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PhD Course in Plant and Animal Sciences
– XXX cycle –
"*Doctor Europaeus*"

PhD Thesis

Evaluation of the antagonistic activity of *Bacillus amyloliquefaciens* against fungal pathogens of wheat

– AGR/12 –

by

Rocco Caracciolo

Dissertation submitted: 4 May 2018

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To my family

“It is through science that we prove, but through intuition that we discover.”

Henri Poincare

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Under the precious supervision of **Prof. Emilio Montesinos** and his collaborators **Dr. Jordi Cabrefiga** and **Dr. Isabel Mora** I performed the assays for the chemical and molecular characterization of cLPs produced by *B. amyloliquefaciens* strains (§ 4.1 .and § 4.2 – Chapter 3), the evaluation of the antifungal activity of bacterial filtrates against the four pathogens of wheat (§ 3.3 – Chapter 3) and other activities that have allowed me to acquire more skills in the different fields of plant pathology.

List of Abbreviations

%: Percentage.
~: Aproximatelly.
+: Positive.
§: Paragraph.
Ø: Diameter.
Abs: Absorbance.
AMC: Antimicrobial compound.
AMP: Antimicrobial peptide.
ANOVA: Analysis of variance.
BCA: Biocontrol agent.
bp: Base pair.
cfu: Colony-forming unit.
cLP: Cyclic lipopeptide.
CS: Contact suspension.
cv.: Cultivar.
DAPG: 2,4-Diacetylphloroglucinol.
DSA: Disease severity area.
DNA: Deoxyribonucleic acid.
DON: Deoxynivalenol.
DPI: Day post inoculation.
DSGP: Daily seed germination percentage.
e.g.: For example.
EFSA: European Food Safety Authority.
FAO: Food and Agriculture Organization.
FCR: Fusarium crown rot.
FDA: Food and Drug Administration.
FGI: Fungal germination inhibition.
FHB: Fusarium head blight.
FPLC: Fast protein liquid chromatography.
GRAS: Generally recognized as safe.
HCN: Hydrogen cyanide.
HPLC-MS: High performance liquid chromatography-mas spectrometry.
i.e.: That is.
IAA: Indole-3-acetic acid.
IH: Inhibition halo.
IPA: Indole-3-pyruvic acid.
IR: Inhibitio ratio.
ISR: Induced systemic resistance.
JA: Jasmonic acid.
LB: Lysogeny broth.
LBA: Lysogeny broth agar.
LBFA: Lysogeny broth filtrate agar.
LP: Lipopeptide.
LSB: Leaf spot blotch.
MGI: Mycelial growth inhibition.
Mipaaf: Ministero delle politiche agricole alimentari e forestali.
nm: Nanometer.
NRP: Non-ribosomal peptide.
NRPS: Non-ribosomal peptides synthetase.
OD: Optical density.
ORF: Open reading frame.
PB: Phosphate buffer.
PCR: Polymerase chain reaction.
PDA: Potato dextrose agar.
PDB: Potato dextrose broth.
PGPR: Plant growth promoting rhizobacteria.
PR: Pathogenesis-related.
QPS: Qualified Presumption of Safety.
rpm: Revolutions per minute.
SA: Salicylic acid.
SAR: Systemic acquired resistance.
SDW: Sterilized distilled water.
sp.: Species.
STB: Septoria tritici blotch.
subsp.: Subspecies.
syn.: Synonym.
TAE: Tris-acetate EDTA.
TFA: Trifluoracetic acid.
Trp: Tryptophan.
v/v: Volume percent.
ver.: Version.

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Summary

Wheat is one of the most important crop all over the world and its widespread diffusion makes it constantly exposed to many abiotic and biotic stresses. Among the latter, pathogenic fungi represent a serious threat to the cultivation of this cereal. Nowadays the use of biocontrol agents (BCAs) is one of the most successful strategies for the prevention and control of plant diseases.

This study deals with (i) the *in vitro* evaluation of the antifungal activity of three strains of *B. amyloliquefaciens* against *Fusarium graminearum, Fusarium culmorum, Bipolaris sorokiniana* and *Septoria tritici*, (ii) the molecular and chemical characterization of the compounds involved in the activity, (iii) the *in vivo* evaluation of the antifungal activity against *B. sorokiniana* and (iv) the *in vivo* wheat growth promotion carried out by *B. amyloliquefaciens* strains.

The inhibitory action showed by the filtrates and the extracts of the bacterial growth broths against all the tested pathogens highlighted the nature of the molecules involved, that are released, liposoluble and thermostable. The analysis of the presence of *ituA, fenD, surfA, bmyB* and *mycA* genes confirmed the potential production of cyclic lipopeptides (cLPs), such as iturin, fengycin, surfactin, bacillomycin and mycosubtilin, that are reported as antifungal compounds. The production of cLPs was confirmed with the characterization of the culture filtrates by FPLC and the antagonistic assays performed with the fractions obtained. It was confirmed that fengycins were mainly responsible of the antifungal activity, especially against *B. sorokiniana,* that was *in vivo* inhibited by *B. amyloliquefaciens* cells and their filtrates.

