciens* FZB42. Experiment conditions were: 21°C and 160 rpm for 72 h. LB; Luria Burtani medium, RE: tomato root exudates concentrated.

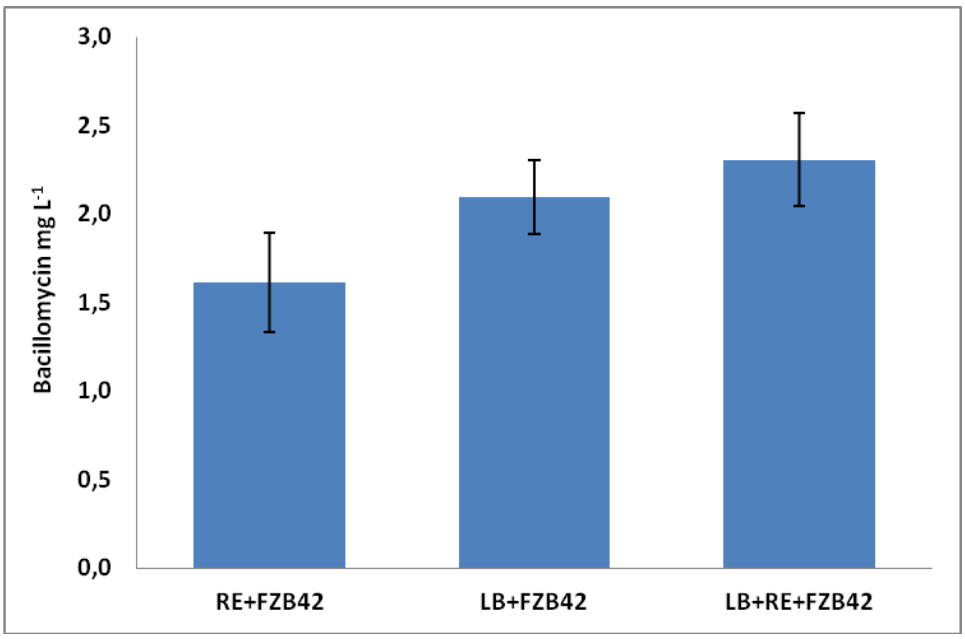


Figure 34 Effect of tomato root exudates on bacillomycin production by *B. amyloliquefaciens* FZB42 as a compared with LB medium. Experiment conditions were: 21°C and 160 rpm for 72 h. LB; Luria medium, RE: tomato root exudates concentrated.

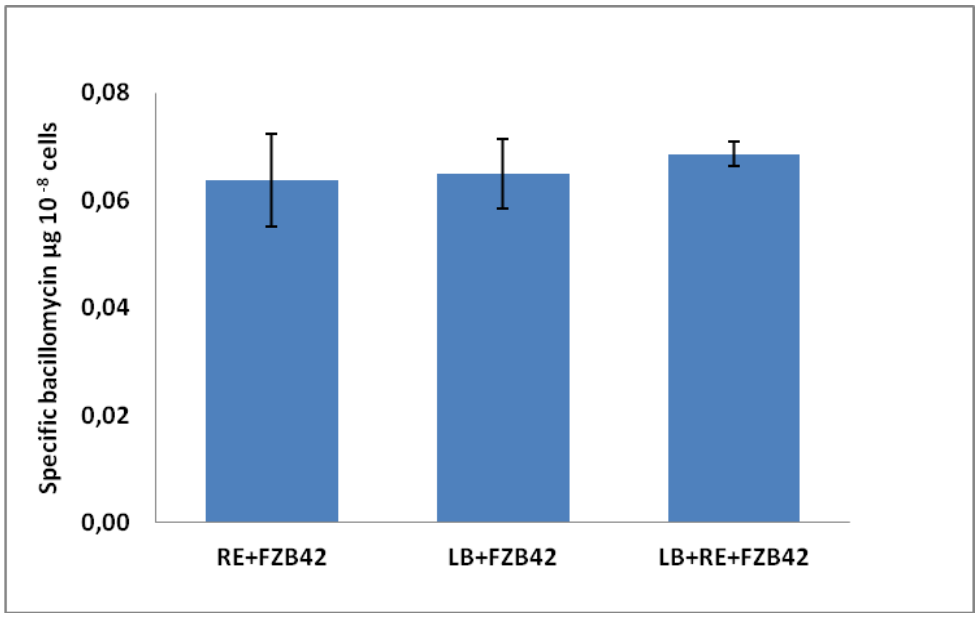


Figure 35 Specific bacillomycin calculated as µg 10^8 cells of *B. amyloliquefaciens* FZB42. Experiment conditions were: 21°C and 160 rpm for 72 h. LB; Luria Burtani medium, RE: tomato root exudates concentrated.

The utilization of root exometabolites by plant growth–promoting rhizobacteria may influence their growth and lipopeptide production. There are arguments to believe that the antifungal activity of rhizobacteria introduced into the plant rhizosphere depends on the sugar

and organic acid composition of the root exudates of these plants and those observations show that the ability of rhizobacteria to colonize the rhizosphere may be related to their ability to utilize the root exudates. This was reported in different studies which explain that the antifungal activity and growth depend on the root exometabolites (Kravchenko *et al*., 2003; James and Gutterson, 1986; Gutterson, 1990). The rhizosphere effect is thought to be caused by the root exudates-dependent growth of rhizosphere microorganisms which represent the primary sources of carbon and energy (Xiao *et al.*, 2015; Dennis *et al*., 2010; Baudoin *et al*., 2003; Hirsch, 2013; Lugtenberg, 1999).

V.4. Influence of different nutrients on Bacillus growth

The stimulation of *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 growth using tomato root exudates raises the question which components of the exudates are the primary source of carbon and energy. Hence, a new experiment was conducted to try to more deeply understand the results obtained in the previous experiment. In order to easily check the influence of the different organic compounds of the tomato root exudates on *Bacillus* growth, the Biolector was used. A first set of experiments was performed on different sugars: glucose, fructose, maltose, xylose and sucrose and a second one on different organic acids: succinic, fumaric, malic, oxalic and citric and one amino acid: glutamic acid. The optimal growth was different in function of the carbon sources and the time for both strains. The results in figure 36 show that the optical density of these media inoculated with *B. amyloliquefaciens* FZB42 was 0.906, 0.718, 0.687, 0.390 and 0.249 for maltose, glucose, sucrose, fructose and xylose respectively and that the optimal growth was obtained after 18h for glucose and sucrose, 24h for maltose and 30h for fructose and xylose. The results of *B. subtilis* BBG131 showed an optical density of 0.728, 0.663, 0.630 and 0.227 for glucose, maltose, fructose and sucrose respectively. A little growth was observed for this strain on xylose (Figure 37). *B. subtilis* BBG131 took more time than *B. amyloliquefaciens* FZB42 to reach its maximal growth in sugars: 24h, 30h, 48h for glucose, sucrose and maltose, respectively and 42h for fructose.

According to the results obtained, it seems that the two strains are different in their behaviour as regards of the consumption of sugars as an energy source. *B. amyloliquefaciens* FZB42 was able to metabolize more different sugars than *B. subtilis* BBG131. The higher growth

of *B. amyloliquefaciens* FZB42 was obtained with maltose while it was with glucose for *B. subtilis* and the metabolism of glucose took a shorter time. Generally, *B. amyloliquefaciens* FZB42 grew more with maltose, sucrose, and xylose than *B. subtilis* BBG131, while they were almost close in growth when using glucose as a source of carbon.

The results with organic acids showed that such carbon sources presented a lower growth than with sugars for both strains. The values of optimal growth estimated as optical density for *B. amyloliquefaciens* FZB42 were 0.597, 0.470, 0.383, 0.350, 0,238 and 0.163 (figure 38) for glutamic, malic, fumaric, succinic, citric and oxalic acids respectively and also this optimal growth were in different times: 24h for malic and oxalic acids, 18h for glutamic, fumaric and citric acids. Succinic acid took more time than others to be metabolized. *B. subtilis* BBG131 took more time than *B. amyloliquefaciens* FZB42 to metabolize organic acids. There is no significant difference between them in term of optimal density except with malic acid. The values of optical density at the optimal growth were 0.448, 0.394, 0.384, 0.143, 0.114 and 0.106 (figure 39) and these took 42, 48, 24, 48, 30 and 48 hours for malic, succinic, glutamic, fumaric, citric and oxalic acids, respectively.

As for the results of sugars and organic acids, it appears that *B. amyloliquefaciens* FZB42 is able to consume the sugars and organic acids faster than *B. subtilis* BBG131, which is reflected in their growth. These results, could possibly explain the results obtained in root exudates assay. In addition, *B. amyloliquefaciens* FZB42 is able to metabolize xylose and fumaric acid which seems less easy for *B. subtilis* BBG131. They were in agreement with the study of Tan *et al*., (2013) which indicated that the composition of tomato root exudates significantly promoted the strain *B. amyloliquefaciens* T-5 and, on the other hand, the root exudates excreted from *Arabidopsis* roots selectively signaled and induced the beneficial rhizobacterium *B. subtilis* FB17, whereas oxalic acid exhibited significantly reduced efficiency (Rudrappa *et al*., 2008). The same observation was seen with *Pseudomonas polymyxa* SQR-21, which growth was significantly promoted by malic and citric acids from watermelon roots while oxalic acids showed a contrast (Ling *et al*., 2011).

Glucose, fructose, and xylose were the major sugars detected in the tomato root exudates (Vancura and Hovadik, 1965; Vancura and Hanzlikova, 1972). Glucose and fructose remained the major components in all growth stages of tomato while the percentage of xylose dropped dramatically in root exudates. This work explained the differentiation in the ability to

use different sugars as carbon sources depending on the bacteria species and the type of sugars and these results are corroborated by previous works (Russel and Baldwin, 1978; Kamilova, 2006; Lugtenberg, 1999).

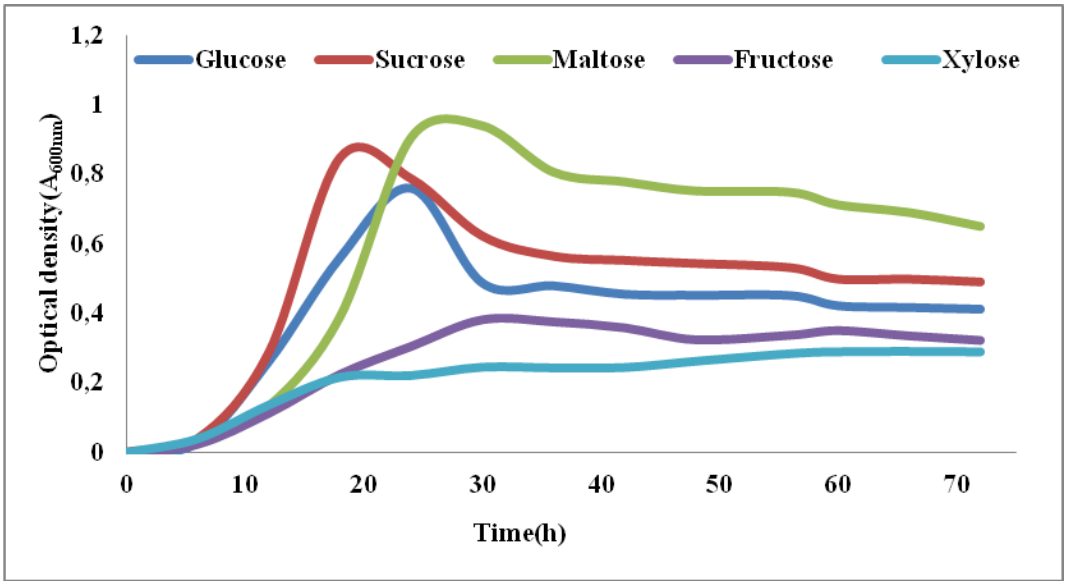


Figure 36 Effect of sugars on the growth of *B. amyloliquefaciens* FZB42. Two g of each sugar (C-equivalent) was dissolved in minimum media. Cultures were incubated at 21°C and 1200 rpm for 72 h.

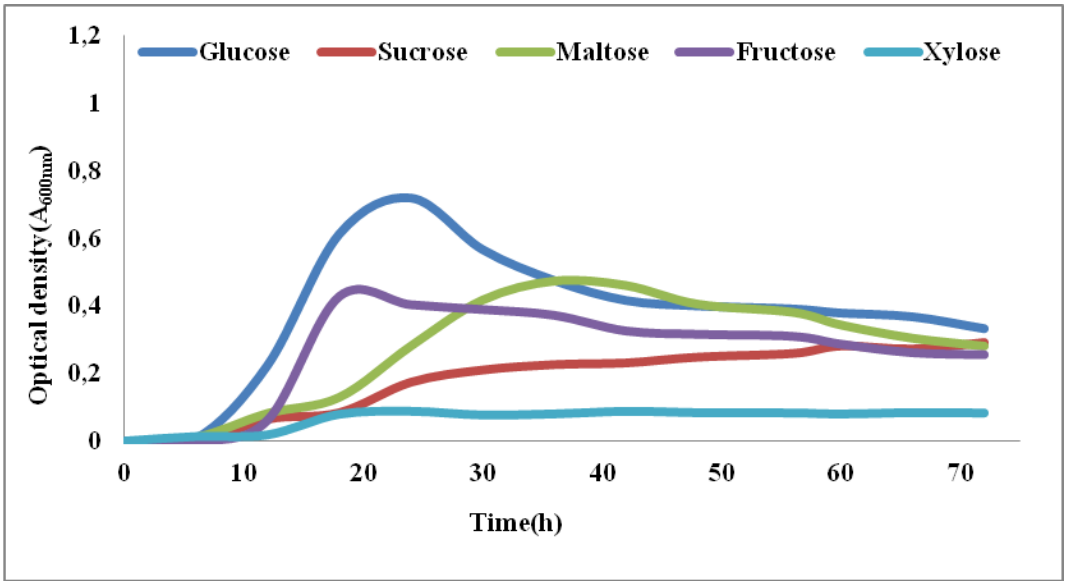


Figure 37 Effect of sugars on the growth of *B. subtilis* BBG131. Two g of each sugar (C-equivalent) was dissolved in minimum media. Cultures were incubated at 21°C and 1200 rpm for 72 h.

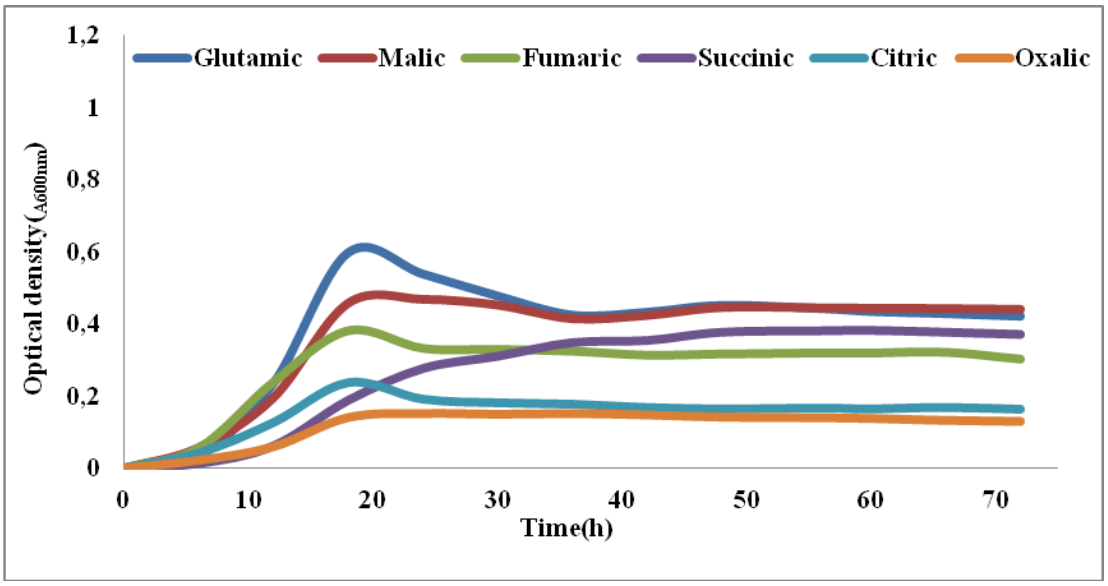


Figure 38 Effect of organic acids on the growth of *B. amyloliquefaciens* FZB42. Two g of each organic acid (C-equivalent) was dissolved in minimum media. Cultures were incubated at 21°C and 1200 rpm for 72 h.

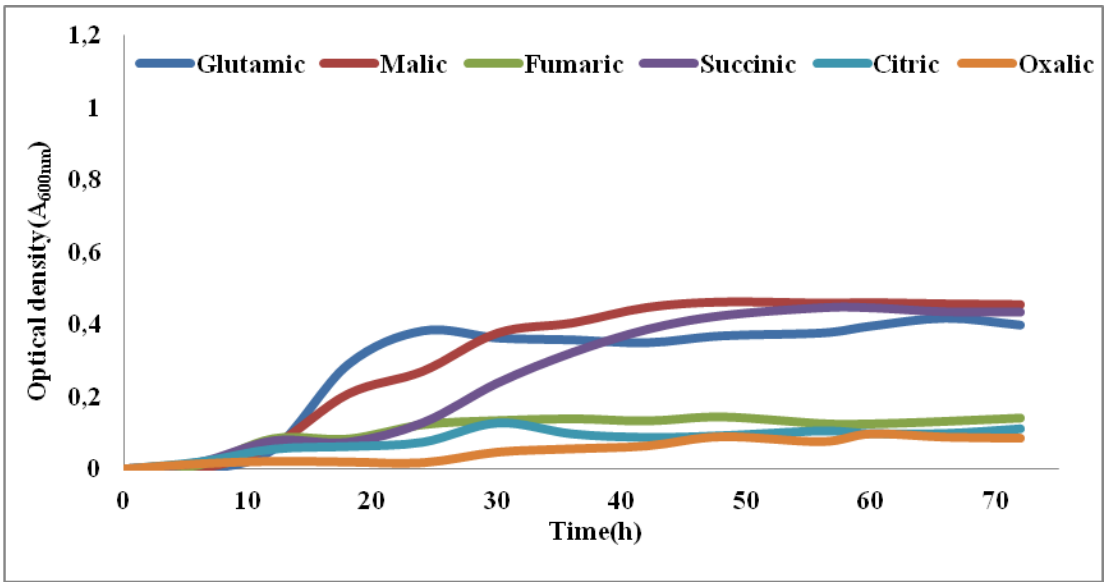


Figure 39 Effect of organic acids on the growth of *B. subtilis* BBG131. Two g of each organic acid (C-equivalent) was dissolved in minimum media. Cultures were incubated at 21°C and 1200 rpm for 72 h.

V.5. Effect of different sugars on bacterial growth and lipopeptide production

The volumes of culture media used in Biolector experiments were too low to use them in the lipopeptides quantification assay. Therefore, experiments with larger volumes were conducted based on the results obtained in the Biolector experiments with tomato root exudates and different carbon sources. These carbon sources were thus tested for their effect on the bacterial growth and the production of lipopeptides by both strains. The results in (figure 40) showed a distinct behaviour of both strains in growth and surfactin production. The values of biomass were calculated as **"**CFU×10^8 mL^{-1}**"** and were 2.04, 2.15, 1.85, 2.27 and 1.20 for *B. amyloliquefaciens* FZB42 and 0.88, 0.68, 0.65, 0.64 and 0.06 for *B. subtilis* BBG131 (figure 40), respectively for glucose, sucrose, fructose, maltose and xylose. For all carbon sources, *B. amyloliquefaciens* FZB42 biomass was higher than for *B. subtilis* BBG131. It approximately was 3 times higher with all sugars tested excepted xylose for which it was 20 times higher. All these values are in harmony with previous results (Biolector experiments).

The surfactin production by *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 was also influenced by the different sugars. Surfactin concentrations were high with glucose, sucrose and fructose used as a carbon source by *B. amyloliquefaciens* FZB42 whereas *B. subtilis* BBG131 presents high surfactin production with glucose and sucrose compared to maltose and xylose (figure 41). Surfactin concentration produced by *B. subtilis* BBG131 in the medium containing glucose was approximately 3.5 higher than that one of *B. amyloliquefaciens* FZB42. In the medium containing fructose, the results showed a contrast: *B. amyloliquefaciens* FZB42 produced more surfactin than *B. subtilis* BBG131. *B. amyloliquefaciens* FZB42 was able to produce surfactin using all sugars while surfactin produced by *B. subtilis* BBG131 was not detected in the medium containing maltose or xylose.

The concentrations of fengycin and bacillomycin produced by *B. amyloliquefaciens* FZB42 in the media containing glucose, sucrose, fructose, maltose or maltose were respectively 7.78, 15.3, 15.11, 3.18 and 0.86 and 3.24, 5.64, 3.07, 1.82 and 0.37 mg L^{-1} (figure 43). The production of fengycin was higher with sucrose and fructose than glucose and maltose. It was very low with xylose. Accordingly to the results of bacillomycin, the highest value was 5.64 mg L^{-1}, when sucrose was used as a carbon sources whereas, the used of xylose as a carbon sources showed the lowest concentrations (0.37 mg L^{-1}). In general, it can be said that lipopeptides can

be produced with significant quantity when glucose, sucrose are used as carbon and energy sources for both strains.

For a clear comparison between the two strains, the results were calculated as specific production (µg 10^{-8} Cells). The specific values of surfactin were 424, 500 and 8.91 µg 10^{-8} cells of *B. subtilis* BBG131, respectively for glucose, sucrose and fructose and they were very high when compared to surfactin produced by *B. amyloliquefaciens* FZB42 in glucose and sucrose (figure 42). The concentrations of surfactin with these carbon sources were respectively 63.58 and 31.98 µg 10^{-8} cells. The specific concentration of fengycin was higher with fructose and sucrose than other sources (figure 44). On the other hand, the highest concentration of iturin was 2.67 µg 10^{-8} cells with sucrose and as usually the lowest concentration was obtained with xylose. In general, these results and the previous results obtained in the Biolector and root exudates experiments supported that *B. amyloliquefaciens* FZB42 has more ability to consume all the carbon sources than *B. subtilis* BBG131. Those observations confirm the ability of *B. amyloliquefaciens* FZB42 to use the components of the root exudates which may be reflected to its ability to be a perfect root colonizer.

There is also a strong influence of carbon sources on lipopeptide production. As previously shown, the strain *B. subtilis* BBG131 shows a specific surfactin production which is, on average, 10 times higher than this of *B. amyloliquefaciens* FZB42.

As it can be seen, carbon sources may affect the synthesis of lipopeptides by bacteria. For instance, Sign *et al*., (2014) showed that the carbon source had a significantly influenced on the lipopeptide production and their antifungal activity, in addition, the data observed by Kravchenko *et al*., (2003) indicated that the antifungal activity of rhizobacteria depends on the sugar and organic acid composition of the root exudates of the plants. Our study indicated that the different carbon sources have a great influence on the growth and the lipopeptide production. Apparently, xylose is the worst usable sugar, while glucose and maltose are the best. It could be interesting in the future study to find some explanations for these differences. This could be performed by physiological and eventually genetic studies to see the link between the genotype and the use of these different carbon sources. The obtained results could also give some informations to explain the different behaviour of the 2 strains regarding the use of the carbon sources.