Wheat growth promotion was evaluated on seedlings after treatment with *B. amyloliquefaciens* suspensions. The three bacterial strains promoted germination of wheat seeds, seedling growth and root development, confirming to be potential biofertilizers for this cereal.

The results obtained confirm that *B. amyloliquefaciens* can be considered a valid BCA against the most important pathogens of wheat, and its use can be recommended in the formulation of commercial bio-pesticides.

Key Words: *Bacillus amyloliquefaciens*, Wheat, Biocontrol, Cyclic Lipopeptides, PGPR.

INTRODUCTION 1

1 Wheat

Cereals are the most cultivated plants in the world with a production of 2,611 million tonnes (FAO, 2017). They are used worldwide, contributing more than 50% of total human nutritional requirements (Mipaaf, 2017). They belong to the *Poaceae* family and caryopsis represent the edible part of their grains. Once it is grinded, the flour obtained is used to be transformed in a wide range of foodstuffs, that satisfy different food habits and traditions. Cereals are used in basic human nutrition, such as wheat and rice, but they are also the main product fed to animals, such as corn, oats and barely.

Among cereals, wheat, is considered the most important one for human consumption. The most common species are *Triticum durum* or durum wheat, used for pastification, and *Triticum aestivum* or common wheat, used for bread and bakery production.

Wheat is cultivated worldwide and its production is gradually increasing year by year; with 220.4 million hectares cultivated (FAOSTAT, 2014) and a world production of 749 million tonnes (FAO, 2017).

The global demand for this cereal, driven by the ever-growing human population's need for food, has led agricultural entrepreneurs to maximize the crop productivity. This process has been implemented through the intensification of the cropping systems, that brings with it several negative inputs. Examples of these are the exhaustion of soil's organic matter (causing the degradation of soil quality and increasing soil erosion); the over-use of chemical pesticides and the rise of greenhouse gas emissions (Gan et al. 2014).

In addition to environmental problems, wheat cultivation is constantly exposed to many abiotic and biotic stresses that negatively affect plant growth and development, reducing crop productivity (Qin et al. 2015; Figueroa et al. 2017). Among the latter, pathogenic fungi represent a serious and steady problem in wheat cultivation and furthermore could be a threat for human and animal health. For this reason, the management of the fungal diseases is one of the biggest priorities in this crop system.

2 Fungal diseases of wheat

In recent years, fungal diseases of wheat have emerged as a serious worldwide plant health and economical problem as they compromise crop yields and grain quality more than bacteria and virus ones (Goutam et al. 2015).
The most important wheat pathogenic fungi are: *Fusarium graminearum, Fusarium culmorum, Bipolaris sorokiniana* and *Septoria tritici.*

2.1 Fusarium head blight

Fusarium head blight (FHB) is caused meanly by *F. graminearum* (Schwabe) Group 2 [teleomorph, *Gibberella zeae* (Schw.) Petch]. Other pathogens that are associated with this disease are *F. culmorum* (Wm. G. Smith) Sacc. and *F. avenaceum* (Corda ex Fr.) Sacc. [teleomorph *G. avenacea* (Cook)] (Buerstmayr et al. 2012). FHB occurs in the principal wheat cultivation areas of Europe, North and South America and Asia, representing a global problem (Lenc et al. 2015; Rawat et al. 2016).
The fungus attacks every part of the plant. The infection starts from the ear, where *F. graminearum* colonizes the extruded anthers, the floral bracts, the caryopsis and finally the rachis. The initial symptoms involve the glumes that turn brown with water-soaked spots (Sutton 1982; Bai and Shaner 1994). The pathogen from the inoculation point can reach the near spikelets through the rachis and in few days the wilting and the premature death of the entire upper part of the ear is observed (Fig. 1).
FHB causes a severe reduction of grain yield and seed quality, reduces seed germination and leads to seedling blight. In addition, the grains infected with *Fusarium* spp. may become contaminated with mycotoxins, in particular with deoxynivalenol (DON), that are a serious threat to human and animal health (Lenc et al. 2015; Salgado et al. 2015; Palazzini et al. 2016; Payros et al. 2016).

Different strategies based on cropping practices, cultivar resistance and foliar fungicides application are used to minimize the crop losses and to control the disease (Willyerd et al. 2012; Clark et al. 2016).

Figure 1 | FHB symptoms on wheat ears (a and b) (Scherm et al. 2013).

2.2 Fusarium crown rot

Fusarium crown rot (FCR) is one of the most chronic and serious diseases of cereals throughout the world. Different species of *Fusarium* can cause FCR, but the most predominant ones are *F. culmorum* (Wm. G. Smith) Sacc. and *F. pseudograminearum* (O'Donnell and Aoki) (teleomorph *G. coronicola*) (Akinsanmi et al. 2004; Erginbas-Orakci et al. 2016).

FCR starts with the proliferation of the pathogen near by the site of infections (the lower part of the plant), leading to crown and root tissues necrotization and leaf sheaths and lower stem browning (James Cook 1992; Beccari et al. 2011; Powell et al. 2017). Finally, diseased plants can also be attacked in the ear, presenting white heads with little or no grain which lead to a severe decrease

in production (Fig. 2). The quality of grains is also compromised due to the production of *Fusarium* mycotoxins (Smiley et al. 2005; Mudge et al. 2006). FCR is managed through the use of chemical products, resistant and tolerant cultivars and agronomics practices (Pariyar et al. 2014; Moya-Elizondo and Jacobsen 2016).

Figure 2 | FCR symptoms on wheat (a) crown, root and (b) ear (white head) (Scherm et al. 2013).

2.3 Leaf spot blotch

Bipolaris sorokiniana (Sacc.) Shoem. syn. *Drechslera sorokiniana* (Sacc.) Subrm and Jain (syn. *Helminthosporium sativum*, teleomorph *Cochliobolus sativus*) is the causal agent of leaf spot blotch (LSB). This disease can be observed in wheat and other cereal cultivation in the warmer and more humid regions of the world. *B. sorokiniana* may be also responsible for other diseases like common root rot, foot rot, seedling blight and seed rot (Acharya et al. 2011; Minotto et al. 2014).

Presence of LSB in wheat crops is characterized by considerable yield production losses.

Infected seeds and crop residues represent the main disease sources of inoculum from which the pathogen starts the infection processes involving both phylloplane and radical apparatus of wheat (Rioux et al. 2016).

LSB aerial symptoms are characterized by oval necrotic lesions surrounded by chlorotic halos, that may reduce leaf photosynthetic area, leading to premature plant senescence (Ghazvini and Tekauz 2007). Seedlings emergence can be also compromised by rotting or with the abortion of germination (Burlakoti et al. 2014). In addition, the fungus may affects roots, interfering in the search and absorption of water and nutrients (Minotto et al. 2014) (Fig. 3).

Cropping practices (crop rotation and tillage), seeds disinfection, use of resistant cultivars and fungicides applications are the only strategies adopted for the prevention of LSB. Indeed, despite many attempts to manage the disease, field results show that *B. sorokiniana* is still the cause of substantial grain yield reductions, emphasizing the need to find out new defense strategies (Acharya et al. 2011).

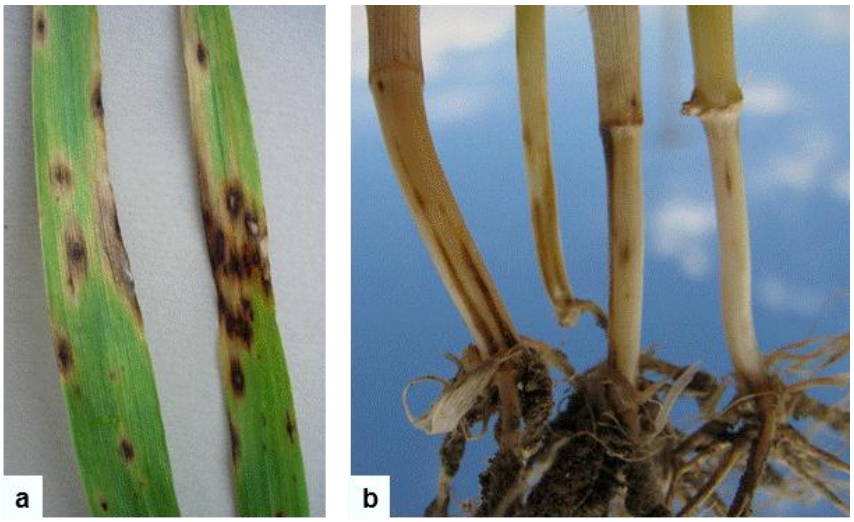


Figure 3 | LSB symptoms on wheat (a) leaves and (b) roots (source: Mary Burrows, Montana State University, bugwood.org).

2.4 Septoria tritici blotch

Septoria tritici (Desm.) [teleomorph *Zymoseptoria tritici* (Desm.) Quaedvlieg and Crous; syn. *Mycosphaerella graminicola* (Fuckel) J. Schröt], is the causal pathogen of septoria tritici blotch (STB). It is widespread globally, especially in European countries were it represents the most damaging wheat foliar disease (Jørgensen et al. 2014). Indeed, STB can lead to a severe reduction of yield production (Ponomarenko et al. 2011).

S. tritici is a hemibiotrophic fungus exhibiting a symptomless period, lasting 8-11 days post inoculation (dpi), during which pathogen hyphae grow between leaf's mesophyll cell (without causing any kind of damage). This stage is followed by a short phase where *S. tritici* increases its biomass and starts to produce cell wall-degrading enzymes, followed by pycnidium formation and a final necrosis development (Siah et al. 2010), that reduce the fitness of the leaves compromising their photosynthetic activity (Fig. 4).

Figure 4 | STB symptoms on wheat leaf (Ponomarenko et al. 2011)**.**

The management of STB is centrally focused on the use of synthetic fungicides and varietal resistance through wheat breeding programs (Ghaffary et al. 2012; Goudemand et al. 2013; Torriani et al. 2015). However, it's reported that chemical and genetic control strategies are compromised in the field, due to the ability of pathogen to frequently develop resistance to fungicides and hosts (Cowger et al. 2000; Cools and Fraaije 2008; Cheval et al. 2017).

The development of new tools and approaches are needed to set up new integrated disease management strategies.