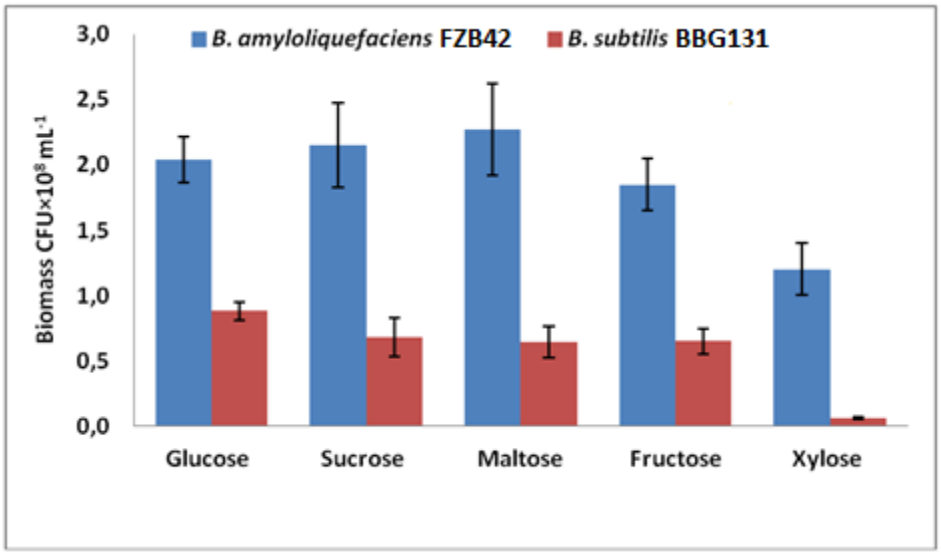


Figure 40 Biomass of *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 grown in different sugars used as carbon source.

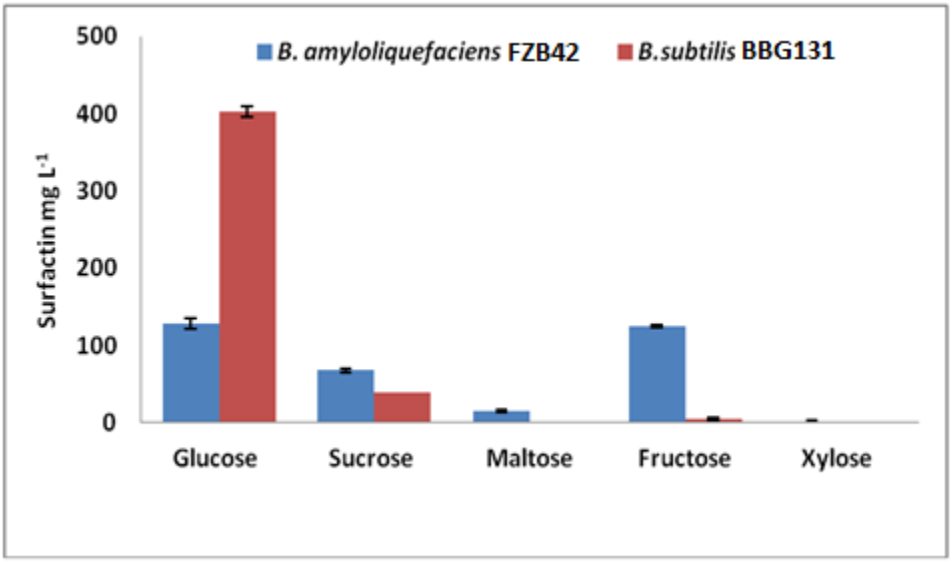


Figure 41 Surfactin produced by *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 in different sugars used as carbon source.

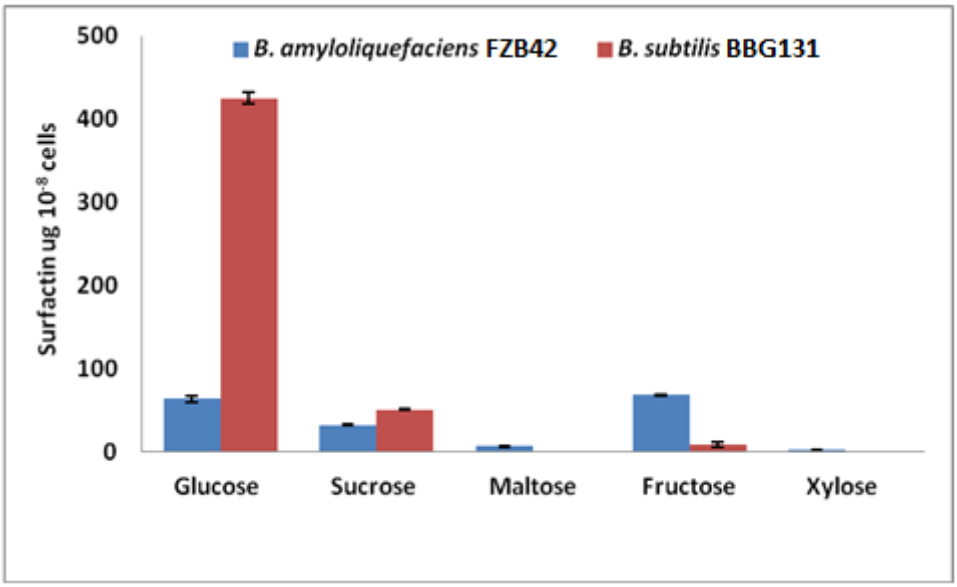


Figure 42 Specific surfactin produced by *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 in different sugars used as carbon source.

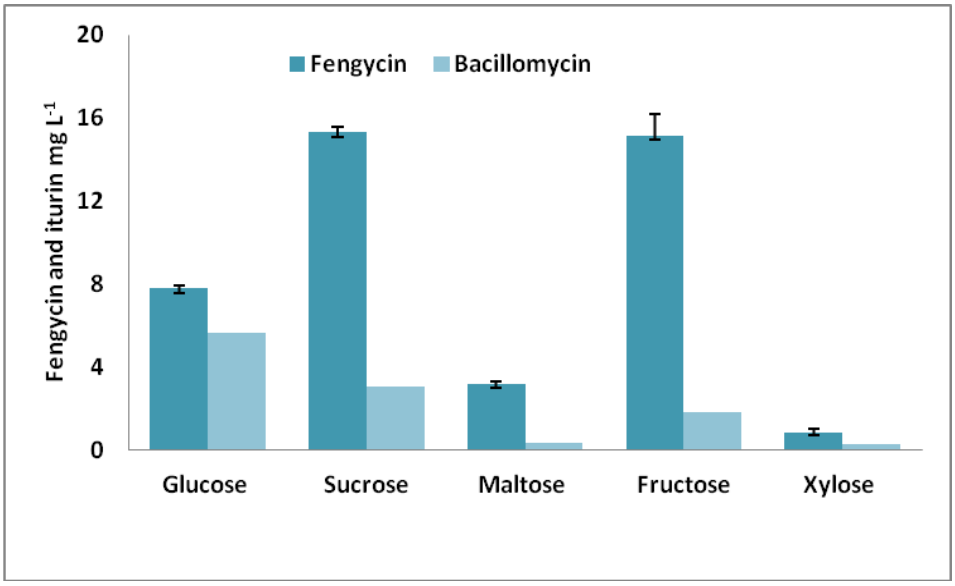


Figure 43 Fengycin and bacillomycin produced by *B. amyloliquefaciens* FZB42 in different sugars used as carbon source.

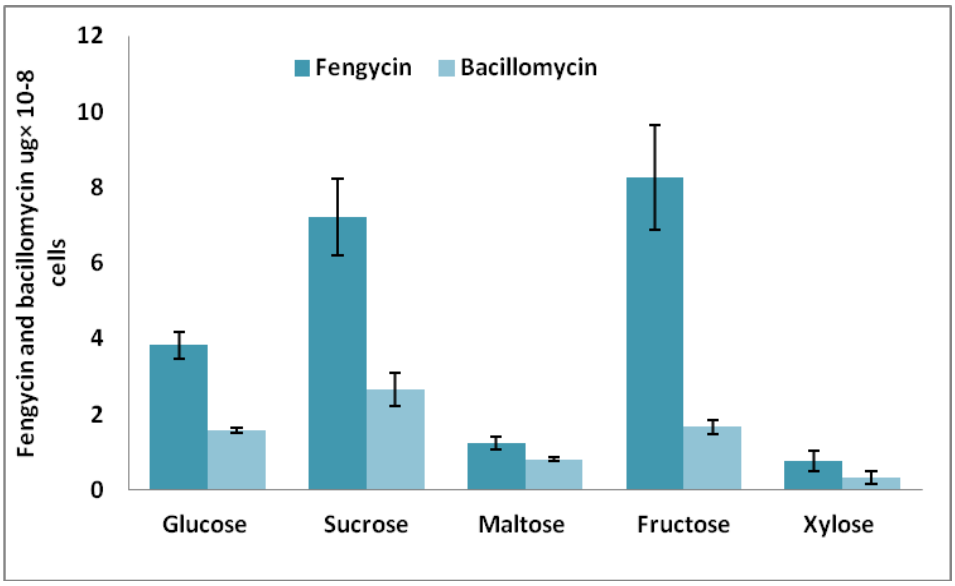


Figure 44 Specific fengycin and bacillomycin produced by *B. amyloliquefaciens* FZB42 in different sugars used as carbon source.

V.6. Influence of carbon sources on biofilm formation by *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131

The proportion of biomass between *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 in the rhizosphere was 25/1, while it was 6/1 when cultivated on the root exudates. Therefore, the rhizosphere colonisation explained by the role of tomato root exudates as a carbon source is not sufficient. Thus, we proposed to study the role of biofilm formation in the rhizosphere colonisation, based on the results obtained in the kinetic experiments: *B. amyloliquefaciens* FZB42 appeared on the tomato root as aggregated cells while *B. subtilis* BBG131 appeared as separated cells.

Bacteria interact physically with plants in various ways. All these interactions commonly appear as colonisation of root and rhizosphere. The bacteria adhere to the external part of plant tissues as individual and aggregated cells. The aggregated cells are defined as biofilms and they display a range of dimensions, locations and compositions (Nongkhlaw *et al*., 2014). The plant microenvironment has different characteristics such as saturation levels, nutrient availabilities and surface chemistries, which are strongly influenced by the form and the activity of biofilms (Ramey *et al*., 2004).

This experiment was performed to study the effect of root exudates and their composition on the biofilm formation by *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131. The results showed a big difference between the two strains. *B. amyloliquefaciens* FZB42 showed a high ability to form the biofilm with LB medium and the root exudates (figure 45). It is important to realize that the biofilm formation had a significant difference with root exudates than with LB medium. The carbon sources (glutamic, glucose, malic and succinic acids) showed a higher ability to form biofilm by *B. amyloliquefaciens* FZB42 than the other carbon sources (figures 46 and 47). On the contrary, *B. subtilis* BBG131 was not able to form a biofilm with all the carbon sources

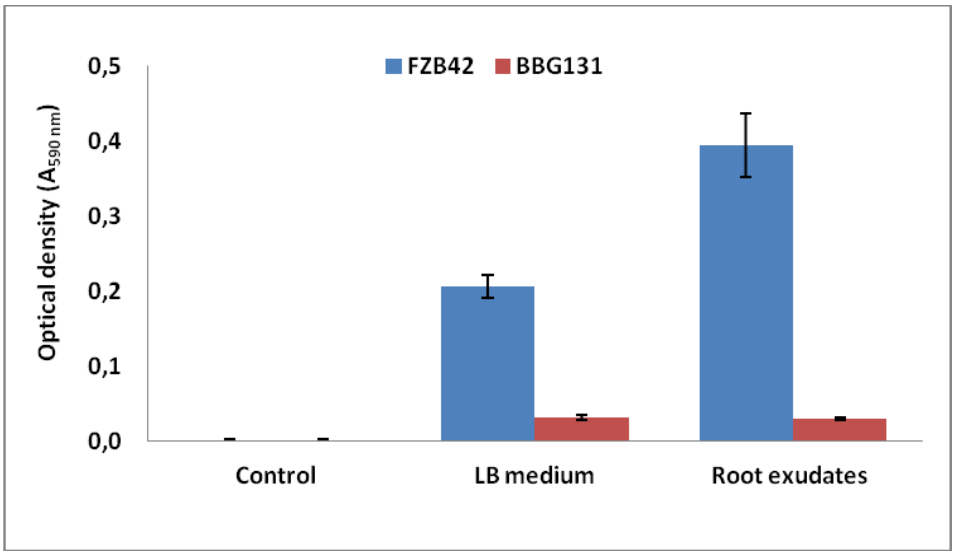


Figure 45 Biofilm formation by *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131. The biofilm was stained with crystal violet after 72 h of incubation at 21°C.

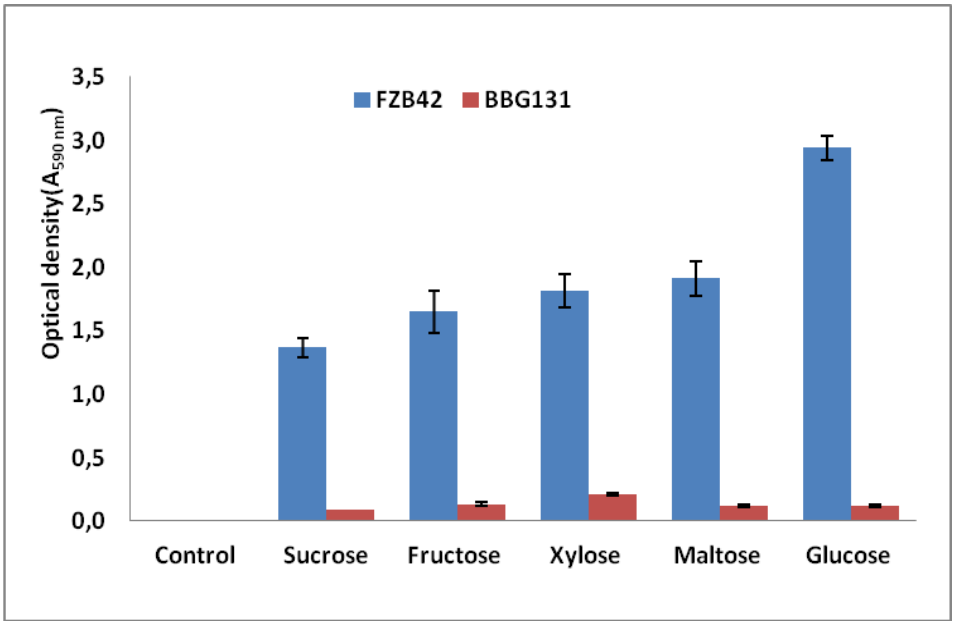


Figure 46 Biofilm formation by *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131. The biofilm was stained with crystal violet after 72 h of incubation at 21°C. The sugars were dissolved in minimum medium at the concentration equivelant to 2 g carbon atoms per liter.

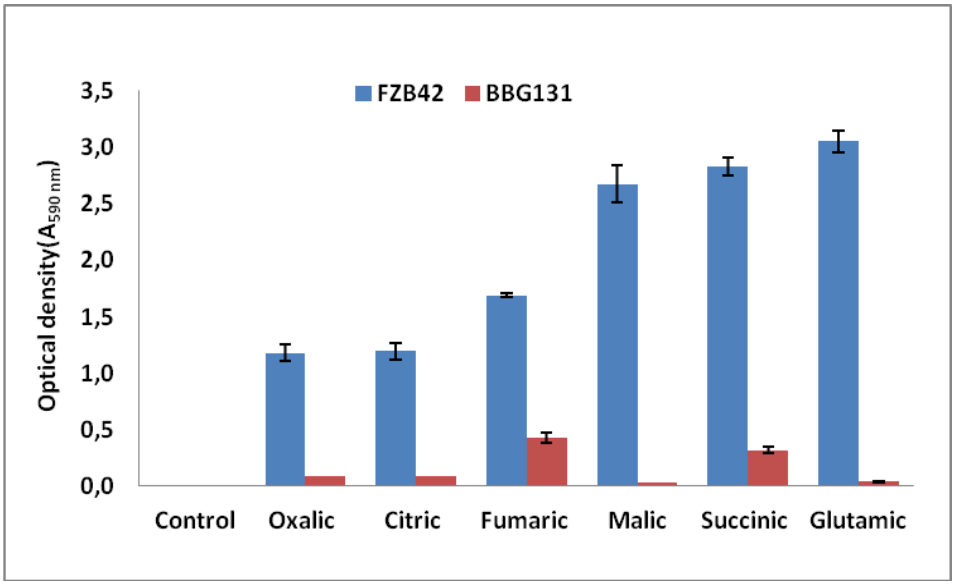


Figure 47 Biofilm formation by *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131. The biofilm was stained with crystal violet after 72 h of incubation at 21°C. The acids were dissolved in minimum medium at the concentration equivelant to 2 g carbon atoms per liter.

V.7. *In vitro* biofilm comparative assays with *B. amyloliquefaciens* FZB42, its derivative deficient in exopolysaccharide production and *B. subtilis* BBG131

V.7.1 Interruption of *epsA* in *B. amyloliquefaciens* FZB42 using fusion with *gfp* gene marker

Based on the results obtained from *in vitro* and *in vivo* experiments, taking into account the importance of exopolysaccharides in biofilm formation, previous reports clearly indicated that mutants which are unable to synthesize EPS are 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). Thus, the following experiment was conducted for the purpose of interrupting a gene (*eps*) implied in EPS synthesis.

A mutant of *B. amyloliquefaciens* FZB42 Eps^- was constructed by cloning a fragment of the *eps* operon in pGEM-T Easy vector which was then transformed into *E. coli* JM109. The purified hybrid plasmid was secondly cloned in p-MUTIN-GFP^+ and also transformed into *E. coli* JM109 for the purpose of obtaining a florescence mutant. Finally, the p-MUTIN-GFP^+::*epsA*$^-$ plasmid was transformed into *B. amyloliquefaciens* FZB42 using electroporation method.

The results of fluorescence microscopic observation showed that the hybrid *epsA-gfp genes* were expressed in all cells as compared with the wild-type strain (figure 48). To confirm the integration of *epsA-gfp* within the corresponding chromosomal locus of FZB42, a fragment (1,917 bp) consisting of a part of *slr*, *epsA*, *epsB* and a part of *gfp* genes was amplified using chromosomal DNA from both strain mutant and mother cell. The agarose gel electrophoresis analysis showed that the amplicon was observed only in the mutant (figure 49).

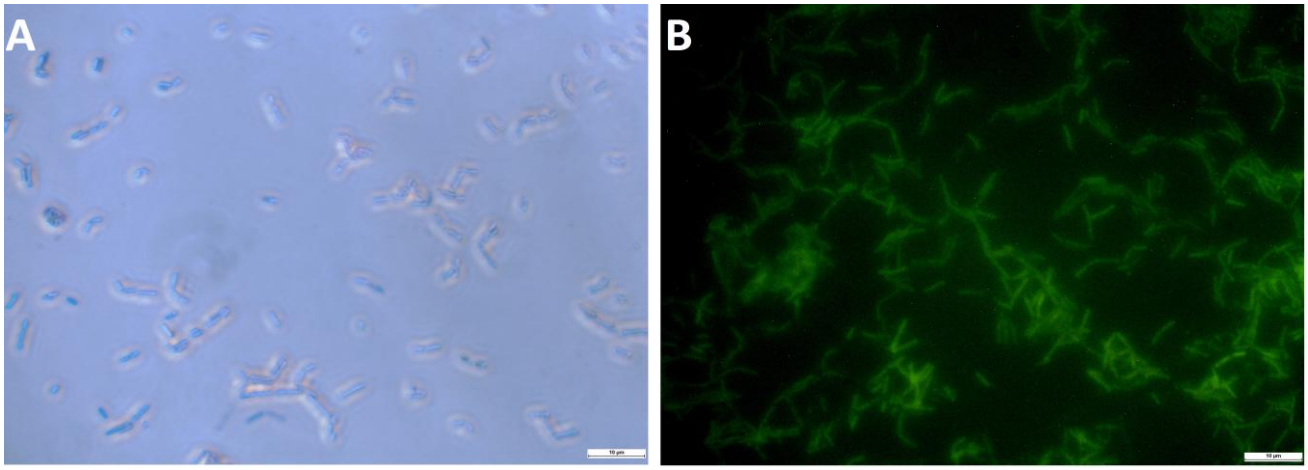


Figure 48 Microscopic observations of (A) Wild-type GFP⁻ *B. amyloliquefaciens* FZB42 treated with acridine orange and observed under ultraviolet light using confocal laser scanning microscopy; and (B) FZB42 Erm-R GFP⁺ Eps⁻ mutant cells using confocal laser scanning microscopy. Bacterial cells were visualized using oily immersion with (100X) magnification (10 µm scale).

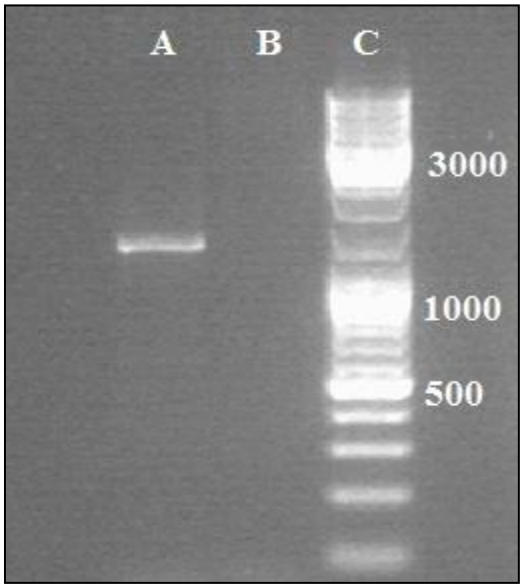


Figure 49 Agarose gel electrophorsis of PCR products obtained from (A) Mutant strain *B. amyloliquefaciens* FZB42 Erm-R GFP⁺ Eps⁻; (B) Wild-type strain FZB42; and (C) O'Gene Ruler (100 – 10,000 bp; Thermo Scientific Fermentas).

V.7.2 Biofilm formation by *B. amyloliquefaciens* FZB42, its Eps⁻ derivative and *B. subtilis* BBG131

This experiment was performed to compare the *B. amyloliquefaciens* FZB42 Eps⁻ mutant to both wild-type and *B. subtilis* BBG131. Firstly, growth kinetics of the three strains in LB medium showed no significant differences between these strains, indicating that there was no effect of *eps* gene interruption on the bacterial growth of the Eps⁻ mutant (figure 50). The Eps⁻ mutant showed the same HPLC profile of lipopeptides as its mother's cell (figure 51).

Thereafter, the three strains were grown in static cultures containing different carbon sources to investigate their ability to form a biofilm.

A big difference was observed between these strains in forming a biofilm in all used media, *B. amyloliquefaciens* FZB42 being largely more efficient than the other ones (figure 52). This result was confirmed by the optical densities of biofilm stained with crystal violet, which showed a significant difference between FZB42 and the other strains under all carbon sources tested, whereas there were no significant differences in optical density values for the Eps^- mutant and BBG131 (figure 46). These results pointed out that the production of exopolysaccharides is necessary for biofilm formation and confirms previous findings (Allison and Sutherland, 1987; Watnick and Kolter, 1999; Sutherland, 2001).