3 Biological control

Management of fungal diseases is based on conventional plant protection and breeding strategies that are quite easy and effective tools, but actually they can lead to different types of problems. Overuse of chemical pesticides increases environmental pollutions and could be considered as human and animal health threat. In addition, political regulations on chemical pesticides restrict their use, and in some cases for the most harmful ones, oblige for their withdrawal from the market (Heydari and Pessarakli 2010). Moreover, breeding practices, targeted on identification and selection of resistant genes, are time-consuming and slow process practices (Goutam et al. 2015). These strategies need high investment costs (Sharma, 2003; Keller et al., 2008) and very often are not determined approaches, as they can lead to pathogens resistance (Torriani et al. 2009; McDonald et al. 2015). Nowadays the use of microorganisms is considered as the best and safest method for plant disease control (Palazzini et al. 2016).

According to Cook and Baker (1983) biological control is the inhibition of growth, infection or reproduction of one organism accomplished by or through one or more organisms other than men. In plant pathology, this term and its abbreviated synonym biocontrol refers to the use of antagonistic microorganisms to suppress diseases. These organisms are employed as natural enemies for eradication and control of pests and pathogens and they are named as biocontrol agents (BCAs). They can be applied directly on plant surfaces (e.g. in the rhizosphere, phylloplane and fruit surfaces) and soil, or from their growth extracted and fermented compounds can be obtained. These may be the constituents of different formulations with specific activities or with multiple effects on the host as well as on the target pest and pathogen. Complex mixtures of natural ingredients can be referred to as bio-pesticides or

biofertilizer, depending on the primary benefit provided to the host (Cook 1993). For these reasons, before developing a product base on antagonistic microorganism, it is essential to understand the way it acts. It is also necessary to focus the attention on its production, formulation and strategies of applications, in order to maximize its effects and exploit all its potential (Burges 1998; Alabouvette et al. 2006).

Several reviews have outlined the traits that characterize the ideal microbial antagonist (Larkin and Fravel 1998; Droby et al. 2009; Sharma et al. 2009; Ulloa-Ogaz et al. 2015). According to Wilson and Wisniewski (1989), later reported and extended by Abano and Sam-Amoah (2012), the aspects that need to be focused during the selection of the BCA are the following:

1. must be genetically stable;
2. should be effective at low concentrations;
3. should be able to colonize the surface of vegetables and persist in the site effectively;
4. must not be demanding in terms of required nutrients;
5. must have better ability than the pathogen to obtain nutrients;
6. must be able to survive under adverse environmental conditions;
7. should be effective against a wide spectrum of pathogens under different conditions;
8. should be amenable to production on inexpensive growth media;
9. should be amendable to formulations with a long shelf life;
10. should be easy to store without being hazardous to human health;
11. must be resistant to chemicals used in the postharvest environments;
12. must be environmentally friendly;
13. must be compatible with commercial processing practices;
14. should not be detrimental to the quality of crops it preserves.

Antagonistic effects can be promoted not only through a direct interaction between BCAs and pathogens, mainly during the saprophytic phase, but also from an indirect action through induced resistance of the host plant (Cook 1993; Schouten et al. 2004).

Beyond good agronomic and cultural practices, biological control represents a viable and safe method to manage plant diseases, that requires a greater and continuous understanding of the complex interactions among microorganisms, plants, people and the environment.

3.1 Microbial interactions

One of the precepts on which plant pathology is based is the interaction between a susceptible host plant, a virulent pathogen and a favorable environment, from which the degree of severity of disease is determined (Stirling and Stirling, 1997). This is reported schematically in the disease triangle (Francl 2001) (Fig. 5).

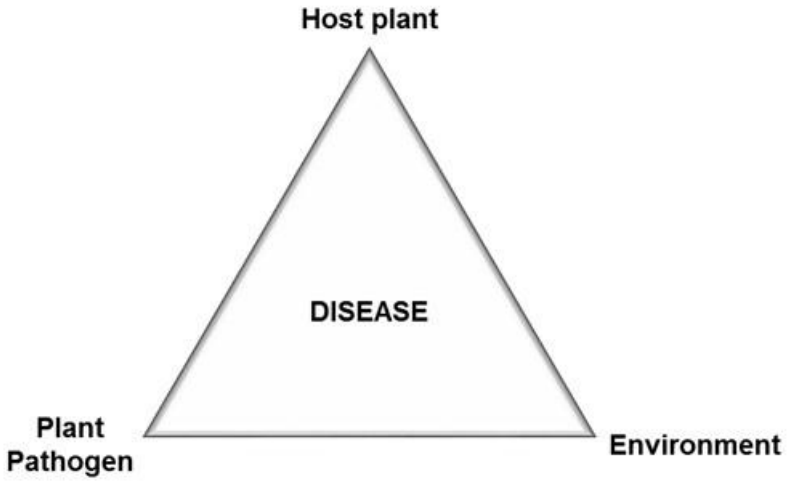


Figure 5 | The disease triangle.

During the life cycle, plants and pathogens interact with a wide variety of organisms. This can affect plant health in various ways, so it is important to

understand all the mechanisms behind these interactions, that are also foundations of the biological control.

The types of interaction involving plants and microorganisms refer to: mutualism, protocooperation, commensalism, neutralism, antagonism, competition, parasitism and predation.

Mutualism is an association based on a close physical and biochemical contact among species where all of them take benefits from this interaction. This kind of association is typical between plants and mycorrhizal fungi. Protocooperation can be considered as a form of mutualism, but where the organisms involved do not depend strictly to each other for the survival. This happens between Rhizobium bacteria and legume plants. Mutualistic interactions can contribute to biological control stimulating host defense mechanisms and improving plant nutrition (Biermann and Linderman 1983; Fitter and Garbaye 1994; Chisholm et al. 2006; Heydari and Pessarakli 2010).

Commensalism is a symbiotic association between two living organisms, where one organism benefits and the other is neither harmed nor benefited (Fitter and Garbaye 1994). Most plant-associated microorganisms fall into this kind of interaction, as their presence can favor host development and defense (Cook 1993; Chisholm et al. 2006).

Neutralism occurs when the population density of a species does not inflict on that of another. This can be traced to the dynamics of populations of a pathogen and another microorganism (Berg et al. 2005; Chisholm et al. 2006). On the other hand, antagonism describes microbial interactions leading to a negative outcome for one or both species involved.

Competition, instead, leads to decreasing growth, activity and/or fecundity of the interacting organisms. Non-pathogenic microorganism can compete with the pathogenic ones for nutrients and site in host plants (Cook 1993).

Parasitism occurs when one organism, named parasite (usually the physically smaller one), benefits on the other, called host. This symbiotic coexistence

takes place over a prolonged period. This interaction can be found in biocontrol thanks to microbial agents that parasitize plant pathogens (Cook 1993; Lo et al. 1997; Pal and Mc Spadden Gardener 2006). Finally, predation is when an organism hunts and kills another one for consumption and sustenance. In plant pathology this phenomenon can be detected in some fungal species that nourish from nematodes and arthropods (Cook 1993).

3.2 Biocontrol mechanisms

Application of BCAs for disease management greatly increases interactions within the crop system. Indeed, pathogens are contended with presence and activities of other microorganisms that they face off.

Biological control is carried out according to the synergistic action of different mechanisms, such as: predation, parasitism, competition for nutrients and space, production of antimicrobial substances and induced resistance (Stirling and Stirling 1997) (Fig. 6). However, they are still always dependent on environmental conditions in which they occur (Heydari and Pessarakli 2010).

These interactions are foundations of the antagonistic process, that can be highlighted in two different ways: direct and indirect antagonism. The first is the physical contact and/or the high-degree selectivity for the pathogen by the mechanisms exerted by BCA. It occurs by competition and/or production of antibiotics and secondary metabolites. The second, instead, does not directly involve the pathogen, but is based on the improvement and stimulation of host plant defense mechanisms by biocontrol organisms.

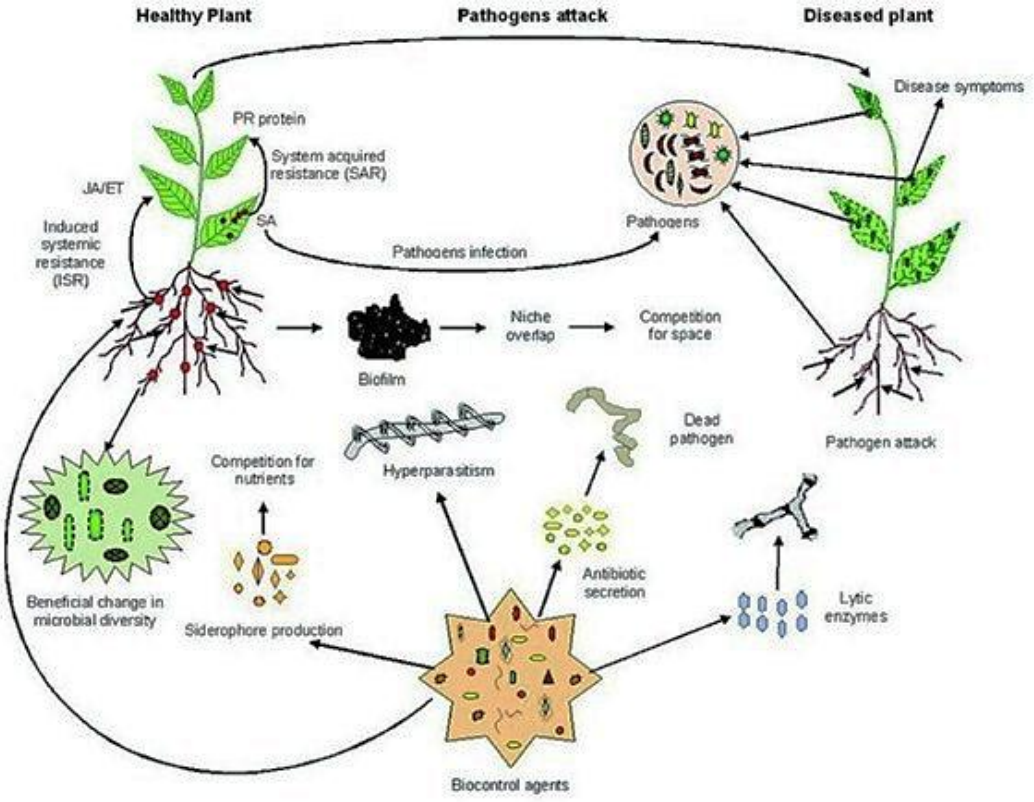


Figure 6 | Biocontrol mechanisms of BCAs for plant diseases management (Singh 2014).