The biofilm formation is an important process which represents the basis of root colonisation and aggregate communities on soil particle surface by rhizobacteria (Davey and O'Toole, 2000; Tan *et al.*, 2013). Biofilm formation was lower with concentrated root exudates than with other carbon sources, due to the fact 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 could be explained by the lack of both environmental harsh and nutrient deficiency and starvation in this concentrated tomato root exudates.

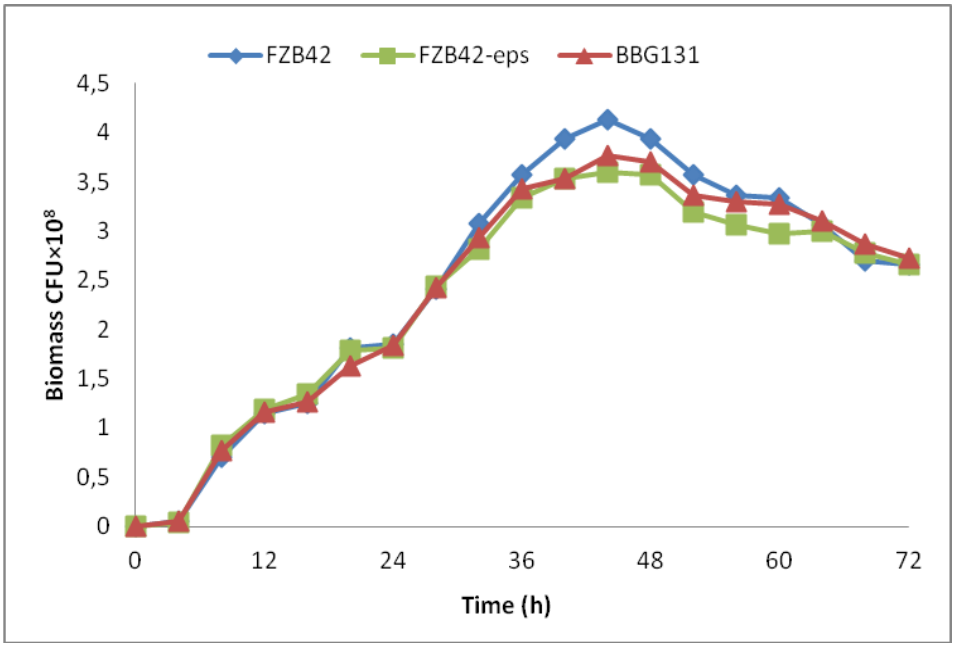


Figure 50 Kinetic of bacterial growth of *B. amyloliquefaciens* FZB42, Eps⁻ mutant and *B. subtilis* BBG131 in LB medium during 72 h at 37°C

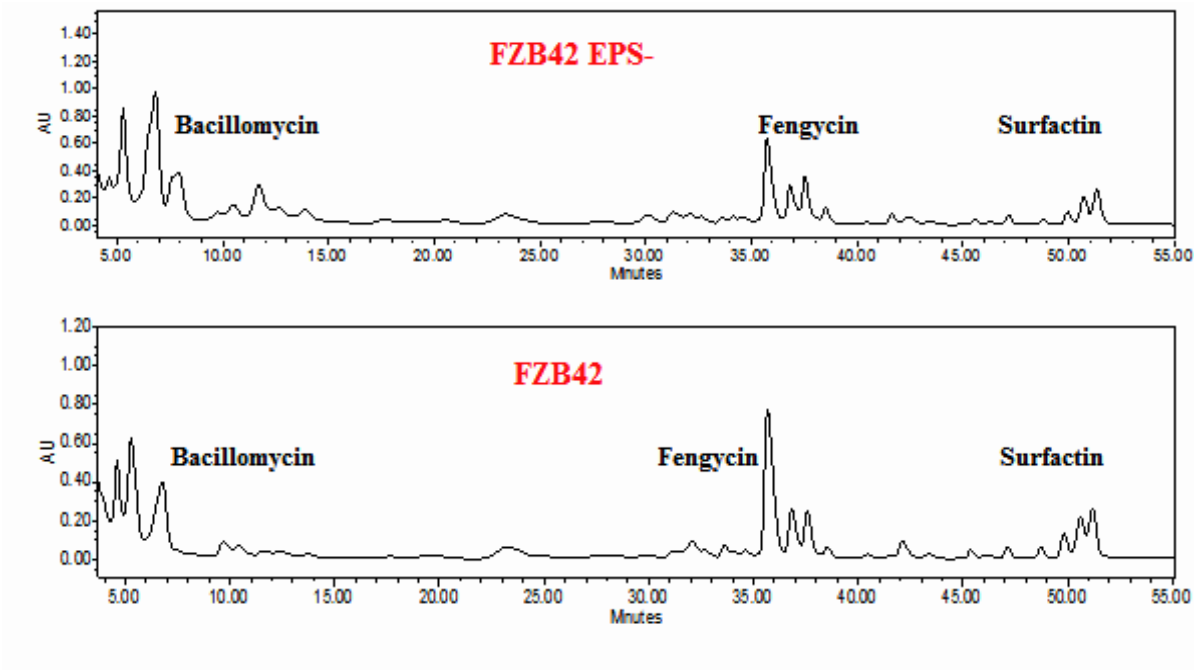


Figure 51 HPLC profile for lipopeptides determination for Eps⁻ mutant and mother cell.

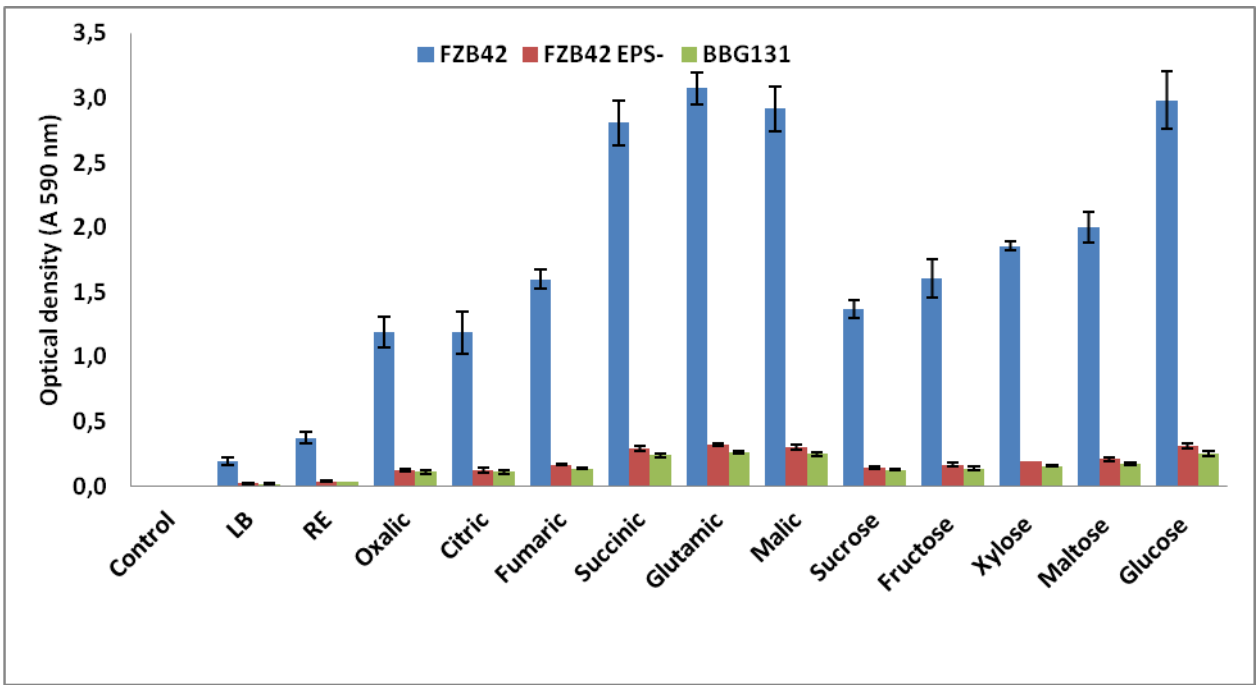


Figure 52 Biofilm formation by *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131. The biofilm was stained with crystal violet after 72 h of incubation at 21°C. The organic compounds were dissolved in minimum medium at the concentration equivelant to 2 g carbon atoms per liter.

V.8. Roots colonisation assays

These assays were realized based on both results of biofilm formation obtained under *in vitro* conditions and several reports which indicated the inability to form a biofilm in the absence of EPS compounds (Allison and Sutherland, 1987; Watnick and Kolter, 1999; Sutherland, 2001). Hence, the same strains; *B. amyloliquefaciens* FZB42, *B. subtilis* BBG131 and the Eps⁻ mutant 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. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 were treated with acridine orange while the Eps⁻ mutant carried the *gfp* gene marker. *B. amyloliquefaciens* FZB42 was the best root colonizer compared with its the Eps⁻ mutant and *B. subtilis* BBG131 (figure 53). For these latter strains, a strong correlation was clearly demonstrated between their low biofilm formation and their weak colonisation ability. Coupled with the microscopic observation, the earlier results of root colonisation expressed as CFU values in the rhizosphere which were 177×10^6, 8.9×10^6 and 7.3×10^6 for *B. amyloliquefaciens* FZB42, *B. subtilis* BBG131 and the Eps⁻ mutant, respectively (figure 54), confirmed the results of microscopic

observations. As it has been noted, all these results concerning biofilm formation under *in vitro* experiments supported that biofilm formation plays a necessary role in roots and rhizosphere colonisation.

As shown in previous reports, the rhizosphere colonisation 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 on the role of biofilm formation in rhizosphere colonisation. The big difference between *B. amyloliquefaciens* FZB42 and the two other strains in forming biofilms under all carbon sources tested confirmed the results obtained in colonisation.

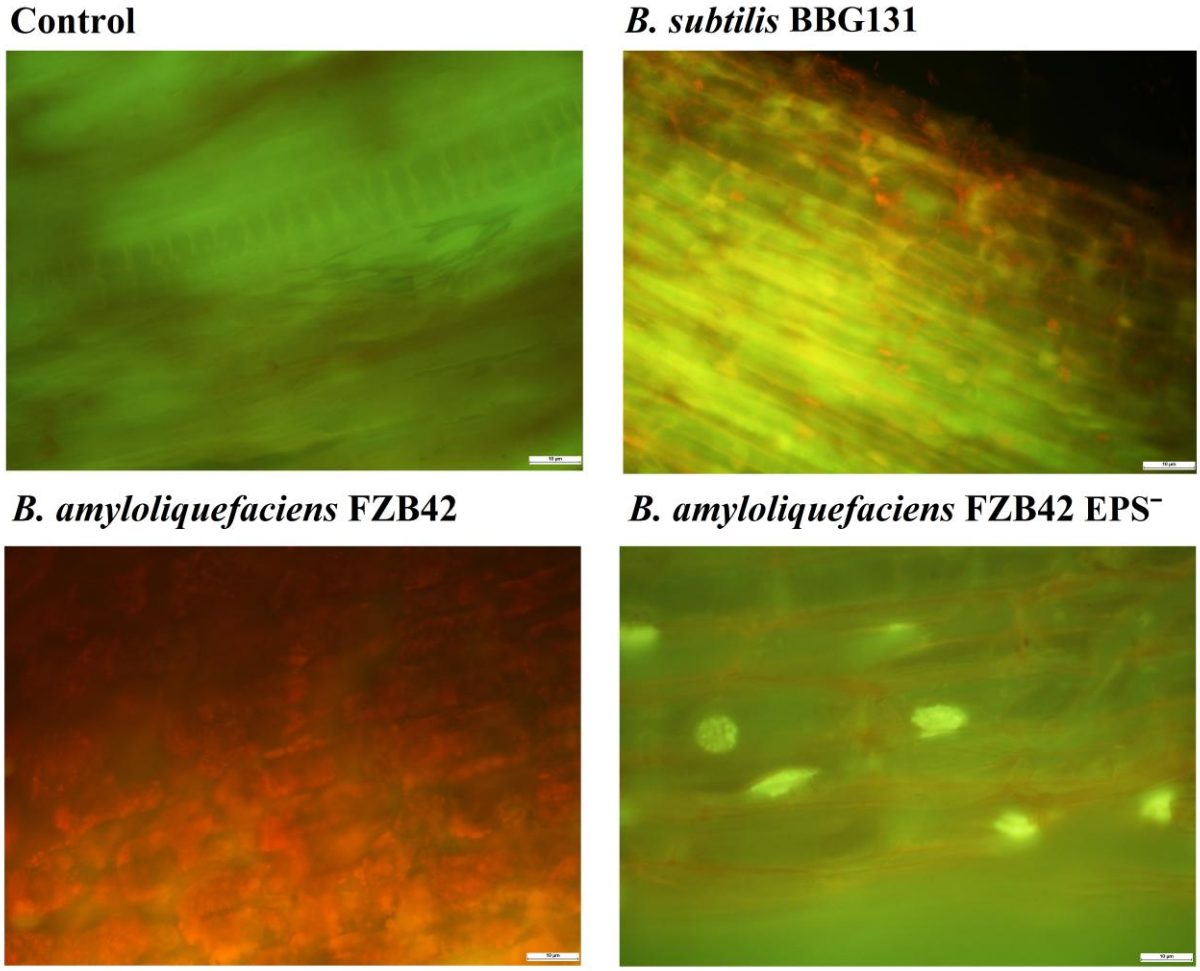


Figure 53 Tomato rhizoplane colonisation by *B. amyloliquefaciens* FZB42, Eps⁻ mutant and *B. subtilis* BBG131 compared with control (non-inoculated sample). Bacterial cells were visualized under confocal laser scanning microscope using oily immersion with (100X) magnification (10 µm scale). The samples were treated with acridine orange dye. After 21 days of colonisation.

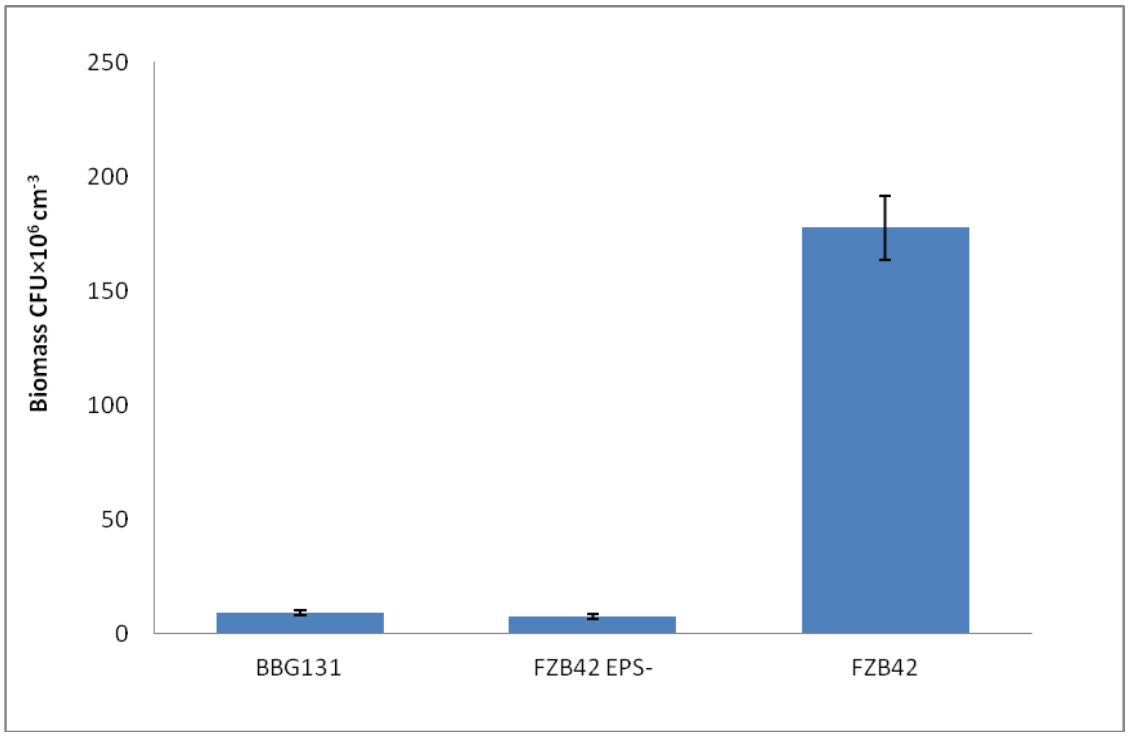


Figure 54 Tomato rhizosphere colonisation by *B. amyloliquefaciens* FZB42, *B. subtilis* BBG131 and Eps⁻ mutant after 21 days.

V.9. *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 stimulate tomato plant growth

It is widely known that the relation between plant growth promoting bacteria and plant is based on the provision of the carbon sources and root exudates represent the majority of them. In contrast, bacteria contribute to facilitate the nutrient availability in addition to supplying some growth factors like hormones (Maheshwari, 2012). Hence, at the same time, an experiment was performed to measure the weight of the aerial parts of plants and the lengths of the stems as a first indicator of the effect of PGPR on plant. Thus, a completely randomized design (CRD) was used to carry out this experiment. The 21-day-old plants were harvested and the roots were removed. The weight of the aerial parts and the lengths of the stems were measured.

A significant difference was shown between the weight and length of plant inoculated with *B. amyloliquefaciens* FZB42 and the control treatment (figures 55 and 56). The average lengths were 18.49, 17.40 and 16.25 cm for *B. amyloliquefaciens* FZB42, BBG131 and the

control treatments, respectively. Weights were 0.361, 0.263 and 0.255 g.Plant^{-1} for *B. amyloliquefaciens* FZB42, *B. subtilis* BBG131 and the control treatments, respectively.

The positive results of *B. amyloliquefaciens* FZB42 obtained in this study may be due to the fact that *B. amyloliquefaciens* FZB42 improves plant growth by different factors: 1) *B. amyloliquefaciens* FZB42 is able to produce IAA, a plant growth hormone which stimulates cell elongation (Idris *et al*., 2004) 2) Phosphate mobilization by the phytase secreted by *B. amyloliquefaciens* FZB42 may provide a key nutrient under conditions of phosphate starvation (Idris *et al*. 2002) 3) Several antibiotics produced by *B. amyloliquefaciens* FZB42 are found to be related with their biocontrol activity against plant pathogens (Koumoutsi *et al*., 2004). Concerning *B. amyloliquefaciens* FZB42, it showed a high capacity for colonizing the rhizosphere and adapting with the root excreted in the rhizosphere as an energy and a carbon source. Plant growth promoting bacteria can improve plant growth by increasing yield, reducing pathogen infection, as well as reducing biotic or abiotic plant stress, without conferring pathogenicity (Glick, 2012; Gagné-Bourque *et al.*, 2015). On the other hand, among diverse mechanisms, root exudates may play a fundamental role in the plant nutrition. They either contain signals that act as regulators of microbial growth and function, or they possess molecules which directly control the rhizosphere processes that enhance nutrient uptake and assimilation, and consequently improve plant nutrition, and hopefully increase crop yields (Dakora and Phillipps, 2002).

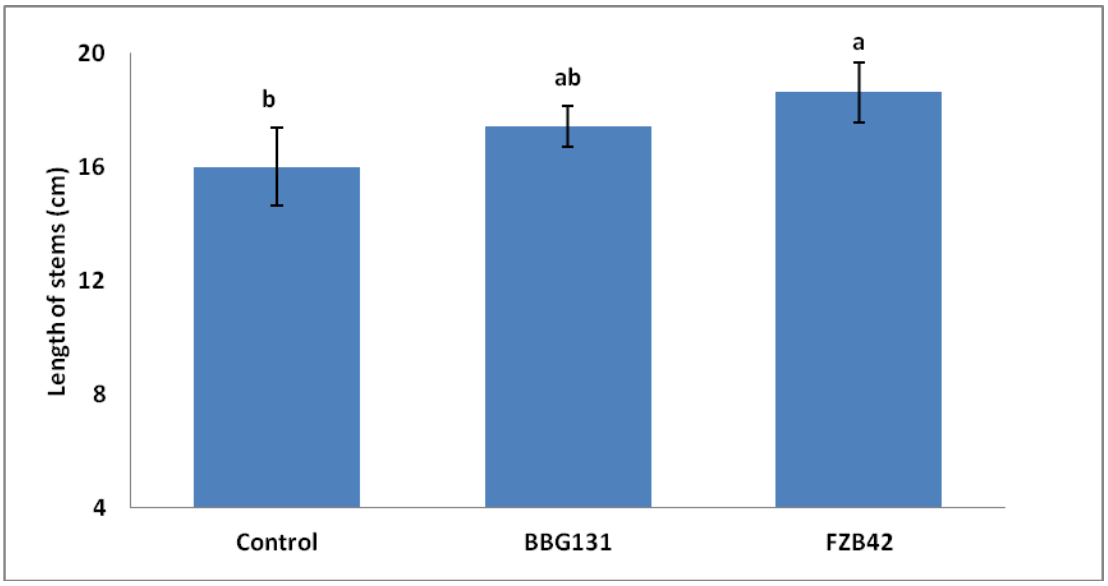


Figure 55 Effect of *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 on the length of the plant stem after 21days

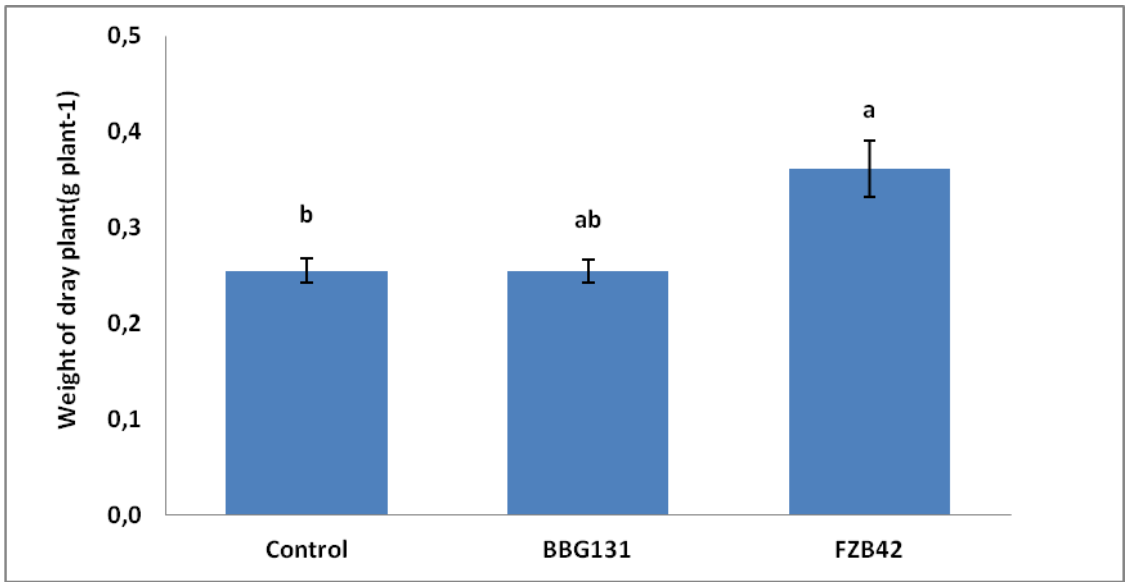


Figure 56 Effect of *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 on the plant weight. *B. amyloliquefaciens* FZB42 showed a significant effect on plant stems length and plant weight after 21-days old than control.

Chapter VI

General discussion

VI. General discussion

As shown in previous studies, the rhizosphere colonisation by plant growth promoting rhizobacteria is the most important step for the microbial biocontrol agents (Weller et al. 2002; Vessey 2003; Pii et al., 2015). Our experiments shed light on the role of different factors affecting rhizosphere colonisation. These factors were distinguished in this study to illustrate the rhizosphere colonisation by two *Bacillus* strains.

The first result reported in this study was related to the bacterial species. Preliminary results had shown that *B. amyloliquefaciens* strains could be better root colonizer that *B. subtilis*. It was confirmed in this study by following the root colonization of two model strains *Bacillus amyloliquefaciens* FZB42 and *Bacillus subtilis* BBG131 during 21 days. In this experiment, *B. subtilis* BBG131 growth is stopped after 3 days at a low level of biomass suggesting a limiting growth factor in the rhizosphere. This kinetic experiment also illustrated that a good surfactin production is not sufficient to get a good colonizing strain. Indeed, *B. amyloliquefaciens* FZB42 produces *in vivo* a small quantity of surfactin compared to *B. subtilis* BBG131.

Interestingly, while *B. amyloliquefaciens* FZB42 has the ability to produce three lipopeptide families; surfactin, iturin and fengycin in *in vitro* conditions only surfactin was detected in the tomato rhizosphere. These results were in agreement with Nihorimbere *et al*., (2012) who found that the secretion of iturin and fengycin around tomato root infected by *B. amyloliquefaciens* S499 was very low.

Root exudates include a diverse array of carbon sources like primary metabolites such as phenolic acids, organic acids, sugars and amino acids and secondary compounds (Badri and Vivanco,2009; Li *et al*., 2013). These compounds provide the growth factors as well as nutrient sources for the bacterial growth of the two strains *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131. The difference between the behaviour of both strains may be due to the ability of *B. amyloliquefaciens* FZB42 to better consume the compounds in the root exudates *B. subtilis* BBG131 or to its lower requirement of nutrients than *B. subtilis* BBG131. This was confirmed by a growth experiment performed *in vitro* with tomato root exudates. These results, in agreement with Tan *et al*, (2013), who indicated that the root exudates from tomato plant supported bacterial cell division and enhanced the growth of *B. amyloliquefaciens*. Interestingly,

root exudates could induce a stress response of *B. subtilis* BBG131 which could explain the high level of surfactin produced per biomass unit which was 125 times higher than for *B. amyloliquefaciens.*

Analyses of growth and lipopeptide production of both strains on different carbon sources potentially present in root exudates showed their different behaviour. For all the tested substrate the growth of *B. amyloliquefaciens* is better than for *B. subtilis*. This was more important for sucrose, maltose, xylose, fumaric, citric and oxalic acids. This could result from genetic differences between both strains linked to the transport or catabolic pathway of these compounds. For example, the significant difference between *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 in the use of sucrose as a sole carbon source could result from their non-similarity in the gene encoding sucrose transporter or sucrose phosphate hydrolase.

The biomass obtained with *B. subtilis* BBG131 cultivated on xylose as a sole carbon sources was very low. These results establish clearly that *B. subtilis* BBG131 is unable to use xylose as a sole carbon source. The different between the two strains may be explained by the genetically difference between the two strains in the gene encoding the xylose transporter or in the operon encoding the xylose isomerase (Schmiedel and Hillen, 1996; Gartner *et al.*, 1988).

The results of bacterial growth in the presence root exudates seem to be not sufficient to explain the difference of the two strains in the rhizosphere, because the proportion of growth in the root exudates was 6/1 when it was 25/1 in the rhizosphere. Therefore, there is another factor which may have an impact on the rhizosphere colonisation: biofilm formation.

Both strains were thus first studied to their ability to forming biofilm *in vitro* in the presence of all studied carbon sources. In all tested conditions *B. amyloliquefaciens* FZB42 was able to form a nice biofilm whereas *B. subtilis* BBG131 could not. Biofilm formation is an important process which represents the basis of root colonisation and aggregate communities on soil particle surface by rhizobacteria (Davey and O'Toole, 2000; Tan *et al.*, 2013). Production of exopolysaccharides have been highlighted as one of the main mechanism to get biofilm. In order to confirm, the role of biofilm formation in root colonization, a mutant unable to produce these exopolysaccharides was obtained. This mutant completely lost its ability to colonize the rhizosphere.

Chapter VII

Conclusions and perspectives

VII. Conclusions and perspectives

VII.1 Conclusions

The goal of the study was to evaluate the factors affecting the rhizosphere colonisation to understand the role played by these factors in the induction of the bacteria into the root environments as a colonizer. As shown above, it does not seem that surfactin is the main factor influencing the rhizosphere colonisation. Then it was aimed to find out which other factors probably have a positive effect on the rhizosphere colonisation.

The results obtained by following the kinetic growth and surfactin production during tomato rhizosphere colonisation showed that *B. amyloliquefaciens* FZB42 is a better colonizer than *B. subtilis* BBG131, which could be mainly due to its lower nutrients requirement provided by root exudates.

The different behaviour in the colonisation ability of these two strains is not completely explained by the role of root exudates because the bacterial growth of *B. amyloliquefaciens* FZB42 was 7 times higher than the cells population of *B. subtilis* BBG131 whereas it was 25 times more significant in the kinetic experiment. Therefore, we suggested another factor affecting the rhizosphere colonisation: the biofilm formation.

The good colonisation of the rhizosphere by *B. amyloliquefaciens* FZB42 compared to its Eps^- mutant confirms this hypothesis.

VII.2. Perspectives

Based on the results obtained in this work, here are some perspectives:

1- To study at molecular level, the differences found between the two strains in the consumption of the different carbon sources;
2- To confirm the localization of the lipopeptides in the rhizosphere using spectroscopy mass imaging.
3- To realize field or green house studies to shed light on the rhizosphere colonisation in the presence of phytopathogens.
4- To modify *B. amyloliquefaciens* or *B. subtilis* for obtaining a strain producing high lipopeptides amounts and having the ability to form a biofilm, to check it as a biocontrol agent.

Chapter VIII

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VIII. References

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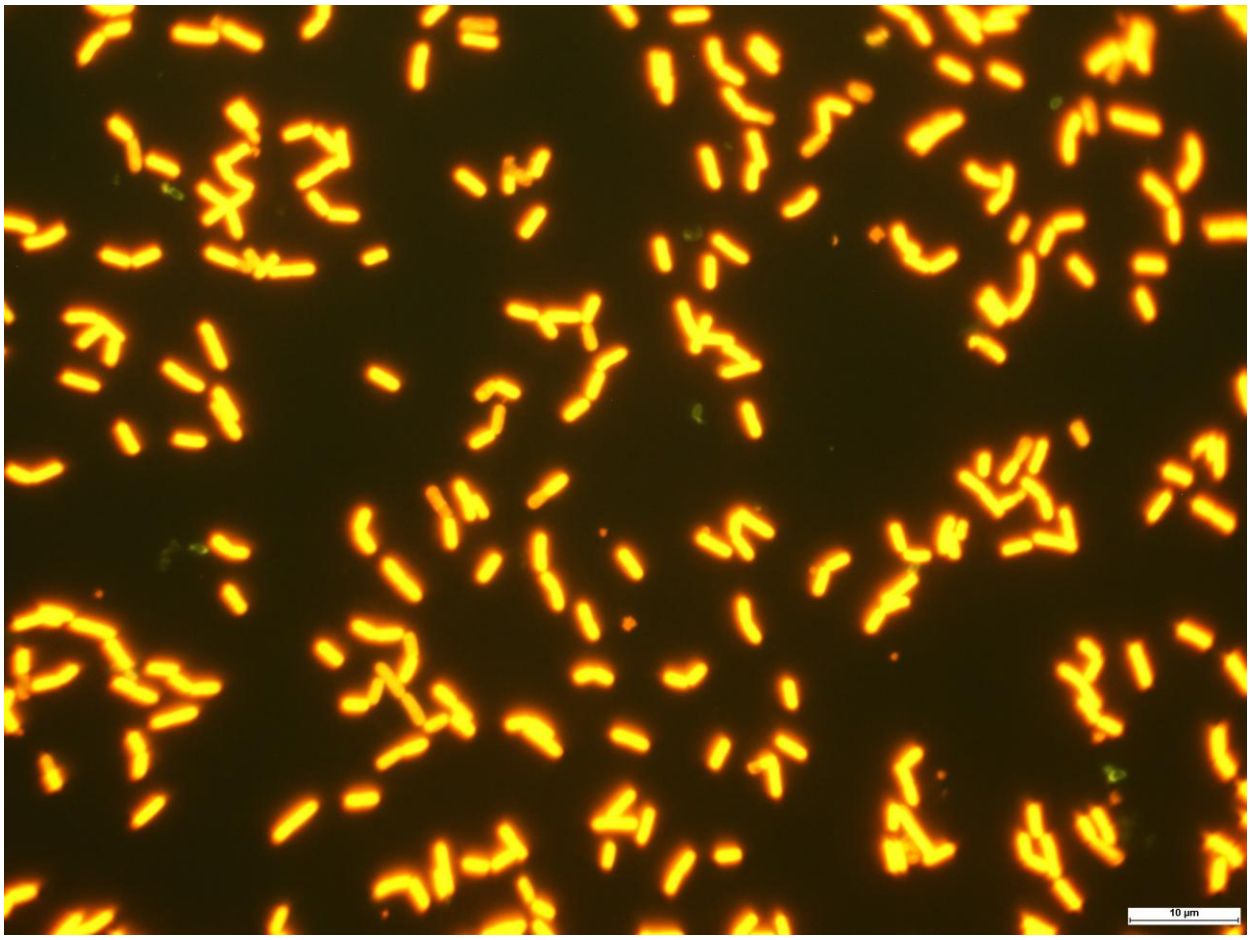
Chapter IX

Appendixes

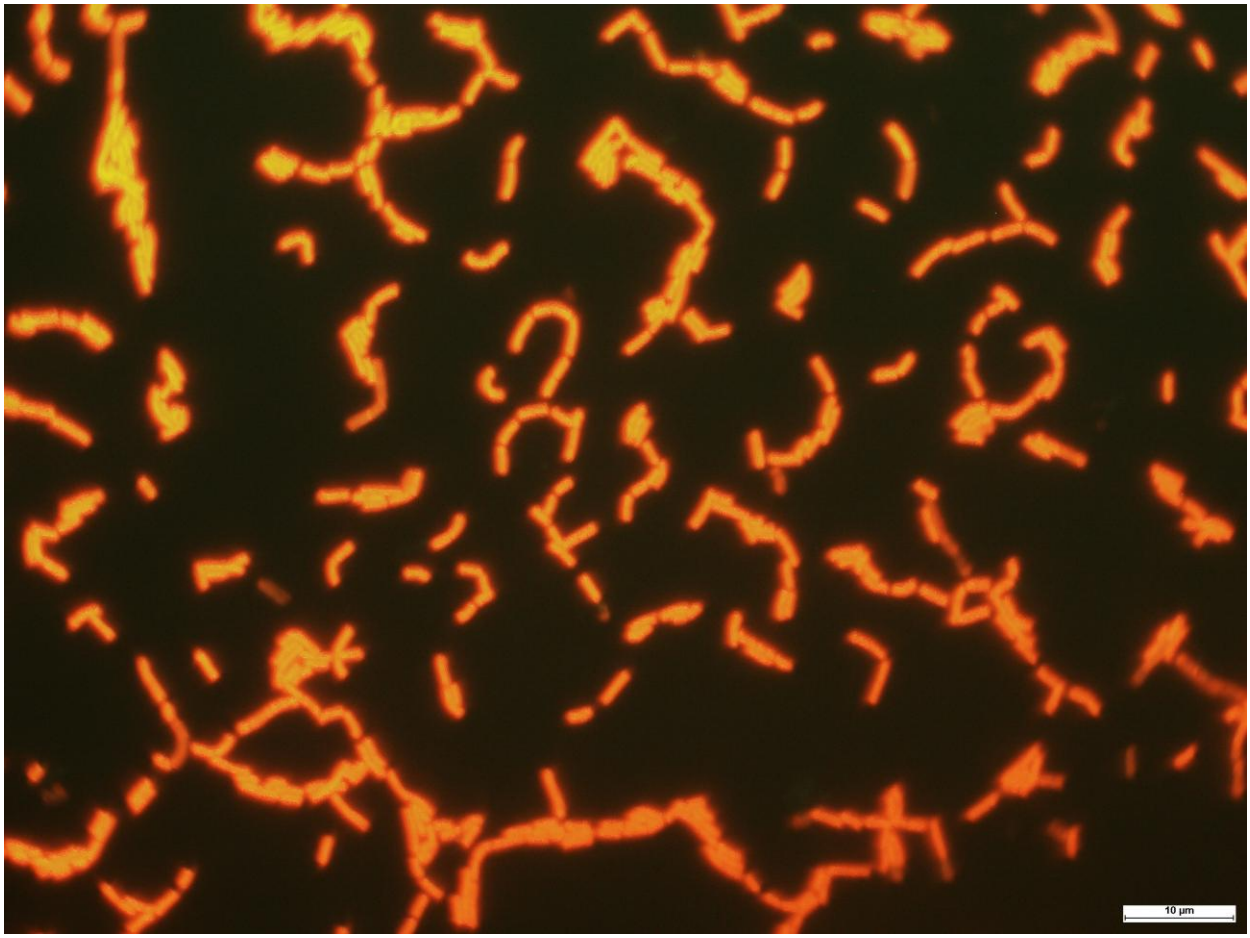
IX. Appendixes:

IX.1. The form of *B. amyloliquefaciens* FZB42 observed under fluorescent microscope with magnification X100.



***Bacillus amyloliquefaciens* FZB42**

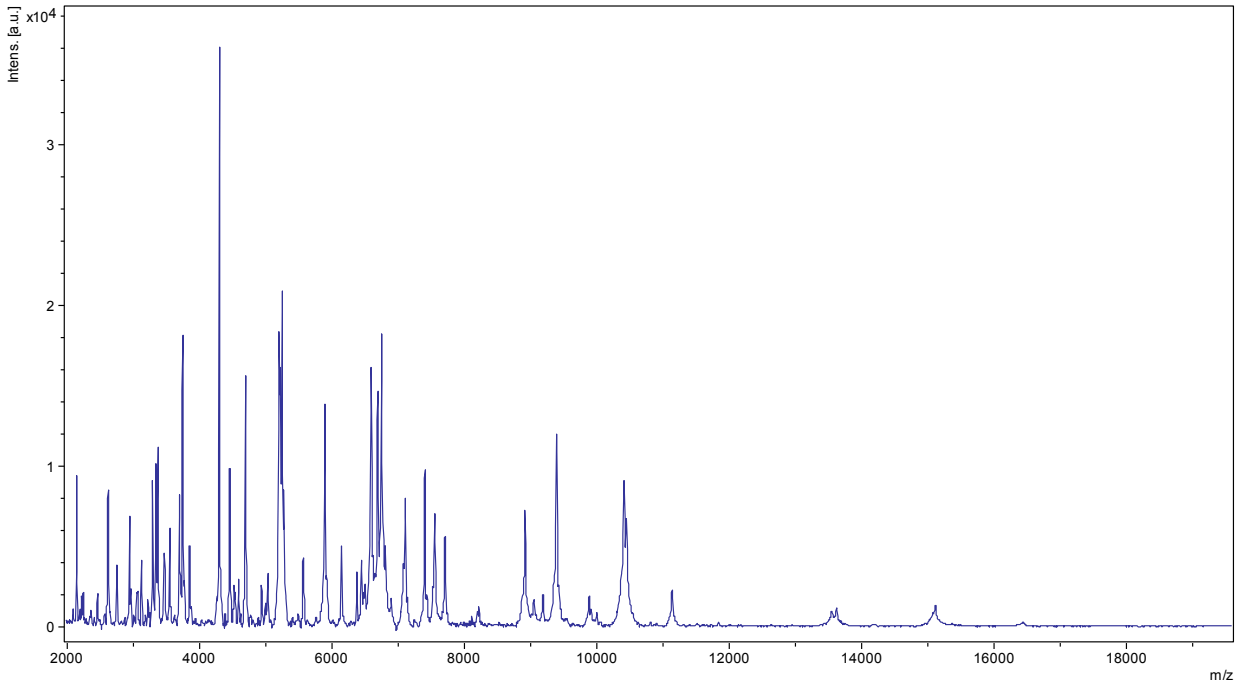
IX.2. The form *B. subtilis* BBG131 observed under fluorescent microscope with magnification X100.



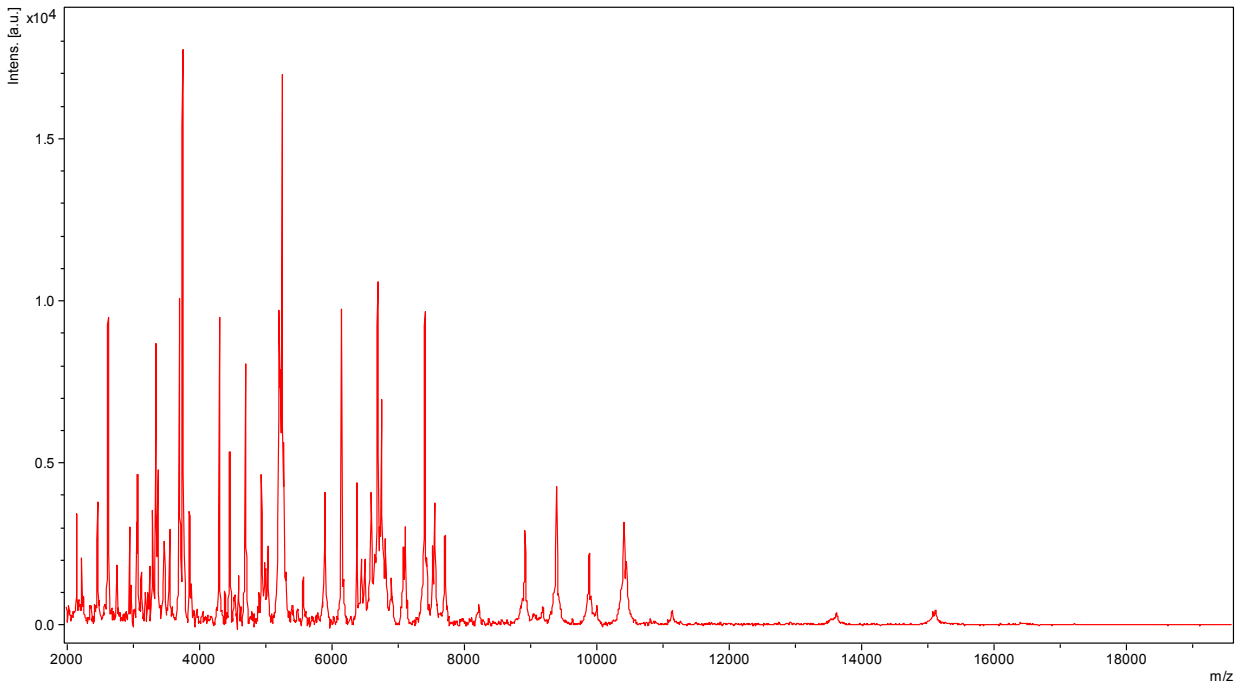
***Bacillus subtilis* BBG131**

IX.3. MALDI-TOF MASS Spectrum of bacteria

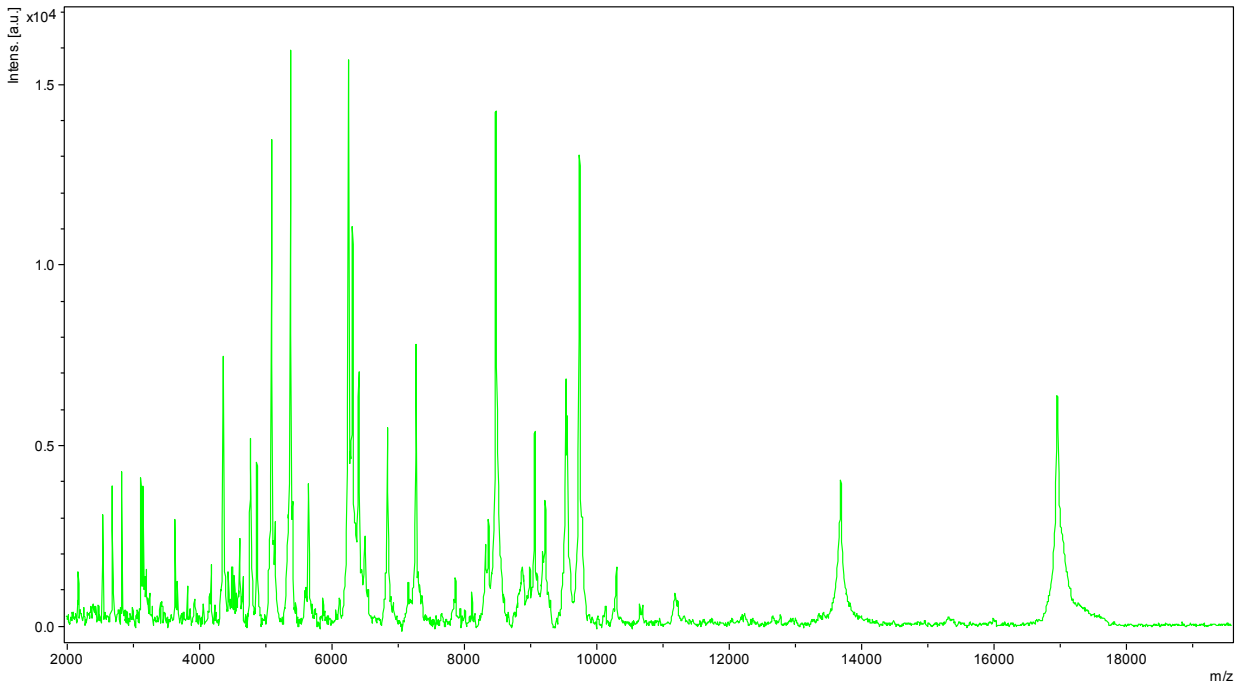
IX. 3.1. Spectrum of *Bacillus amyloliquefaciens* FZB42