Competition: Nutritional sources in soil and plant surfaces (rhizosphere and phylloplane) are limited for the survival of many microorganisms. This phenomenon allows, therefore, to regulate the population dynamics of organisms that share the same ecological niche. Indeed, it has been demonstrated that non-pathogenic microorganisms can protect the plant by rapidly colonizing and thereby exhausting the limited available substances, preventing the growth and development of pathogens (Heydari and Pessarakli 2010). These microbes colonize the sites where water and carbon sources are most readily available.

Soil and rhizosphere represent a region of intense microbial exchanges due to the presence of exudates, leachates and senescent tissues, and where there may also be competition for oxygen. On leaf surface, where nutrients are in short supply, competition is thought to play a significant role for limiting the

incidence and the severity of the disease. The most effective pathogenic competitors are BCAs that have the same nutritional requirements.

Competition for minor but essential elements, such as iron, has been demonstrated to be also important for the biological control of pathogens. Iron is an element found in limited concentrations in soil and rhizosphere. The low concentration depends on soil pH values, which make this metal insoluble (in ferric form) and unavailable for microbial growth. Microorganisms to survive in such condition have begun to secrete metabolic products of iron-binders, called siderophores; bounded iron can be transported from the environment into the microbial cell (Kageyama and Nelson, 2003; Asghar and Nader, 2009). Iron competition is one of the strategies used by fluorescent pseudomonads to limit pathogen growth, reducing the incidence and severity of the disease (Kloepper et al. 1980; Heydari and Pessarakli 2010).

Beyond trophic competition, BCAs can also compete for space. The competition between microorganisms for colonization of the same site is called “site exclusion”. This occurs most frequently among organisms that are taxonomically closer.

Antibiosis: Many BCAs produce and secrete various compounds interfering with pathogen growth and activities; among these, antibiotics are the most effectives ones. Antibiotics are microbial toxins that, even at low concentrations, can poison or kill the organism that has been exposed.

In antibiotic processes, not only antibiotics in *sensu stricto* are involved, but other molecules like: bacteriocins, cell wall degrading enzymes and volatile compounds with antifungal activity (Alabouvette et al. 2006). Many of these compounds, produced by BCAs, have been proved effective against plant pathogens, but among all 2,4-Diacetylphloroglucinol (DAPG), phenazines, pyluteorines, pyrrolnitrines and cyclic lipopeptides are the most promising ones. *Pseudomonas* spp., *Bacillus* spp. and *Trichoderma* spp. are the most

important species that produce these molecules (Yoshihisa et al. 1989; Islam et al. 2005; Pal and Mc Spadden Gardener 2006).

For being effective (*in vitro* and *in situ* conditions) an antimicrobial compound (AMC) must be produced in sufficient quantity (Heydari and Pessarakli 2010). Furthermore, for the selection of a potential BCA, it is important to know when and where these molecules are produced, but also their mechanisms of action. Depending on the specific cellular composition, an organism may be more susceptible to an AMC than another one. Indeed, each molecule has its own mechanism of action: some attack the cellular membranes, others have inhibitory effects on the ribosome and others alter cellular constituents (McSpadden Gardener and Fravel 2002; Sturz 2006; Ulloa-Ogaz et al. 2015).

Many microorganisms perform direct antagonism through production and release of lytic enzymes which are able to hydrolyse a wide range of polymeric compounds (proteins, cellulose, hemicellulose and DNA). Chitinase and β-1,3 glucanase, are the most important lytic enzymes as they can degrade the main components of the cell walls (chitin and β-1,3 glucan) of many pathogenic fungi, leading to their deaths (Chernin and Chet 2002; Singh 2014).

In addition to previously described metabolites, other AMCs that control plant diseases are volatile compounds, such as hydrogen cyanide (HCN). It is typically produced by some fluorescent pseudomonads and it acts by blocking the cytochrome oxidase pathway, becoming toxic, even in small concentrations, to all aerobic microorganisms (Ramette et al. 2003; Heydari and Pessarakli 2010).

Induction of host resistance: Plants respond to abiotic and biotic stresses by activating defense responses. Physical stress, interaction with pathogenic and non-pathogenic organisms and organic and synthetic chemical molecules, can induce or affect the defenses of the host plant, which reacts through biochemical changes. These processes can be localized or systemic

and depend on the type and amount of stimulation agents. There are two pathways leading plants to elicit defensive responses: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Vallad and Goodman 2004).

SAR is mediated by salicylic acid (SA), which is produced by the host following infection of a pathogen. This leads to the expression of pathogenesis-related (PR) proteins, such as phytoalexins, which act directly by lysing the pathogen cells, leading to the death of the localized cells of the affected tissues or to the reinforcement of cell walls, allowing them to withstand infection (Ryals et al. 1996; Durrant and Dong 2004; Vallad and Goodman 2004).

ISR is mediated by jasmonic acid (JA) and/or ethylene, that are produced by the host plant after bounding with non-pathogenic microorganisms.

It has been reported that host defenses are constantly stimulated throughout its life cycle this is due to the involvement of a substantial number of microbial products (Ryu et al. 2003).

Several strains of *Pseudomonas* spp., *Trichoderma* spp. and *Bacillus* spp. can elicit ISR, allowing plants to withstand pathogen attacks to the leaves and roots without offering a direct protection (Harman et al. 2004; Kloepper et al. 2004; Pal and Mc Spadden Gardener 2006; Choudhary et al. 2007).

The characteristics previously analyzed make BCAs a potential resource for the control of plant diseases in modern agriculture. Moreover, a part of them can also be considered as potential biofertilizers, due to their ability to solubilize insoluble substances present in the soils and to produce phytohormones promoting a greater growth and development of crops. The microorganisms that combine these two activities are referred to Plant Growth Promoting Rhizobacteria (PGPR) (Beneduzi et al. 2012).

Bacillus spp., together with *Pseudomonas* spp. are the most important bacteria belonging to this group.

4 *Bacillus* spp.

4.1 General information

Bacillus is a bacterial genus belonging to Firmicutes phylum and Bacillaceae family. It was described for the first time by Cohn in 1872, after the discovery of a strain by Ehrenberg in 1835. *Bacillus* spp. are Gram+ bacteria, obligate aerobes or facultative anaerobes (Alina et al. 2015). Size and cell morphology may differ between and within species, as they can show up rod-shaped, straight or slightly curved; occurring singly, in pairs or in chains and occasionally as long filaments. The terminal electron acceptor is molecular oxygen (O_2). Many *Bacillus* species are able to secrete large quantities of enzymes (they are oxidase and catalase positive) and some of them can also produce pigments in presence of specific nutrients (Khaneja et al. 2010). Many *Bacillus* species appear to be mobile by means of peritrichous flagella; they can metabolize carbohydrates by fermentation and they are typically resistant to penicillin (with the exception of *B. anthracis*).

According to Bergey's Manual (Logan and De Vos 2009) over 90 *Bacillus* species have been described. These include both pathogenic (for humans and animals) and beneficial (probiotics) species, widely adapted to grow in different contexts (Earl et al. 2008).

This group of bacteria is mostly ubiquitous in nature, being distributed in all the ecological environments (soil, air and water) (Zhang et al. 2008). This skill is certainly linked to the ability of this genus to form a highly resistant dormant endospore in response to environmental stresses, like: heat, salinity, nutrient depletion, radiation exposure, pH variations and other conditions that can lead to the impairment of bacterial metabolic and physiological processes. There is only one endospore in the sporangia cell that can be distinguished from the rest of vegetative cell, as they are refractile and less colored (Alina et al. 2015).

Based on its position within the bacteria it is possible to use it as a distinctive trait among species.

Cohn (in 1872) and Koch (in 1876) describe for the first time resistance, development and formation cycle of bacterial spore (Logan and De Vos 2009). It is formed inside the bacterial cell, so why called endospore, and subsequently released as a free spore after the lysis of the mother cell.

Endospores are formed by vegetative cells after receiving an adverse environmental signal, and only when they found appropriate vital conditions germinate, originating a new vegetative cell. This feature combined with their metabolic diversity allowed these bacteria to spread and colonize a wide variety of environments (Wipat and Harwood 1999).

B. subtilis is the type species for this genus and has been used as a model organism for the prokaryotic research concerning the investigation of the sporulation system and the capacity of these bacterium to produce antibiotics and enzymes of industrial importance (Devine 1995). For these reasons, in 1997, *B. subtilis* strain 168 was the first *Bacillus* species to have the complete genome sequenced (Kunst et al., 1997). Its genome consists of 4,214,810 base pairs (bp) and includes about 4,100 genes encoding proteins; of these, 192 have proved to be indispensable, others 79 are considered essential or highly determinant (Kobayashi et al. 2003).

The main studied *Bacillus* species are divided into two specific groups. The first one, referring to *Bacillus subtilis* group, includes: *B. subtilis*, *B. pumilus*, *B. licheniformis* and *B. amyloliquefaciens* among others. The second group, referring to *Bacillus cereus* group, includes: *B. cereus*, *B. thuringiensis*, *B. mycoides* and *B. anthracis* (Priest et al. 1988; Mora 2013).

4.2 *Bacillus* in industry

Bacillus species have always been an important industrial resource and they have been used in most of industrial sectors: food, pharmaceutical, chemical,

energy, medical and agricultural. Their application has been possible due to their biological and metabolic characteristics. That being the ability to grow at very high rates with very short fermentation cycles, to form spores and produce different types of proteins on extracellular medium (Mora 2013). Moreover, all the developments on the biochemical, physiological and genetic knowledge of *Bacillus* species allow to have a greater development and industrial application of these organisms (Schallmey et al. 2004).

In addition, most of the *Bacillus* species, such as *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* have been granted "Qualified Presumption of Safety" (QPS) status by the European Food Safety Authority (EFSA) and as "Generally recognized as safe" (GRAS) by the Food and Drug Administration (FDA) of the US (Hazards 2013; U.S. Governmen Publishing Office 2017).