IX. 3.2. Spectrum of *Bacillus subtilis* BBG131

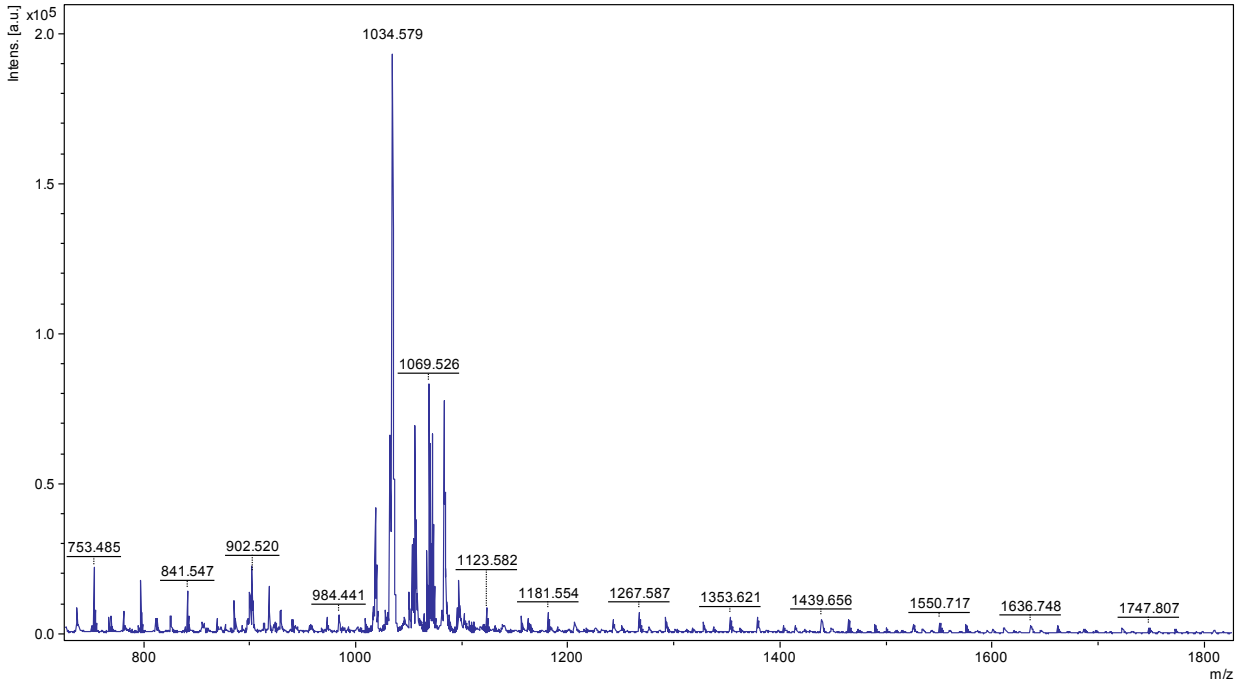


IX. 3.3. Spectrum of *Escherichia coli*

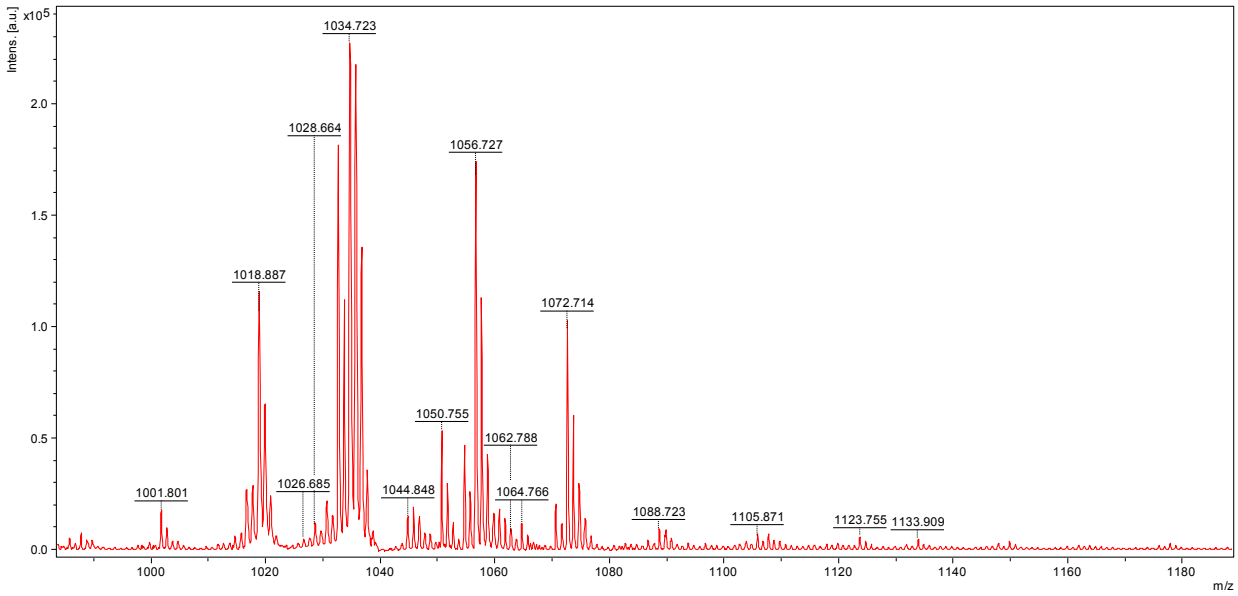


IX.4. MALDI-TOF MASS Spectrum of surfactin in the rhizosphere

IX. 4.1. Surfactin produced by *Bacillus amyloliquefaciens* FZB42 in the tomato rhizosphere

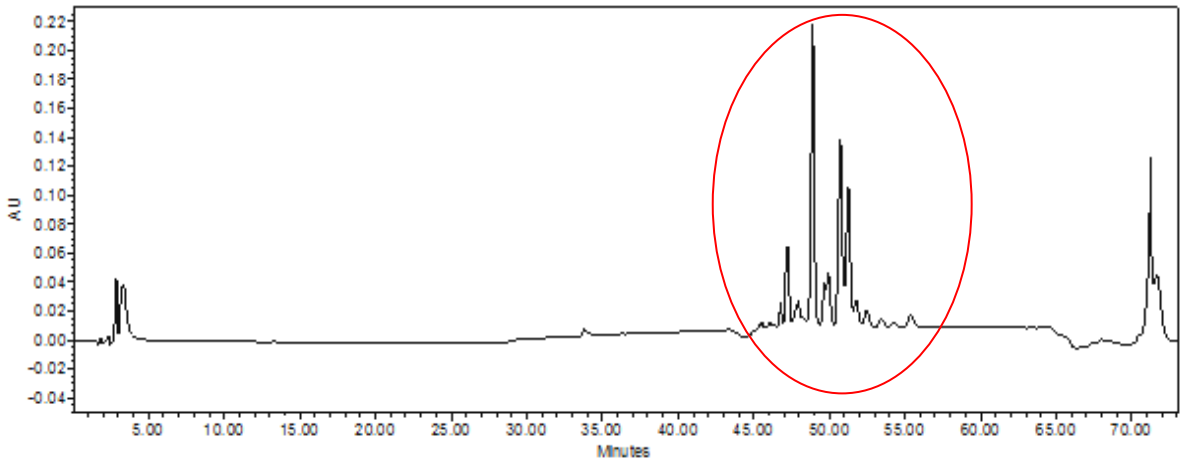


IX. 4.2. Surfactin produced by *Bacillus subtilis* BBG131 in the tomato rhizosphere

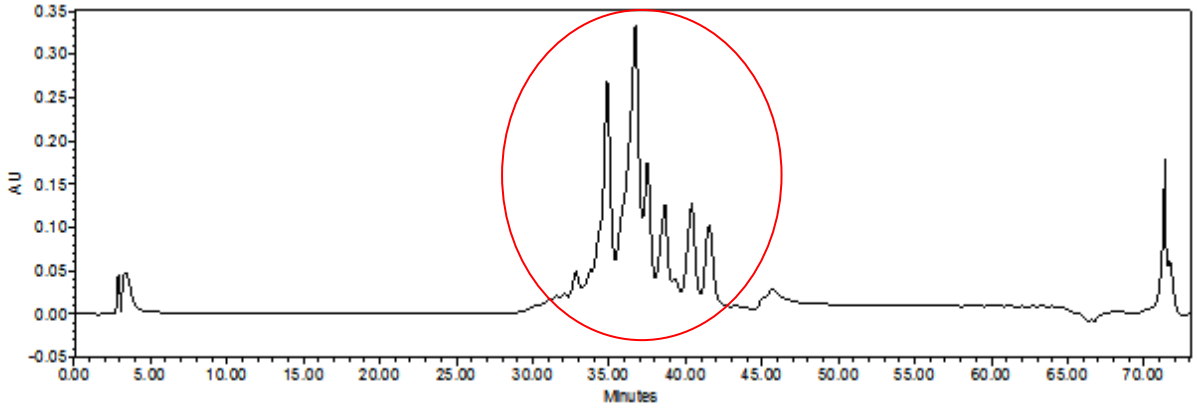


IX.5. Lipopeptides standards

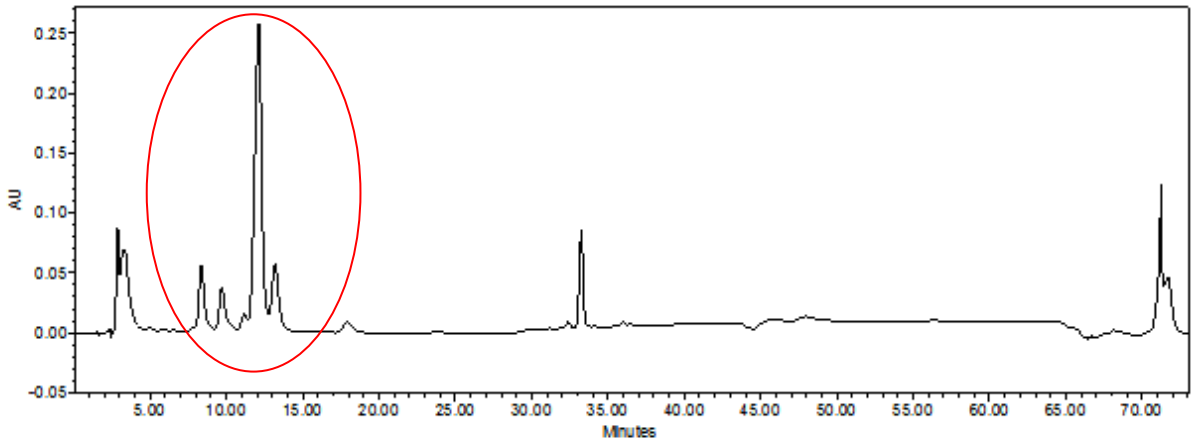
IX. 5.1. Surfactin standard spectrum



IX. 5.2. Fengycin standard spectrum



IX. 5.3. Iturin standard spectrum



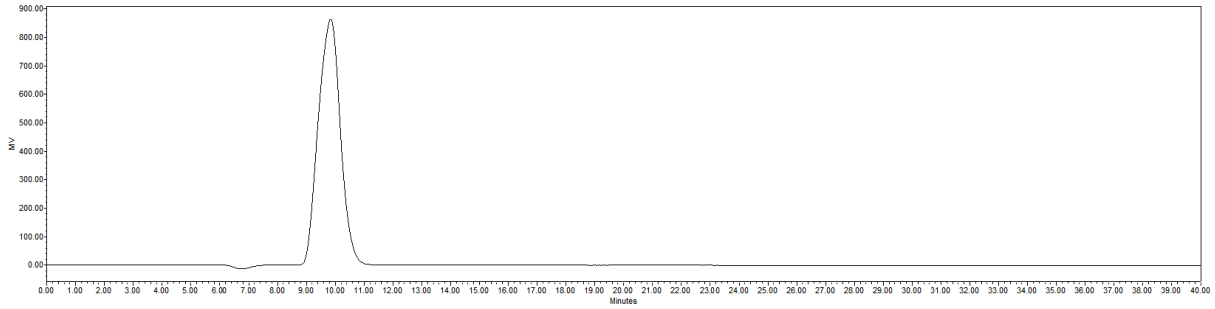
IX.6. Lipopeptides calculation

Lipopeptide (mg L^{-1}) = ((area of lipopeptide/area of standard) × standard concentration × dilution factor))

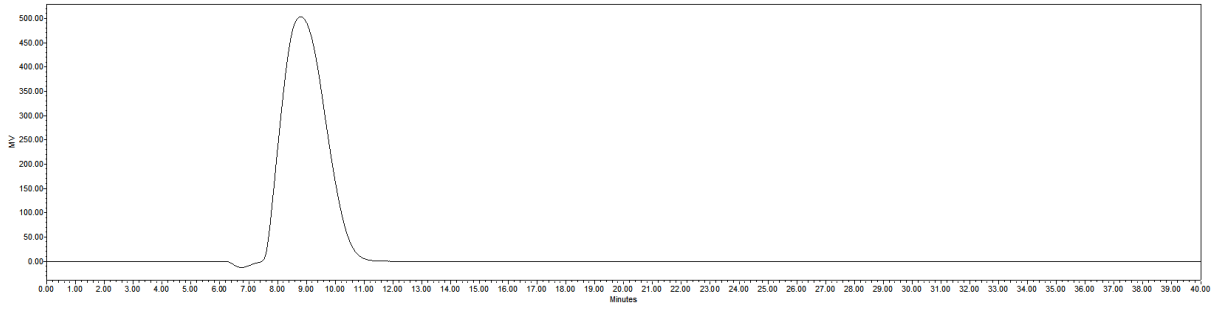
The dilution factor of 1mL concentrated to 100 µL = 100 µL/1000 µL = 0.1 and so on.

IX.7. Spectrum of sugars (HPLC-Refractometer)

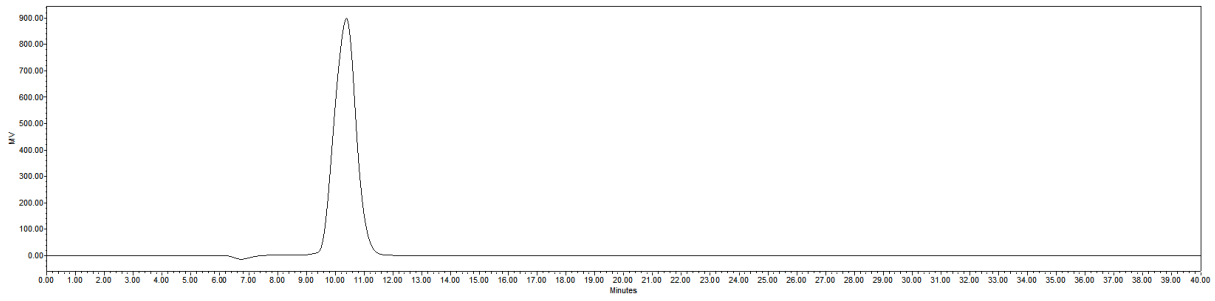
IX. 7.1. Glucose spectrum



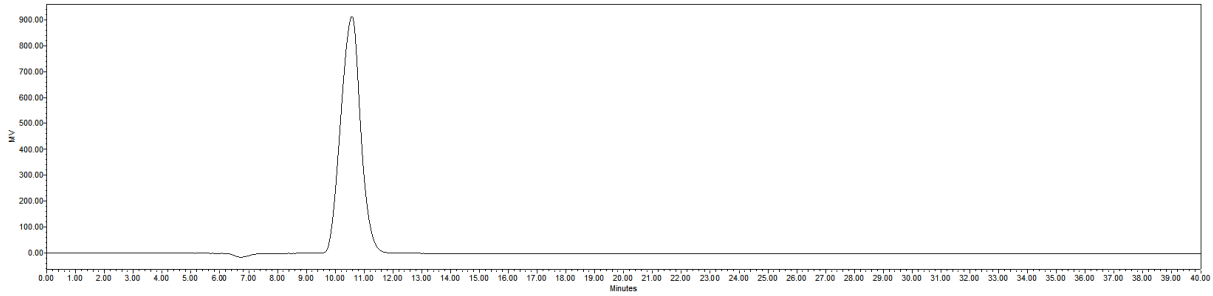
IX. 7.2. Sucrose spectrum



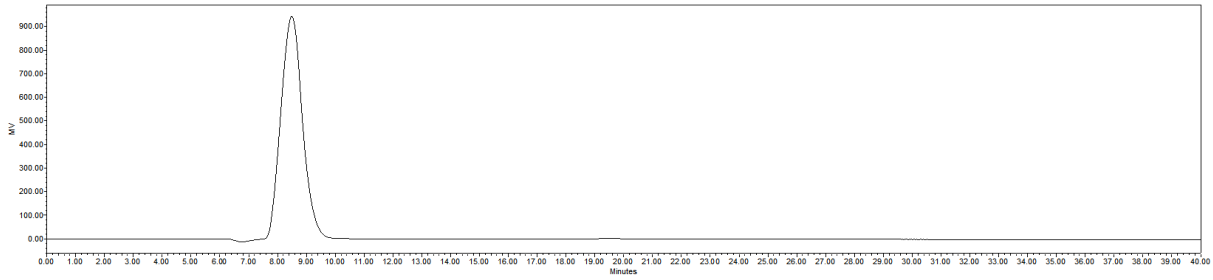
IX. 7.3. Maltose spectrum



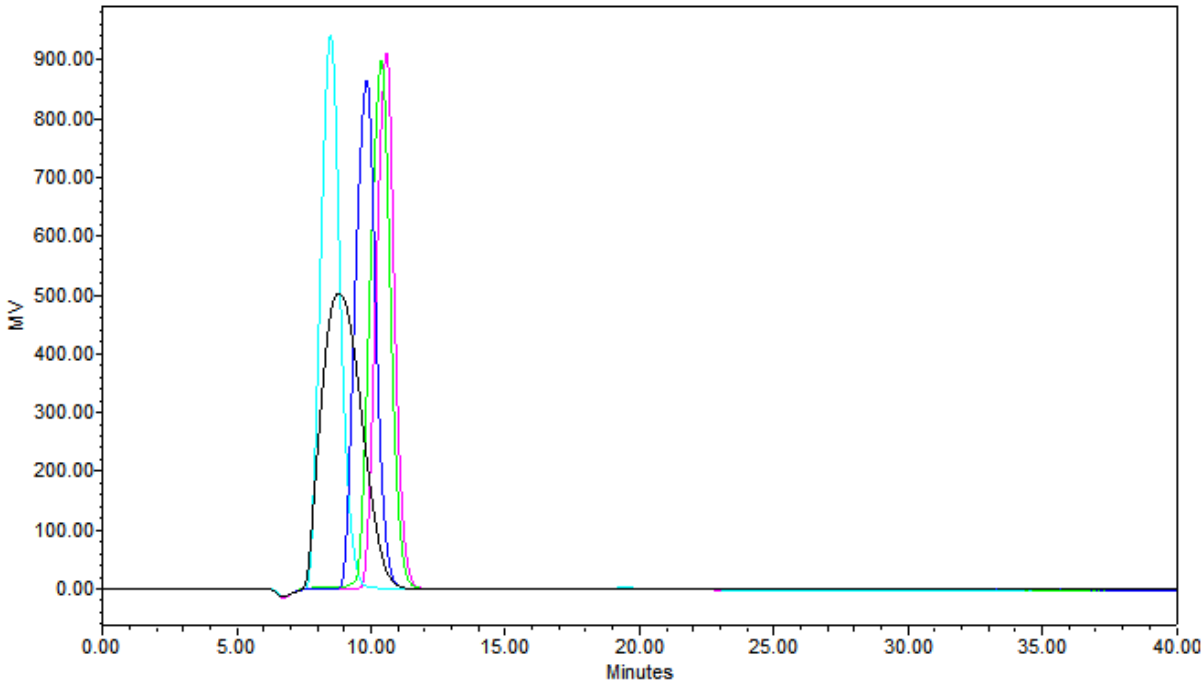
IX. 7.4. Fructose spectrum



IX. 7.5. Xylose spectrum

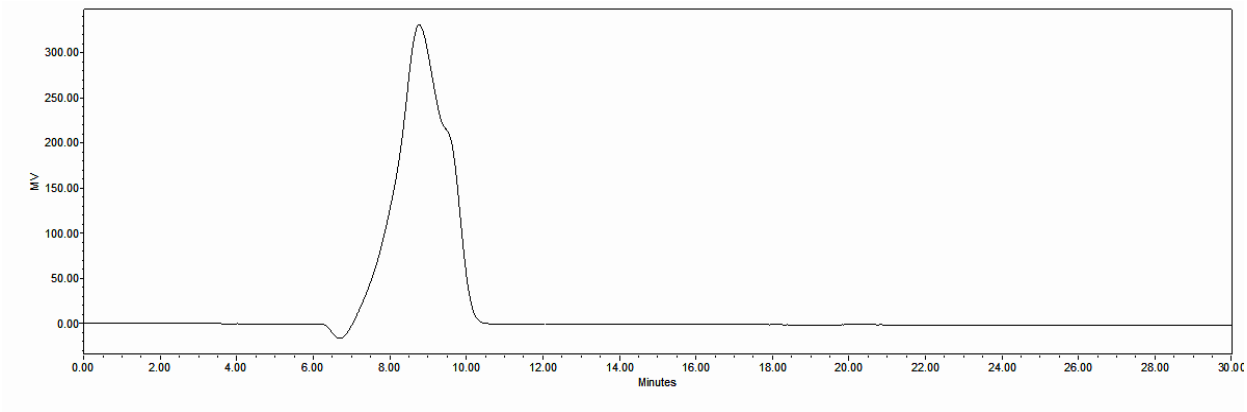


IX. 7.6. Sugars standard spectrum

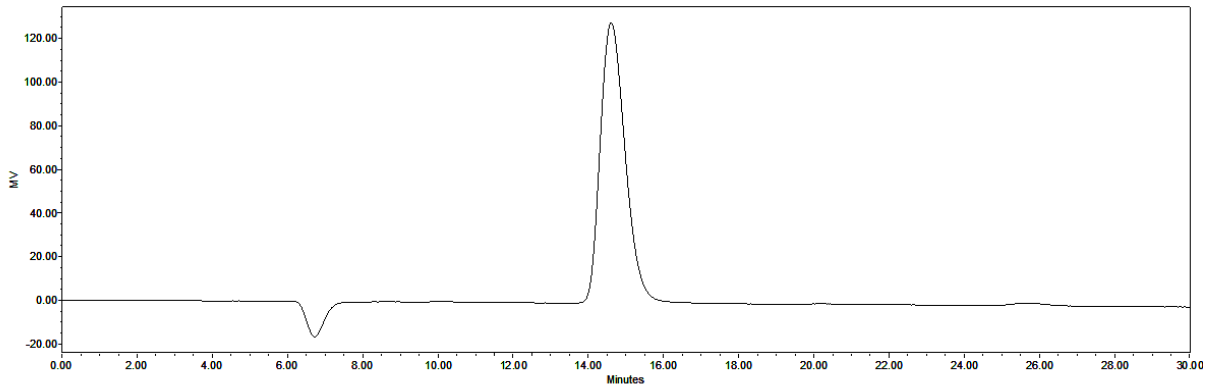


IX.8. Spectrum of organic acids (HPLC-Refractometer)

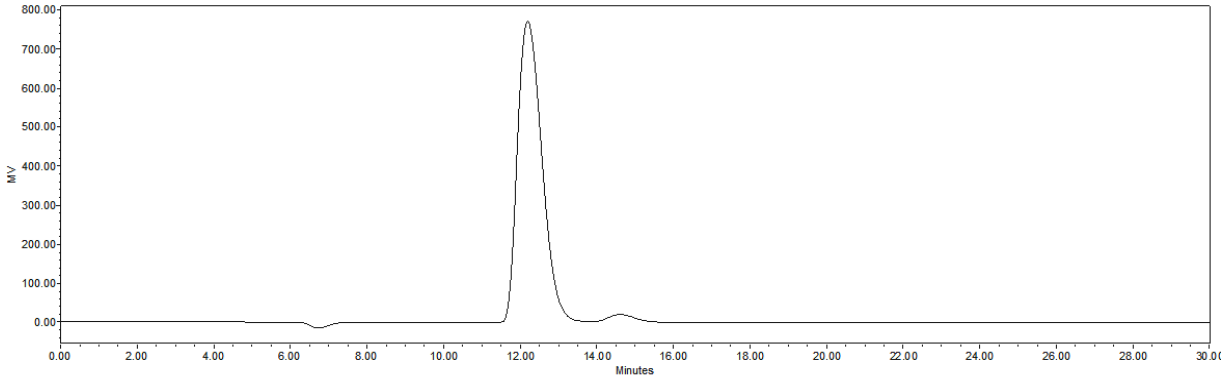
IX. 8.1. Oxalic spectrum



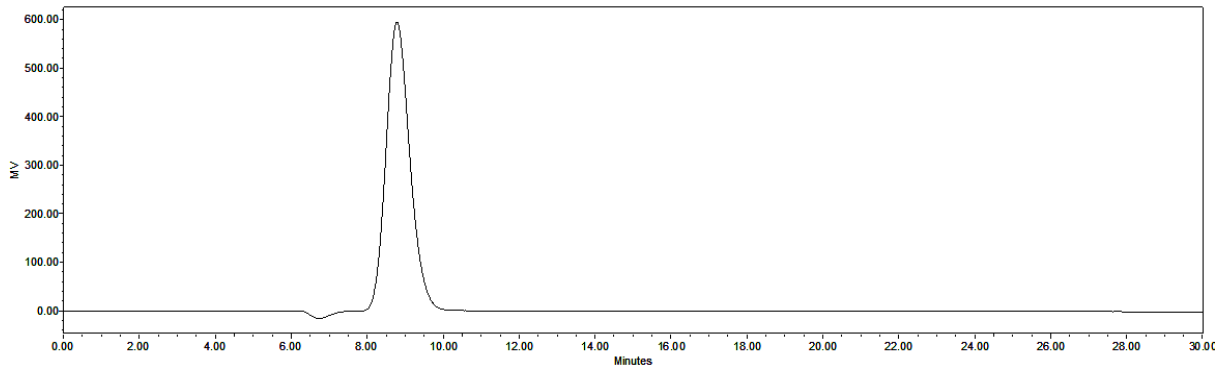
IX. 8.2. Fumaric spectrum



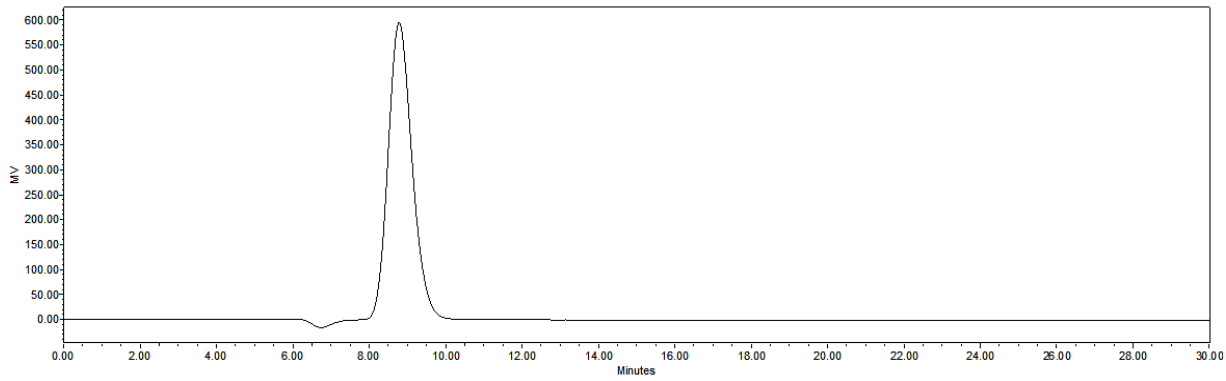
IX. 8.3. Succinic spectrum



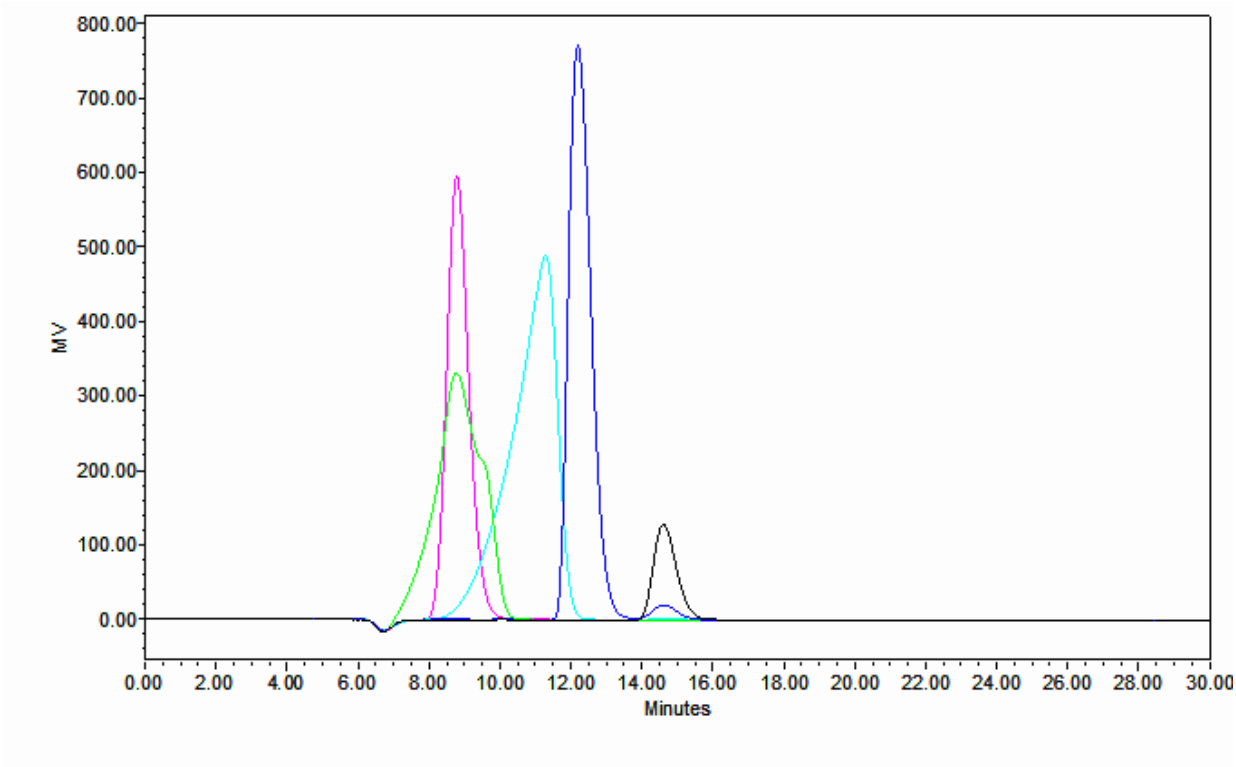
IX. 8.4. Citric spectrum



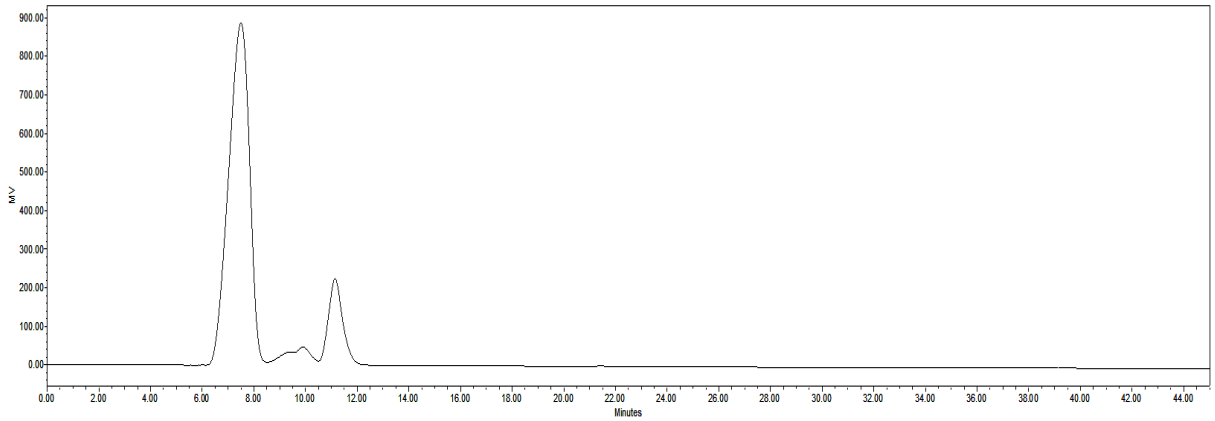
IX. 8.5. Malic spectrum



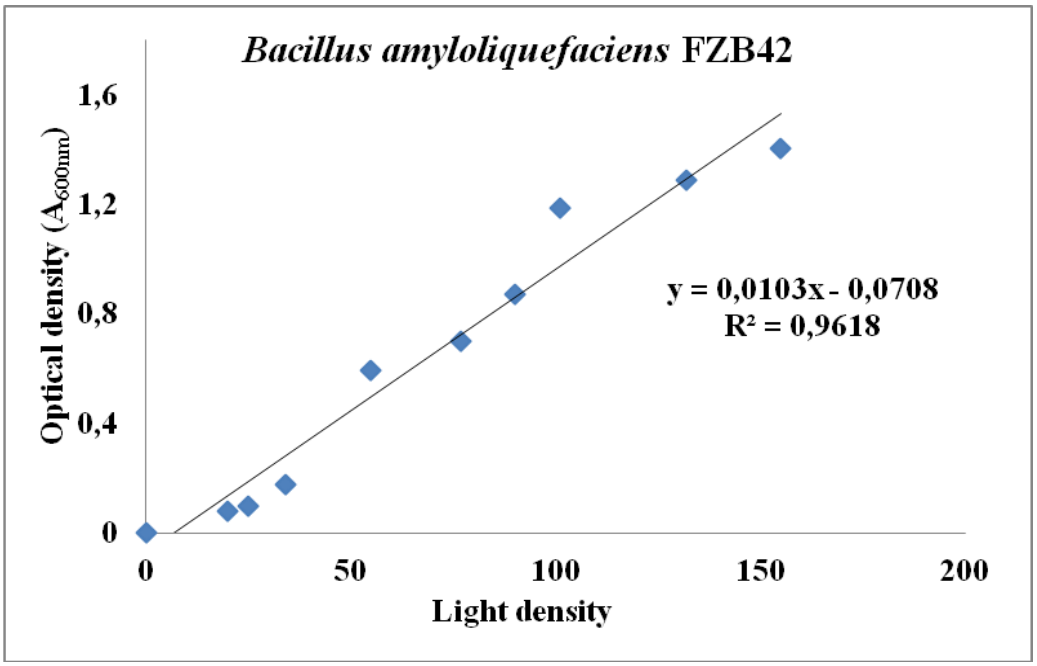
IX. 8.6. Organic acids spectrum

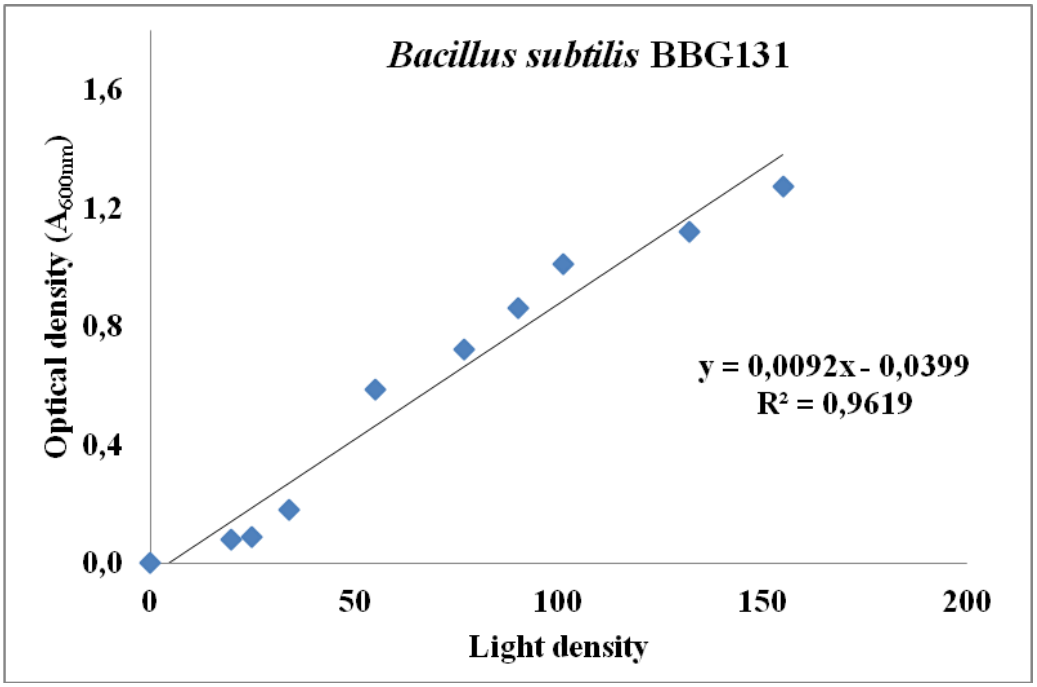


IX.9. Root exudates spectrum



IX.10. Equations used for Biolector results calibration





IX.11. Primers design using primer3 program

IX. 11.1. Sequence *slr-epsA-epsB*/KpnI-XmaIII

Sequence modified with *Xma*III site (*Eco*52I):

***Kpn*I**

AGAGACACCTGCTTCAACTGCTAGCTGATTAATAGGGTACCCTTTTCTTCTGCGGTATAAACGAATTATTCTTCCAA

TCATTGTGCTGAATTCTCCCCTGTATACTGGCGTTTTTTTGTTCATTATAAGAAATTTTTCGTTCTTTATAAAATTTA

AAATTATAAGGTAAGTGCAGTAAATAAGAGGAAAATCATGATAATGTTCTTTAAAAAGAACTAAATGGCTTAATT

TGAAATTTTCAAATTTCGACCTTTTCTTTTATAATCCAATCATTAACAGAAGGGGGCGTTTAAGCCTGATGCAATAA

GGATGAGGCTGTAATTACATGAATGAGAATATGAGTTTTAAAGAATTATTTGACATTATTAAACACAGATTTTTAC

TGATTTTTATCATGACAGCAGTTGTAACGCTGGTGACGGGATACATCCAATTCCGGGTGATTTCGCCCGTTTATCAG

GCATCAACCCAAGTGCTCATTCATGAAACAAGCGGTGAAAAAAATTCGAATCTCAGCGACGTTCAGCTGAATCTTC

ATTACAACAATACGTTTCAAACGATAATGAAAAGCCCGGTAGTGCTTGAGAAAGTGAAGCAGACGCTGCATCTTT

CTGAGACGGCATCCGCTTTAAAAGCAAAGATCACGACAAGCAGCGAAACCGATTCAGAGATCATAACCATAGCGG

TGCAGGACGAAAATCCGAAACAGGCCGCCGCTATAGCGAACACGCTGATGAAGACATTTAAAAAAGAAGTCCGTG

ACAGGATGAATATAAAAGGCGTCATTGTTTTGTCTGAGGCAAAAGCATCGGAAAGCCCGATGGTCAAGCCTTCGC

GCATCAGGAATATCATGATGGCGTTCGGTGCGGCTCTCATGGCGGGTGTGACGCTTGCGTTTTTTCTCCATTTTCTT

GATGAAACCGTTAAAAGCGAGCGGCAGCTCAGCGAAAAAACAGACTTGCCTGTTTTAGGGGTTGTGTATGACATC

AAAAATCAGCAGACACGGTCTGATGAAAAACATTTCGGGGAGTGAGGCGTTTGGGATTCAGAAAAAAGAAATCA

AGAAGGGGACTGGCTCAAATATCCGTTTTACATCACAAATCATTGGTGGCTGAACAATACCGCACCATTCGGACAA

ATATTGAGTTCTCCTCTGTTCAGATCGATTTGCGCTCTATTCTCGTCACTTCTTCCGTTCCGGGAGAAGGAAAATCA

TTCAGCGCCGCCAACCTTGCCGCGGTATTTGCGCAGCAGGAAAAAAAGGTGCTGCTCGTCGATGCGGATTTACGAA

AATCGATGATACACGAG*ATCGATC*AGCTTGAAAATGTACAAGGCCTTACGAATGTGTTAGTCGGAATCGATTCCCT

CGGCGAATCGATGCAAAAATCGATGATTGATAACCTTTACGTCTTATCGATCCGGCCG

(G) ***Kpn*I**

AGAGACACCTGCTTCAACTGCTAGCTGATTAATAGGGTACCCTTTTCTTCTGCGGTATAAACGAATTATTCTTCCAA

TCATTGTGCTGAATTCTCCCCTGTATACTGGCGTTTTTTTGTTCATTATAAGAAATTTTTCGTTCTTTATAAAATTTA

AAATTATAAGGTAAGTGCAGTAAATAAGAGGAAAATCATGATAATGTTCTTTAAAAAGAACTAAATGGCTTAATT

TGAAATTTTCAAATTTCGACCTTTTCTTTTATAATCCAATCATTAACAGAAGGGGGCGTTTAAGCCTGATGCAATAA

GGATGAGGCTGTAATTACATGAATGAGAATATGAGTTTTAAAGAATTATTTGACATTATTAAACACAGATTTTTAC

TGATTTTTATCATGACAGCAGTTGTAACGCTGGTGACGGGATACATCCAATTCCGGGTGATTTCGCCCGTTTATCAG

GCATCAACCCAAGTGCTCATTCATGAAACAAGCGGTGAAAAAAATTCGAATCTCAGCGACGTTCAGCTGAATCTTC

ATTACAACAATACGTTTCAAACGATAATGAAAAGCCCGGTAGTGCTTGAGAAAGTGAAGCAGACGCTGCATCTTT

CTGAGACGGCATCCGCTTTAAAAGCAAAGATCACGACAAGCAGCGAAACCGATTCAGAGATCATAACCATAGCGG

TGCAGGACGAAAATCCGAAACAGGCCGCCGCTATAGCGAACACGCTGATGAAGACATTTAAAAAAGAAGTCCGTG

ACAGGATGAATATAAAAGGCGTCATTGTTTTGTCTGAGGCAAAAGCATCGGAAAGCCCGATGGTCAAGCCTTCGC

GCATCAGGAATATCATGATGGCGTTCGGTGCGGCTCTCATGGCGGGTGTGACGCTTGCGTTTTTTCTCCATTTTCTT

GATGAAACCGTTAAAAGCGAGCGGCAGCTCAGCGAAAAAACAGACTTGCCTGTTTTAGGGGTTGTGTATGACATC

AAAAATCAGCAGACACGGTCTGATGAAAAACATTTCGGGGAGTGAGGCGTTGGGATTCAGAAAAAAGAAATCA

AGAAGGGGACTGGCTCAAATATCCGTTTTACATCACAAATCATTGGTGGCTGAACAATACCGCACCATTCGGACAA

ATATTGAGTTCTCCTCTGTTCAGACCCATTTGCGCTCTATTCTCGTCACTTCTTCCGTTCCGGGAGAAGGAAAATCA

TTCAGCGCCGCCAACCTTGCCGCGGTATTTGCGCAGCAGGAAAAAAAGGTGCTGCTCGTCGATGCGGATTTACGAA

AACCGACGATACACGAGACCTATCAGCTTGAAAATGTACAAGGCCTTACGAATGTGTTAGTCGGAAACGCTTCCCT

CGGCGAAACGGTGCAAAAAACGCTGATTGATAACCTTTACGTCTTAACGAGCCGGCCGACGCCGCCGAATCCGGC

TGAGCTTTTATCTTCAAAGGCGATGGGAGAGCTGATTCAGGAGATGTACAGCCGGTACAGTCTGGTTATTTTCGAT

TCACCTCCGCTTTTAGCTGTGGCGGACGGACAGATTTTAGCGAACCAGACAGATGGAAGCGTTCTCGTCGTCTTGA

GCGGAAAAACGAAAATGGATACCGTCCAAAAGGCGAGAGACGCGCTCCAGCAGTCAAAGGCAAAGCTTTTGGGG

GCGCTGCTGAATAAAAAGAAAATCAAAAAAACGGAGCACTACTCGTATTGA

***Xma*III**

FUSION AMPLICON *Kpn*I-*Xma*III + *gfp*$^{+}$:

TTGGGATTCAGAAAAAAGAAATCAAGAAGGGGACTGGCTCAAATATCCGTTTTACATCACAAATCATTGGTGGCT

GAACAATACCGCACCATTCGGACAAATATTGAGTTCTCCTCTGTTCAGACCCATTTGCGCTCTATTCTCGTCACTTC

TTCCGTTCCGGGAGAAGGAAAATCATTCAGCGCCGCCAACCTTGCCGCGGTATTTGCGCAGCAGGAAAAAAAGGT

GCTGCTCGTCGATGCGGATTTACGAAAACCGACGATACACGAGACCTATCAGCTTGAAAATGTACAAGGCCTTAC

GAATGTGTTAGTCGGAAACGCTTCCCTCGGCGAAACGGTGCAAAAAACGCTGATTGATAACCTTTACGTCTTAACG

AGC*CGGCCGGAAGGAGATATACATATGGCTAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTA*

GATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCTACATACGGAAAGCTTACCCTTAAA

TTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTGACCTATGGTGTTCAATGCTTTTCCCG

TTATCCGGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAACGCACTATATCTTTC

AAAGATGACGGGAACTACAAGACGCGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGT

ATTGATTTTAAAGAAGATGGAAACATTCTCGGACACAAACTCGAGTACAACTATAACTCACACAATGTATACATCACGGCAG

ACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTCGCCACAACATTGAAGATGGATCCGTTCAACTAGCAGACCATTA

TCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCGACACAATCTGCCCTTTCGAAA

GATCCCAACGAAAAGCGTGACCACATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTACACATGGCATGGATGAGCTC

TACAAATAATGAATTCGAGCACTAGTGCAGCCCGCCTAATGAGCGGGCTTTTTTCCATGCAAGCTAATTCCG

Translate Tool - Results of translation

Open reading frames are highlighted in red. Please select one of the following frames - in the next page, you will be able to select your initiator and retrieve your amino acid sequence:

5'3' Frame 1

L G F R K K K S R R G L A Q I S V L H H K S L V A E Q Y R T I R T N I E F S S

V Q T H L R S I L V T S S V P G E G K S F S A A N L A A V F A Q Q E K K V L

L V D A D L R K P T I H E T Y Q L E N V Q G L T N V L V G N A S L G E T V Q

K T L I D N L Y V L T S R P E G D I H **Met** A S K G E E L F T G V V P I L V E L

D G D V N G H K F S V S G E G E G D A T Y G K L T L K F I C T T G K L P V P

W P T L V T T L T Y G V Q C F S R Y P D H **Met** K R H D F F K S A **Met** P E G

Y V Q E R T I S F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F

K E D G N I L G H K L E Y N Y N S H N V Y I T A D K Q K N G I K A N F K I R

H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S T Q S A

L S K D P N E K R D H **Met** V L L E F V T A A G I T H G **Met** D E L Y K **Stop**

Stop I R A L V Q P A **Stop Stop** A G F F P C K L I P

IX. 11.2. primer design of *slr-epsA-epsB*/KpnI-XmaIII

Primer3 Output

```
PRIMER PICKING RESULTS FOR Amplicon slr-epsA-epsB KpnI-XmaIII

No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any th   3' th  hairpin  seq
LEFT PRIMER         10   20   58.55   55.00     0.00    0.00     0.00
GGTACCCTTTTCTTCTGCGG   SLR-KpnI-FZB42-FWD4
RIGHT PRIMER      1399   18   60.88   66.67    25.73    6.34     0.00
CGGCCGGCTCGTTAAGAC     ESPB-XmaIII-FZB42-REV4
SEQUENCE SIZE: 1411
INCLUDED REGION SIZE: 1411

PRODUCT SIZE: 1390, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00


    1 GATTAATAGGGTACCCTTTTCTTCTGCGGTATAAACGAATTATTCTTCCAATCATTGTGC

                >>>>>>>>>>>>>>>>>>>

   61 TGAATTCTCCCCTGTATACTGGCGTTTTTTTGTTCATTATAAGAAATTTTTCGTTCTTTA

  121 TAAAATTTAAAATTATAAGGTAAGTGCAGTAAATAAGAGGAAAATCATGATAATGTTCTT

  181 TAAAAAGAACTAAATGGCTTAATTTGAAATTTTCAAATTTCGACCTTTTCTTTTATAATC

  241 CAATCATTAACAGAAGGGGGCGTTTAAGCCTGATGCAATAAGGATGAGGCTGTAATTACA

  301 TGAATGAGAATATGAGTTTTAAAGAATTATTTGACATTATTAAACACAGATTTTTACTGA

  361 TTTTTATCATGACAGCAGTTGTAACGCTGGTGACGGGATACATCCAATTCCGGGTGATTT

  421 CGCCCGTTTATCAGGCATCAACCCAAGTGCTCATTCATGAAACAAGCGGTGAAAAAAATT

  481 CGAATCTCAGCGACGTTCAGCTGAATCTTCATTACAACAATACGTTTCAAACGATAATGA

  541 AAAGCCCGGTAGTGCTTGAGAAAGTGAAGCAGACGCTGCATCTTTCTGAGACGGCATCCG

  601 CTTTAAAAGCAAAGATCACGACAAGCAGCGAAACCGATTCAGAGATCATAACCATAGCGG

  661 TGCAGGACGAAAATCCGAAACAGGCCGCCGCTATAGCGAACACGCTGATGAAGACATTTA

  721 AAAAAGAAGTCCGTGACAGGATGAATATAAAAGGCGTCATTGTTTTGTCTGAGGCAAAAG
```

```
 781 CATCGGAAAGCCCGATGGTCAAGCCTTCGCGCATCAGGAATATCATGATGGCGTTCGGTG

 841 CGGCTCTCATGGCGGGTGTGACGCTTGCGTTTTTTCTCCATTTTCTTGATGAAACCGTTA

 901 AAAGCGAGCGGCAGCTCAGCGAAAAAACAGACTTGCCTGTTTTAGGGGTTGTGTATGACA

 961 TCAAAAATCAGCAGACACGGTCTGATGAAAAACATTTCGGGGAGTGAGGCGTTTGGGATT

1021 CAGAAAAAAGAAATCAAGAAGGGGACTGGCTCAAATATCCGTTTTACATCACAAATCATT

1081 GGTGGCTGAACAATACCGCACCATTCGGACAAATATTGAGTTCTCCTCTGTTCAGACCCA

1141 TTTGCGCTCTATTCTCGTCACTTCTTCCGTTCCGGGAGAAGGAAAATCATTCAGCGCCGC

1201 CAACCTTGCCGCGGTATTTGCGCAGCAGGAAAAAAAGGTGCTGCTCGTCGATGCGGATTT

1261 ACGAAAACCGACGATACACGAGACCTATCAGCTTGAAAATGTACAAGGCCTTACGAATGT

1321 GTTAGTCGGAAACGCTTCCCTCGGCGAAACGGTGCAAAAAACGCTGATTGATAACCTTTA

1381 CGTCTTAACGAGCCGGCCGACGCCGCCGAAT

      <<<<<<<<<<<<<<<<<<
```

```
KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

Statistics
Pair Stats:
considered 1, primer in pair overlaps a primer in a better pair 1, ok 1
libprimer3 release 2.3.6
```

IX. 11.3. primer design of *-slr-epsA-epsB-gfp* for verification

Primer3 Output

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any    3' seq
LEFT PRIMER        224   19   54.96   52.63  2.00  0.00 ACTCATCTTCCGTGTCTCC
RIGHT PRIMER      2140   20   54.58   50.00  2.00  0.00 GTCTTGTAGTTCCCGTCATC
SEQUENCE SIZE: 2545
INCLUDED REGION SIZE: 2545

PRODUCT SIZE: 1917, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00

    1 CAGTAACGCTTCCGTTCTAAAAATGATCTGACTTCCTGAACGGACAAGCCGAGTTCTTTG

   61 GCTTCCTGCATCAGAGCTTTCCATTCCTCAATGTTGGATTCAGTCAGTTTTCTGTTGCGG

  121 TAGGGTGCGGGCTCCGGGCGTTTTTCAGTCAGTTTGTTCGTAAAGGTGAACAGTTCTTCT

  181 TTCGGCATGCCCGACTGCACGGCCTGCACAAGGTGGATGCGCCACTCATCTTCCGTGTCT
                                                 >>>>>>>>>>>>>>>>>

  241 CCCATGTGATGCAGCATCGTTTCGGCATCAAAAAGCTCCGTCAGATCGACCTGCAGGGTG
      >>

  301 GCAGATACCTTTTTCAGAAATTGGATGGATGGATTTGAGTGAACCCCCCGTTCTATCTTG

  361 CTTAAATAAGATTTAGAGACACCTGCTTCAACTGCTAGCTGATTAATAGAGTAGCCTTTT

  421 CTTCTGCGGTATAAACGAATTATTCTTCCAATCATTGTGCTGAATTCTCCCCTGTATACT

  481 GGCGTTTTTTTGTTCATTATAAGAAATTTTTCGTTCTTTATAAAATTTAAAATTATAAGG

  541 TAAGTGCAGTAAATAAGAGGAAAATCATGATAATGTTCTTTAAAAAGAACTAAATGGCTT

  601 AATTTGAAATTTTCAAATTTCGACCTTTTCTTTTATAATCCAATCATTAACAGAAGGGGG

  661 CGTTTAAGCCTGATGCAATAAGGATGAGGCTGTAATTACATGAATGAGAATATGAGTTTT
```

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 721 AAAGAATTATTTGACATTATTAAACACAGATTTTTACTGATTTTTATCATGACAGCAGTT

 781 GTAACGCTGGTGACGGGATACATCCAATTCCGGGTGATTTCGCCCGTTTATCAGGCATCA

 841 ACCCAAGTGCTCATTCATGAAACAAGCGGTGAAAAAAATTCGAATCTCAGCGACGTTCAG

 901 CTGAATCTTCATTACAACAATACGTTTCAAACGATAATGAAAAGCCCGGTAGTGCTTGAG

 961 AAAGTGAAGCAGACGCTGCATCTTTCTGAGACGGCATCCGCTTTAAAAGCAAAGATCACG

1021 ACAAGCAGCGAAACCGATTCAGAGATCATAACCATAGCGGTGCAGGACGAAAATCCGAAA

1081 CAGGCCGCCGCTATAGCGAACACGCTGATGAAGACATTTAAAAAAGAAGTCCGTGACAGG

1141 ATGAATATAAAAGGCGTCATTGTTTTGTCTGAGGCAAAAGCATCGGAAAGCCCGATGGTC

1201 AAGCCTTCGCGCATCAGGAATATCATGATGGCGTTCGGTGCGGCTCTCATGGCGGGTGTG

1261 ACGCTTGCGTTTTTTCTCCATTTTCTTGATGAAACCGTTAAAAGCGAGCGGCAGCTCAGC

1321 GAAAAAACAGACTTGCCTGTTTTAGGGGTTGTGTATGACATCAAAAATCAGCAGACACGG

1381 TCTGATGAAAAACATTTCGGGGAGTGAGGCGTTTGGGATTCAGAAAAAAGAAATCAAGAA

1441 GGGGACTGGCTCAAATATCCGTTTTACATCACAAATCATTGGTGGCTGAACAATACCGCA

1501 CCATTCGGACAAATATTGAGTTCTCCTCTGTTCAGACCCATTTGCGCTCTATTCTCGTCA

1561 CTTCTTCCGTTCCGGGAGAAGGAAAATCATTCAGCGCCGCCAACCTTGCCGCGGTATTTG

1621 CGCAGCAGGAAAAAAAGGTGCTGCTCGTCGATGCGGATTTACGAAAACCGACGATACACG

1681 AGACCTATCAGCTTGAAAATGTACAAGGCCTTACGAATGTGTTAGTCGGAAACGCTTCCC
```

```
1741 TCGGCGAAACGGTGCAAAAAACGCTGATTGATAACCTTTACGTCTTAACGAGCCGGCCGG

1801 AAGGAGATATACATATGGCTAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTC

1861 TTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAG

1921 GTGATGCTACATACGGAAAGCTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTG

1981 TTCCATGGCCAACACTTGTCACTACTTTGACCTATGGTGTTCAATGCTTTTCCCGTTATC

2041 CGGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGG

2101 AACGCACTATATCTTTCAAAGATGACGGGAACTACAAGACGCGTGCTGAAGTCAAGTTTG
                          <<<<<<<<<<<<<<<<<<<

2161 AAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAA

2221 ACATTCTCGGACACAAACTCGAGTACAACTATAACTCACACAATGTATACATCACGGCAG

2281 ACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTCGCCACAACATTGAAGATGGAT

2341 CCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTT

2401 TACCAGACAACCATTACCTGTCGACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGC

2461 GTGACCACATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTACACATGGCATGGATG

2521 AGCTCTACAAATAATGAATTCGAGC
```

KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS

		start	len	tm	gc%	any	3'	seq
1	LEFT PRIMER	327	19	55.18	47.37	3.00	0.00	GGATGGATTTGAGTGAACC
	RIGHT PRIMER	2140	20	54.58	50.00	2.00	0.00	GTCTTGTAGTTCCCGTCATC

PRODUCT SIZE: 1814, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00

```
 2 LEFT PRIMER        470   19   55.28   47.37  8.00  0.00 CCCTGTATACTGGCGTTTT
   RIGHT PRIMER      2425   20   55.40   50.00  6.00  3.00 GTCGACAGGTAATGGTTGTC
   PRODUCT SIZE: 1956, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 0.00

 3 LEFT PRIMER        470   19   55.28   47.37  8.00  0.00 CCCTGTATACTGGCGTTTT
   RIGHT PRIMER      2422   20   55.41   50.00  4.00  1.00 GACAGGTAATGGTTGTCTGG
   PRODUCT SIZE: 1953, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 0.00

 4 LEFT PRIMER        297   19   54.68   47.37  4.00  0.00 GGTGGCAGATACCTTTTTC
   RIGHT PRIMER      2140   20   54.58   50.00  2.00  0.00 GTCTTGTAGTTCCCGTCATC
   PRODUCT SIZE: 1844, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00

Statistics
         con    too     in     in          no     tm     tm  high  high         high
         sid   many    tar   excl    bad     GC    too    too   any    3'  poly   end
        ered     Ns    get    reg    GC% clamp    low   high compl compl     X  stab    ok
Left    3568      0      0      0  3015      0      0    310     0     2    17    36   188
Right   3553      0      0      0  2830      0      4    337     0     4     0    49   329
Pair Stats:
considered 153, unacceptable product size 143, ok 10
primer3 release 1.1.4


(primer3_results.cgi release 0.4.0)
```

IX. 11.4. Sequence of *slr-epsA-epsB-epsC* cloned in pGEM-T Easy plasmid

>pG::*slr-epsB*/FZB42-A_M13uni-21 -- 12..1009 of sequence:

TAGGGCGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTC**GATT**

CATGACTGCAGTTGTAACGCTGGTGACGGGATACATCCAATTCCGGGTGATTTCGCCCGTTTATCAGGCATCAACCC
AAGTGCTCATTCATGAAACAAGCGGTGAAAAAAATTCGAATCTCAGCGACGTTCAGCTGAATCTTCATTACAACAAT
ACGTTTCAAACGATAATGAAAAGCCCGGTAGTGCTTGAGAAAGTGAAGCAGACGCTGCATCTTTCTGAGACGGCATC
CGCTTTAAAAGCAAAGATCACGACAAGCAGCGAAACCGATTCAGAGATCATAACCATAGCGGTGCAGGACGAAAATC
CGAAACAGGCCGCCGCTATAGCGAACACGCTGATGAAGACATTTAAAAAAGAAGTCCGTGACAGGATGAATATAAAA
GGCGTCATTGTTTTGTCTGAGGCAAAAGCATCGGAAAGCCCGATGGTCAAGCCTTCGCGCATCAGGAATATCATGAT
GGCGTTCGGTGCGGCTCTCATGGCGGGTGTGACGCTTGCGTTTTTTCTCCATTTTCTTGATGAAACCGTTAAAAGCG
AGCGGCAGCTCAGCGAAAAAACAGACTTGCCTGTTTTAGGGGTTGTGTATGACATCAAAAATCAGCAGACACGGTCT
GATGAAAAACATTTCGGGGAGTGAGGCGTTTGGGATTCAGAAAAAAGAAATCAAGAAGGGGACTGGCTCAAATATCC
GTTTTACATCACAAATCATTGGTGGCTGAACAATACCGCACCATTCGGACAAATATTGAGTTCTCCTCTGTTCAGAC
CCATTTGCGCTCTATTCTCGTCACTTCTTCCGTTCCGGGAGAAGGAAAATCATTCAGCGCCGCCCACCTTGCCGCGG
TATTTGCGCAGCAGGAAAAAAAGGTGCTGCTCGTCGATGCGGATTTACGAAAACCGACGATACACGAGACCTATCAG
CTTGAAATGGAC **[936 nt]**

>pG::*slr-epsB*/FZB42-A_M13rev-29 -- 14..980 of sequence:

GGACCTATAGAATACTCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGA
ATTC*ACTAGT***GATT**

TGATATCCTATAACAACGGAAAGTAAAACGAGATACGTATCCAATGCAAAAATCATTGAAAGTCTTCTCCGGTAAGT
CAATCTTTCCCCTCCTCATTTATCGGTTTAGCTGATACGGTTTTCGTAAAAACAGACATAACAGCTAAGCCGATAAC
AATGCATCATCTTAAGATAAAATTGGGCATTTAACCCGTCCTCACACCCCGCCACCGACGACCAGCATCTAAGGCGG
CGAAAAGCGCCGCCCACAGCAGAGCCGAGTGCCTCCGTTGATAAAGGTAAGGAGACATCACCTGCACCGCTCAATAC
GAGTAGTGCTCCGTTTTTTTGATTTTCTTTTTATTCAGCAGCGCCCCCAAAAGCTTTGCCTTTGACTGCTGGAGCGC
GTCTCTCGCCTTTTGGACGGTATCCATTTTCGTTTTTCCGCTCAAGACGACGAGAACGCTTCCATCTGTCTGGTTCG
CTAAAATCTGTCCGTCCGCCACAGCTAAAAGCGGAGGTGAATCGAAAATAACCAGACTGTACCGGCTGTACATCTCC
TGAATCAGCTCTCCCATCGCCTTTGAAGATAAAAGCTCAGCCGGATTCGGCGGCGTCGGCCCGCTCGTTAAGACGTA
AAGGTTATCAATCAGCGTTTTTTGCACCGTTTCGCCGAGGGAAGCGTTTCCGACTAACACATTCGTAAGGCCTTGTA

CATTTTCAAGCTGATAGGTCTCGTGTATCGTCGGTTTTCGTAAATCCGCATCGACGAGCAGCACCTTTTTTTCCTGC
TGCGCAAATACCGCGGCAAGGTTGGCGGCGCTGAATGATTTTCCTTCTCCCGGAACGGAAGAAGTGACGAGAATAGA
GCGCAAATGGGTCTGAACAGAGGAGAACC **[876 nt]**

Antiparallel sequence:

GGTTCTCCTCTGTTCAGACCCATTTGCGCTCTATTCTCGTCACTTCTTCCGTTCCGGGAGAAGGAAAATCATTCAGC
GCCGCCAACCTTGCCGCGGTATTTGCGCAGCAGGAAAAAAAGGTGCTGCTCGTCGATGCGGATTTACGAAAACCGAC
GATACACGAGACCTATCAGCTTGAAAATGTACAAGGCCTTACGAATGTGTTAGTCGGAAACGCTTCCCTCGGCGAAA
CGGTGCAAAAAACGCTGATTGATAACCTTTACGTCTTAACGAGCGGGCCGACGCCGCCGAATCCGGCTGAGCTTTTA
TCTTCAAAGGCGATGGGAGAGCTGATTCAGGAGATGTACAGCCGGTACAGTCTGGTTATTTTCGATTCACCTCCGCT
TTTAGCTGTGGCGGACGGACAGATTTTAGCGAACCAGACAGATGGAAGCGTTCTCGTCGTCTTGAGCGGAAAAACGA
AAATGGATACCGTCCAAAAGGCGAGAGACGCGCTCCAGCAGTCAAAGGCAAAGCTTTTGGGGGCGCTGCTGAATAAA
AAGAAAATCAAAAAAACGGAGCACTACTCGTATTGAGCGGTGCAGGTGATGTCTCCTTACCTTTATCAACGGAGGCA
CTCGGCTCTGCTGTGGGCGGCGCTTTTCGCCGCCTTAGATGCTGGTCGTCGGTGGCGGGGTGTGAGGACGGGTTAAA
TGCCCAATTTTATCTTAAGATGATGCATTGTTATCGGCTTAGCTGTTATGTCTGTTTTTACGAAAACCGTATCAGCT
AAACCGATAAATGAGGAGGGGAAAGATTGACTTACCGGAGAAGACTTTCAATGATTTTTGCATTGGATACGTATCTC
GTTTTACTTTCCGTTGTTATAGGATATCA

Reconstructed sequence: [1627 nt]

CATGACTGCAGTTGTAACGCTGGTGACGGGATACATCCAATTCCGGGTGATTTCGCCCGTTTATCAGGCATCAACCC
AAGTGCTCATTCATGAAACAAGCGGTGAAAAAAATTCGAATCTCAGCGACGTTCAGCTGAATCTTCATTACAACAAT
ACGTTTCAAACGATAATGAAAAGCCCGGTAGTGCTTGAGAAAGTGAAGCAGACGCTGCATCTTTCTGAGACGGCATC
CGCTTTAAAAGCAAAGATCACGACAAGCAGCGAAACCGATTCAGAGATCATAACCATAGCGGTGCAGGACGAAAATC
CGAAACAGGCCGCCGCTATAGCGAACACGCTGATGAAGACATTTAAAAAAGAAGTCCGTGACAGGATGAATATAAAA
GGCGTCATTGTTTTGTCTGAGGCAAAAGCATCGGAAAGCCCGATGGTCAAGCCTTCGCGCATCAGGAATATCATGAT
GGCGTTCGGTGCGGCTCTCATGGCGGGTGTGACGCTTGCGTTTTTTCTCCATTTTCTTGATGAAACCGTTAAAAGCG
AGCGGCAGCTCAGCGAAAAAACAGACTTGCCTGTTTTAGGGGTTGTGTATGACATCAAAAATCAGCAGACACGGTCT
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Sequences producing significant alignments:

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3 Select seq gb|CP007244.1|	Bacillus amyloliquefaciens subsp. plantarum TrigoCor1448, complete genome	2950	2950	100%	0.0	99%	CP007244.1
4 Select seq	Bacillus amyloliquefaciens subsp. plantarum NAU-B3, complete genome	2924	2924	100%	0.0	99%	HG514499.1

Select for downloading or viewing reports	Description	Max score	Total score	Query cover	E value	Ident	Accession
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Alignment statistics for match #1

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IX.12 List of publications

- **10th The European Symposium in Biochemical Engineering Sciences and 6th International Forum on Industrial Bioprocesses in collaboration with ACS. Lille, September 7-11, 2014. France. Poster presentation titled, (High-throughput screening of root exudates effect on bacterial growth of *Bacillus amyloliquefaciens* FZB42 and *Bacillus subtilis* BBG131 and their lipopeptides production)**
- **6th Congress of European Microbiologists (FEMS). Maastricht, June 7-11, 2015. The Netherlands. Poster presentation titled: (Effect of root exsudates and different carbon sources on bacterial growth of *Bacillus amyloliquefaciens* and *Bacillus subtilis* and their lipopeptides production).**
- **10th International PGPR Workshop. Liège, June 9, 2015. Belgium. Poster presentation titled: (Rhizosphere colonisation influenced by root exudates compositions and surfactin production by *Bacillus amyloliquefaciens* FZB42 and *Bacillus subtilis* BBG131).**

- **Annual conference/microbiology society. Liverpool, Mars 20-24. 2016. United Kingdom. Poster presentation titled: (Rhizosphere colonisation by Bacillus amyloliquefaciens FZB42 and Bacillus subtilis BBG131is influenced by different factors)**
- **The 68th International Symposium on Crop Protection. Gent, May, 2016. Belgium. Oral presentation titled:(Study of factors influencing the colonisation of tomato rhizosphere by *Bacillus* strains)**

- **Different rhizosphere colonisation potential of *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131 as affected by surfactin production and tomato root exudates (submitted publication)**

High-throughput screening of root exudates effect on bacterial growth of *Bacillus amyloliquefaciens* FZB42 and *Bacillus subtilis* BBG131 and their lipopeptides production

Ameen Al-Ali[a b] , François Krier[a], Philippe Jacques[a]*

[a] Research Institute Charles Viollette, ProBioGEM team, Université Lille 1 Sciences et Technologie, Polytech'Lille, Avenue Paul Langevin, 59655 Villeneuve d'Ascq cedex, France

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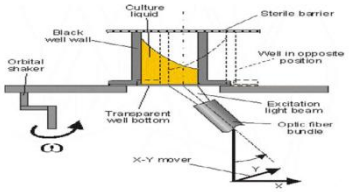
[b] Babylon University, Irak, department of soil and biology sciences

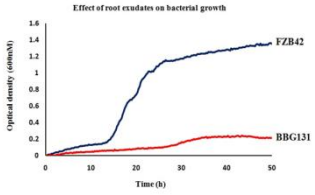
Rhizosphere is the region that is closed to the roots of plant. Plant root exudates consist of a complex mixture of organic acids, sugars and amino acids which are used as substrates for the growth of bacteria colonizing this rhizosphere. In this work, two *Bacillus* strains producing different lipopeptides were studied. *Bacillus amyloliquefaciens* FZB42 is a natural wild-type strain which produces three families of lipopeptides (fengycin, surfactin and bacillomycin) and which shows a high potential as biopesticide for the control of plant diseases. *Bacillus subtilis* BBG131 is a genetically engineered microorganism which overproduces surfactin. A previous study has shown a different colonizing potential of these strains in the tomato rhizosphere. *B. amyloliquefaciens* FZB42 appears as a good colonizer while *B. subtilis* BBG131 not. In order to explain these behavior differences, the effect of root exudates components (five different sugars) was evaluated on the growth and surfactin production by these strains by using the micro fermentation system named Biolector.

As it was long and difficult to obtain a high volume of root exudates, it was necessary to perform the bacterial cultures in small volumes. We choosed to use the Biolector system which allowed us a fast screening of the effects of different culture media in the same time. We followed the kinetic of bacterial growth, the oxygen consumption and the pH values for each condition.



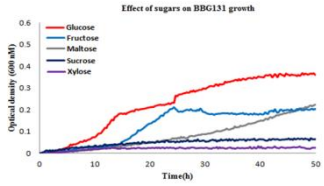
Biolector chamber incubator description

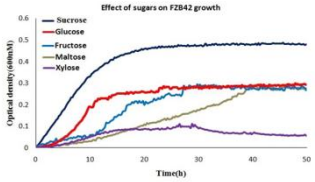
Effect of root exudates on bacterial growth



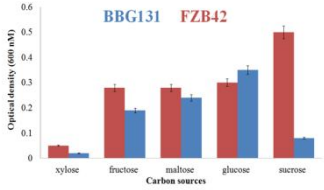
Effect of tomato *Solanum lycopersicum* root exudates on BBG131and FZB42 growth. Root exudates allowed the growth of two strains but the final biomass with FZB42 after 48h at 37°C was higher than with BBG131.

Effect of sugars on FZB42 and BBG131 growth



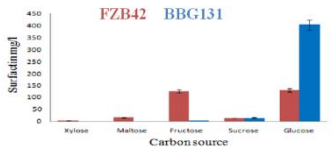


Growth kinetic of FZB42 and BBG131 in a minimal medium containing different sugars as sole carbon source. The results showed different behavior of these strains especially with sucrose (red arrows)



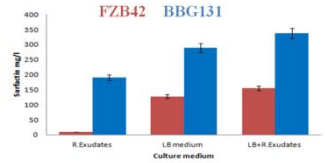
Final optical density of FZB42 and BBG131 after 48h incubation at 37°C with different sugars used as carbon sources. The main difference was obtained with sucrose : the FZB42 final biomass is five times higher than with BBG131.

Effect of sugars and root exudates on surfactin production by FZB42 and BBG131



Effect of sugars on surfactin production by FZB42 and BBG131.. Surfactin was not detected in presence of xylose, maltose and fructose with BBG131. The best sugar for surfactin production was glucose and, with glucose, production of surfactin by BBG131 is four times higher than with FZB42.

The two strains showed different behaviors with the root exudates and the different the sugars.
With root exudates, final biomass of FZB42 is higher than with BBG131 but surfactin production by BBG131 was much higher than with FZB42.
Sucrose is a good substrate for FZB42 growth and for BBG131.
Surfactin is produced with the five sugars by FZB42 but only with sucrose and glucose by BBG131.
Surfactin production by BBG131 is higher than with FZB42 with glucose.



Effect of root exudates on surfactin production by FZB42 and BBG131 after 72h at 37°C. R.exudates: only root exudates, LB : Luria Burtani medium, LB + R.exudates : Luria Burtani medium + root exudates (3/1). Root exudates are efficient for surfactin production. With only root exudates as culture medium, surfactin production by BBG131 is much higher than with FZB42.

Root exudates have different influences on bacterial growth and surfactin production by *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131. With the root exudates and the tested sugars, the FZB42 growth is better than BBG131 growth. On the contrary, with these substrates, surfactin production is much higher with BBG131 than with FZB42. These results seem to confirm that FZB42 is a good root colonizer and BBG131 is a good surfactin producer.
The Biolector micro system is a very efficient tools to quickly test the influence of a lot of different substrates at the same time on bacterial growth.

Aknowledgements : thanks to REALCAT platform for the use of the Biolector system. *European Symposium on Biochemical Engineering Sciences and 6th International Forum on Industrial Bioprocesses . Lille, September 7-10, 2014, France*

Effect of root exsudates and different carbon sources on bacterial growth of *Bacillus amyloliquefaciens* and *Bacillus subtilis* and their lipopeptides production

Ameen Al-Ali[a b] , François Krier[a], Philippe Jacques[a]*

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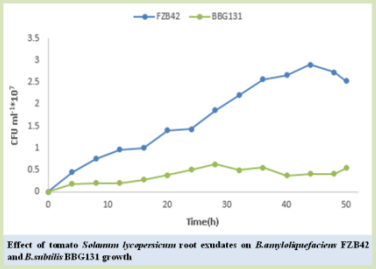
[b] Babylon University, Irak, department of soil and biology sciences

Several natural compounds produced by various microorganisms, are commonly synthesized by non ribosomal peptides synthetases (NRPS). Among these, lipopeptides which play an important role in sustainable agriculture as biocontrol agents to kill or suppress plant pathogens. Different strains of *B. subtilis* and *B. amyloliquefaciens* isolated from the rhizosphere are the most efficient biocontrol agents. They produce different families of cyclic lipopeptides such as Iturin, Fengycin and Surfactin. The root exudates, organic compounds released from living plants, is the most important part of the rhizosphere. They play an important role to induce bacterial colonization in the rhizosphere.

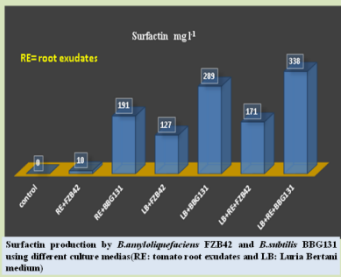
The aim of this work was to study the effect of root exudates and some of their compositions on the bacterial growth and lipopeptides production by *B. subtilis* BBG131 and *B. amyloliquefaciens* FZB42, as a first step to understand the relation between bacteria and plant in the rhizosphere.

Root exudates (RE): were collected from hydroponic culture of tomato *Solanum lycopersicum*. Tomatoes seeds were sterilized using 70% ethanol for two minutes and sodium hypochlorite for 15 minutes, then they were washed five times with sterilized water. All the solutions from 21-days old hydroponic culture were recovered and filtered through 0.22µm filter and then concentrated using speed vacuum.

Tomato plant in hydroponic culture



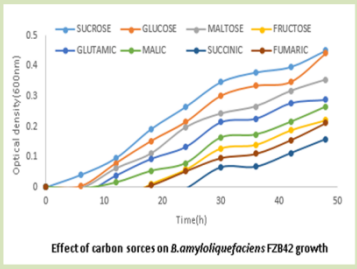
Effect of tomato *Solanum lycopersicum* root exudates on *B.amyloliquefaciens* FZB42 and *B.subtilis* BBG131 growth



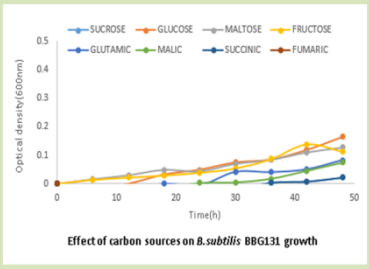
Surfactin production by *B.amyloliquefaciens* FZB42 and *B.subtilis* BBG131 using different culture medias(RE: tomato root exudates and LB: Luria Bertani medium)

The two strains have the ability to grow in tomato root exudates, but the final biomass of FZB42 was higher than with BBG131. Surfactin production was higher with BBG131 than with FZB42.

Effect of carbon sources on *B.amyloliquefaciens* FZB42 and *B.subtilis* BBG131 growth



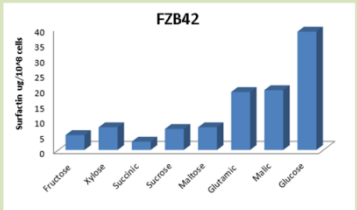
Effect of carbon sorces on *B.amyloliquefaciens* FZB42 growth



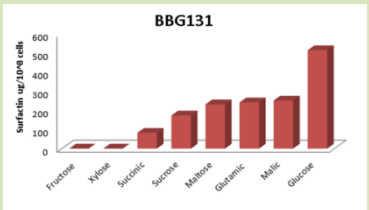
Effect of carbon sources on *B.subtilis* BBG131 growth

Eight carbon sources were used in this study to find out their effect on bacterial growth as stimulated the rhizosphere nutrition condition because these carbon sources are found in root exudates. In general, the results showed a significant difference between the two strains to consumption the different carbon sources. These results may lead to that FZB42 is more adaptable in the rhizosphere than BBG131.

Effect of carbon sources on surfactin production by *B.amyloliquefaciens* FZB42 and *B.subtilis* BBG131



Effect of carbon sources on surfactin production by *B.amyloliquefaciens* FZB42



Effect of carbon sources on surfactin production by *B.subtilis* BBG131

The two strains showed different behaviors to use the eight carbon sources. Final biomass of FZB42 is higher than with BBG131 but surfactin production by BBG131 was much higher than with FZB42.
Sucrose is a good substrate for FZB42 growth and for BBG131.
Surfactin is produced with the five sugars by FZB42 but only with sucrose and glucose by BBG131.
Surfactin production by BBG131 is higher than with FZB42 with glucose.

Root exudates have different influences on bacterial growth and surfactin production by *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131. With the root exudates and the tested carbon sources, the FZB42 growth is better than BBG131 growth. On the contrary, with these substrates, surfactin production is much higher with BBG131 than with FZB42. These results seem to confirm that FZB42 is a good root colonizer and BBG131 is a good surfactin producer. As a results of bacterial growth in root exudates and different carbon sources for the two strains it seems that the surfactin production is not necessary for root colonization.

6th Congress of European Microbiologists. Masstricht, June 7-11, The Netherlands

Rhizosphere colonization influenced by root exudates compositions and surfactin production by *Bacillus amyloliquefaciens* FZB42 and *Bacillus subtilis* BBG131

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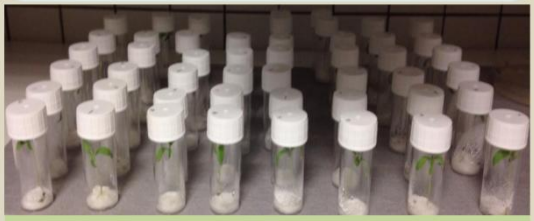
Introduction:

Plant growth promoting bacteria (PGPR) are an indispensable part of rhizosphere biota that when grown in association with the host plants can stimulate the growth of the host. PGPR promote plant growth directly by either facilitating resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents. Different strains of *B. subtilis* and *B. amyloliquefaciens* are classified as PGPR. Among these, several strains produce different non-ribosomal peptides such as cyclic lipopeptides. Surfactin, fengycin and Iturin are cyclic lipopeptides produced by *B.subtilis*, and *B. amyloliquefaciens*. They play an important role in the rhizosphere to suppress the plant pathogens and thus, it improves the relationship between PGPR and host plant. The colonization is the most important criterion to PGPR and is influenced by different factors: plants species, PGPR and their lipopeptides production, rhizosphere conditions, root exudates and their compositions.

In this work, we highlighted to study the effect of root exudates, and some of their compositions on the rhizosphere colonization, in addition, to study the surfactin role on this criterion. Two strains, *B. subtilis* BBG131, and *B. amyloliquefaciens* FZB42 were chosen to evaluate their behavior in the rhizosphere and their growth in the root exudates and some of carbon sources found in root exudates. *B. subtilis* BBG131 produces only surfactin and *B. amyloliquefaciens* produces three lipopeptides families: Iturin, Fengycin, and Surfactin.

Materials and methods

Root exudates (RE): were collected from the hydroponic culture of tomato *Solanum lycopersicum*. Tomatoes seeds were sterilized using 70% ethanol for two minutes and sodium hypochlorite for 15 minutes, then they were washed five times with sterilized water. All the solutions from 21-days old hydroponic culture were recovered and filtered through 0.22µm filter and then concentrated using speed vacuum.



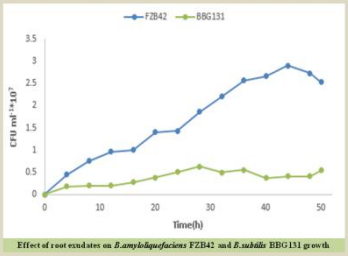
Sterilized hydroponic culture of tomato plant *Solanum lycopersicum*

Tomato plant in perlite for study the rhizosphere colonization

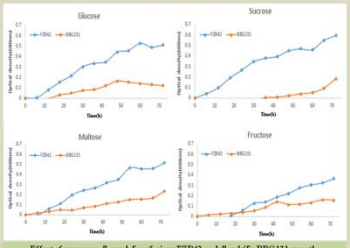
- To stimulate the natural conditions in the rhizosphere for the purpose of studying two species of bacteria in isolation from other microorganisms, an experiment was carried out in greenhouse using sterilized tubes containing perlite.
- Three grams of perlite were put in each tube and Hoagland solution were used as a plant nutrition solution as nine milliliters in each tube.
- After seeds germination, two seeds were put in each tube and every three days three tubes were used for bacterial count plate and other three tubes were used for surfactin extraction, using a mix of pure acetonitrile and 0.1% formic acid.

Results

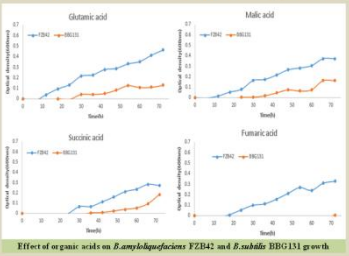
Effect of carbon sources and tomato root exudates on *B.amyloliquefaciens* FZB42 and *B.subtilis* BBG131 growth



Effect of root exudates on *B.amyloliquefaciens* FZB42 and *B.subtilis* BBG131 growth



Effect of sugars on *B.amyloliquefaciens* FZB42 and *B.subtilis* BBG131 growth

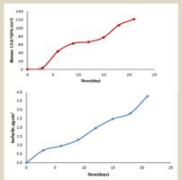


Effect of organic acids on *B.amyloliquefaciens* FZB42 and *B.subtilis* BBG131 growth

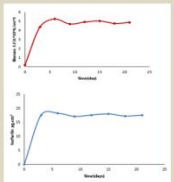
Root exudates and different carbon sources were tested to evaluate their effect on bacterial growth and surfactin production. The two strains, *B.amyloliquefaciens* and *B.subtilis* showed the ability to grow into tomato root exudates. The results showed a significant different in bacterial growth between *B.amyloliquefaciens* and *B.subtilis*. Glucose, sucrose and maltose were the best carbon sources for *B.amyloliquefaciens FZB42*. In all carbon sources the final biomass *of B. subtilis BBG131* was less than with *B.amyloliquefaciens* FZB42. There was no growth of *B.subtilis* BBG131 with fumaric acid.. The biomass of *B.amyloliquefaciens* FZB42 was higher than biomass of *B. subtilis* BBG131. These different results to consumption of the carbon sources can be explained by the different behavior of the two strains in the rhizosphere and their growth with root exudates.

Root and rhizosphere colonized by *B.amyloliquefaciens* FZB42 and *B.subtilis* BBG131 growth

- *B.amyloliquefaciens* FZB42 colonized well the rhizosphere through 21 days as a comparison with *B. subtilis* BBG131.
- The production of surfactin was not necessary to colonize the rhizosphere.

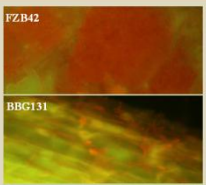


Rhizosphere colonization and surfactin production by *B.amyloliquefaciens* FZB42



Rhizosphere colonization and surfactin production by *B.subtilis* BBG131 through 21days

- Rhizoplane colonization showed that the *B. amyloliquefaciens* FZB42 colonized almost all the tomatoes rhizoplane.
- *B. subtilis* BBG131 colonized the rhizoplane by microcolonies and it appears as a longitudinal edge of the wall of root cells.



Root colonization by *B.amyloliquefaciens* FZB42 and *B.subtilis* BBG131 after 21days

Conclusion:

The two strains showed the ability to consume the root exudates and carbon sources. The different between *B.amyloliquefaciens* FZB42 and *B.subtilis* BBG131 was clear, *B.amyloliquefaciens* FZB42 presented a high growth in all carbon sources studied as compared with *B.subtilis*. The high different between the two strains reflect on their behavior in the rhizosphere. *B.amyloliquefaciens* FZB42 showed a better colonization than *B.subtilis* BBG131 while the surfactin production showed a contrary. It seems that surfactin production is not the sole factor influencing on the colonization.

10th International PGPR Workshop Liège, Belgium – 2015

Rhizosphere colonization by *Bacillus amyloliquefaciens* FZB42 and *Bacillus subtilis* BBG131 is influenced by different factors

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Introduction:

Many of bacterial genera isolated from rhizosphere have an important effect on stimulating plant growth in the rhizosphere. These bacteria classified as plant growth promoting rhizobacteria (PGPR) have a direct effects on host plant by either facilitating resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels and indirect effects by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents. Several strains of *Bacillus amyloliquefaciens* and *Bacillus subtilis* which produce different non-ribosomal peptides such as cyclic lipopeptides can be classified as PGPR. Surfactin, fengycin and iturin are cyclic lipopeptides produced by these strains. These molecules play an important role in the rhizosphere to suppress the plant pathogens and thus, they improve the relationship between PGPR and host plant. The rhizosphere colonization is the crucial criterion for the bacteria used as a biocontrol agent and may be influenced by biotic and abiotic factors. The current work represents a comparative study between the two strains: *Bacillus amyloliquefaciens* FZB42 and *Bacillus subtilis* BBG131 in an attempt to understand the nature of the conditions which affect the colonization. *B.amyloliquefaciens* FZB42 produces three lipopeptides families: Iturin, Fengycin, and Surfactin while, *B. subtilis* BBG131 produces only surfactin. The colonization of rhizosphere by the two strains was studied and the behavior of the two strains in the rhizosphere was evaluated using root exudates and their composition as an important factor influencing the rhizosphere colonization.

Materials and methods

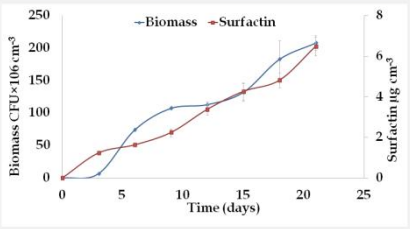
- To study the comportement of the two strains *B. amyloliquefaciens* FZB42 and *B. subtilis* BBG131, tomato plant was used as a host plant.
- Tomato seeds were sterilized, respectively, using ethanol 70% and sodium hypochlorite and were washed three times in sterilized water.
- The sterilized seeds were left for germination during 4 days on filter papers.
- The germinated seeds were planted in sterilized conditions using tubes containing Hoagland solution and perlite.
- Bacterial population cells were counted using bacterial count plate and surfactin quantification was performed using HPLC.

Tomato plant in the rhizosphere

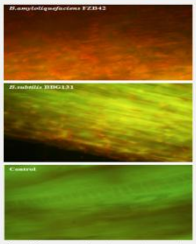
- Based on the results of rhizosphere colonization and stimulated to the rhizosphere conditions, two factors were studied: root exudates and their composition.
- Root exudates were collected from the hydroponic culture of tomato *Solanum lycopersicum*, concentrated and filtered through 0.22 µm filter.
- Root exudates and different carbon sources found in the tomato root exudates were used as a carbon source for bacterial growth.
- The different carbon sources were dissolved in the minimum medium in the reason of 2 g C L^{-1} and the C:N ratio was (8:1).

Results

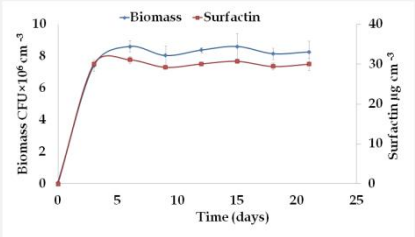
Tomato rhizosphere colonization by *B.amyloliquefaciens* FZB42 and *B.subtilis* BBG131 and surfactin production



Biomass and surfactin produced by *B.amyloliquefaciens* FZB42 in the tomato rhizosphere



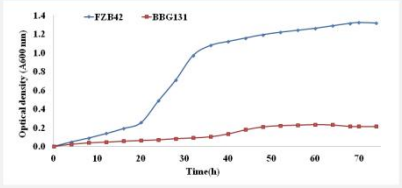
Bacillus on the tomato roots



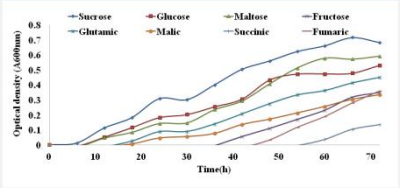
Biomass and surfactin produced by *B. subtilis* BBG131 in the tomato rhizosphere

The biomass of *B.amyloliquefaciens* FZB42 increased during 21 days of experiment and the surfactin quantity produced by this strain was harmonized whith biomass while the population of *B.subtilis* BBG131 in the rhizosphere and surfactin produced by this strain increased only during the first three days and then were stable. *B. amyloliquefaciens* FZB42 colonized almost all the tomatoes rhizosphere while *B. subtilis* BBG131 colonized the rhizosphere by microcolonies. *B. amyloliquefaciens* FZB42 colonized well the rhizosphere but was a low surfactin producer whereas *B. subtilis* BBG131 presented the contrairy. The surfactin production was not necessary for rhizosphere colonization.

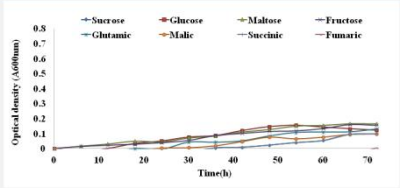
Effect of tomato root exudates and different carbon sources on the bacterial growth



Tomato root exudates and bacterial growth



Carbon sources and *B.amyloliquefaciens* FZB42 growth



Carbon sources and *B.subtilis* growth

Root exudates represent important factors in the rhizosphere and they are composed of different carbon sources such as organic acids and sugars. Some of them have been selected in this study. The results showed a significant difference between the two strains: *B. amyloliquefaciens* FZB42's growth was better than *B.subtilis* BBG131. The two strains showed a different behavior in the consumption of these different carbon sources. The biomass of *B. amyloliquefaciens* FZB42 in the root exudates was 6 times higher than that the one of *B. subtilis* BBG131.

Conclusion:

The different comportments presented in the rhizosphere by the two strains showed a high ability of *B. amyloliquefaciens* FZB42 to colonize the rhizosphere compared to *B. subtilis* BBG131. This difference may be explained by the growth of the two strains in the root exudates and some of their compositions as sole carbon sources in the rhizosphere. But this explication is not enough on regard for the proportion of biomass between the two strains in the rhizosphere and in root exudates: it was (25:1) (FZB42:BBG131) and (6:1) (FZB42:BBG131), respectivly. Thus, this study sheds light on other factors that may influence the colonization in addition to the effect of root exudates and their compositions. In regard of the quantities of surfactin produced by the two strains in the rhizosphere, the role of surfactin is not a crucial factor for rhizosphere colonization .

Annual Conference-Microbiology society. Mars 12-24. 2016 / Liverpool-UK