Bacillus species are able to produce three important industrial products: enzymes, antibiotics and insecticides.

Enzymes, such as proteases, amylases, glucanases and cellulases are produced by different *Bacillus* species, which growing in a liquid substrate under industrial fermentation conditions, can produce up to 20-25 g of enzymes per liter of supernatant (Ferrari et al. 1993; Schallmey et al. 2004). The ability of different species to carry out fermentative processes at different pH and temperature ranges led to the development of various new commercial enzymatic products with different stability properties, allowing these products to deal with a variety of specific applications. Natural mutations and/or selection techniques, along with advanced cloning and protein engineering strategies, have been exploited to improve these products and increase the quality and quantity of their production.

Among *Bacillus* species, *B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens* have a high secreting capacity of extracellular enzymes, that are used in different industrial sectors (Tab. 1).

Table 1 | Industrial application of *Bacillus* spp. enzymes and products.

Enzyme / Product	**Industrial sector**	***Bacillus* spp.**
Protease	Detergent	*B. licheniformis, B. clausii* and *B. amyloliquefaciens*
	Feed additive	*B. thermoprotyolyticus*
Amylase	Detergent	*B. subtilis* and *B. licheniformis*
	Starch	*B. subtilis* and *B. licheniformis*
	Textile	*B. halodurans*
	Feed additive	*B. licheniformis* and *B. amyloliquefaciens*
Cellulase	Bioenergy	*B. subtilis*
β-glucanase	Feed additive	*B. subtilis* and *B. licheniformis*
Lipopeptides	Pharmaceutical	*B. subtilis*
Purine nucleotides	Feed additive	*B. subtilis*
	Manufacturing	*B. subtilis*
Endotoxins	Agrochemical	*B. thuringiensis*

Among *Bacillus* compounds, primary and secondary metabolites are those characterized by a strong antimicrobial activity. Bacitracin A and surfactin are the most widespread antibiotic molecules in various industrial fields. Bacitracin A is a cyclic oligopeptide synthesized from *B. licheniformis* and is mainly used as a dietary antibiotic in poultry consumption, mainly in chicken farms. Surfactin, instead, is a cyclic lipopeptide (cLP) with strong surfactant activity and emulsification capacity and it is produced by *B. subtilis* and related species. It has great potential application in agricultural, chemical, food and pharmaceutical industries to prevent or control spoilage and pathogenic microorganisms, as well as in bioremediation of contaminated sites (Stein 2005; Coutte et al. 2010; Saimmai et al. 2011).

One of the major industrial uses of some *Bacillus* species is as probiotics. In fact, their ability to form spores allows these microorganisms to be used for more than fifty years, gaining more interest than other Gram+ bacteria (i.e.

Lactobacillus spp.). *B. clausii, B. subtilis* and *B. licheniformis* are the most used probiotics, among other *Bacillus* species (Raddadi et al. 2012; Shahcheraghi et al. 2015).

Vitamins (like riboflavin) and purine nucleotides are other *Bacillus* compounds used in pharmaceutical and feed industries (Schallmey et al. 2004; Raddadi et al. 2012).

Furthermore, in the food sector, can be found the employment of a *B. subtilis* strain, called *Bacillus* "nattō". It is used in the preparation of a typical Japanese dish called Nattō, which consists of the fermentation of soybeans with this microorganism (Schallmey et al. 2004; Logan and De Vos 2009). It has been demonstrated that this dish is effective in reducing blood coagulation by fibrinolysis and eliciting host's immune system (Raddadi et al. 2012; Shahcheraghi et al. 2015).

As previously mentioned some species of *Bacillus* can produce carotenoids, in addition to being used as food colorants or supplements. These pigments may also have cosmetic and pharmaceutical purposes as nutraceuticals as well as biological properties such as anti-cancer, anti-inflammatory and anti-oxidative (Raddadi et al. 2012).

At last other important enzymes produced by *Bacillus* spp. are cellulases and xylanases. They are used in various fields such as in the paper and pulp industry, for improving texture of baked goods, in the textile industry and in poultry feed (as food supplements).

4.3 *Bacillus* in agriculture

Most *Bacillus* species used in biotechnological processes are located in *Bacillus subtilis* group. They are implicated in several industrial productions including enzymes, functional foods and biopolymers. In addition to the industrial applications, mentioned above, many *Bacillus* spp. strains. have different uses in agriculture. Their growing use, especially in recent years, has

been due to the setup of new methods of controlling plant diseases in order to replace, or at least supplement, the existing chemical-based strategies.

Moreover, species of *Bacillus* have resulted in being part of the microflora that colonize and inhabit several plant species tissues (including cereals, legumes, vegetables and several fruit trees), where they naturally play roles of protection and growth stimulation for plants (Kumar et al. 2011). This bacterial community is therefore a pool from which, in the last few years, researchers have isolated and selected strains that act as natural BCAs.

They are used both as natural antagonists, carrying out biocontrol action against different phytopathogens and pests, as well as biofertilizers, carrying out a plant growth promotion activity (Mishra and Kumar 2012; Alina et al. 2015) (Fig. 7).

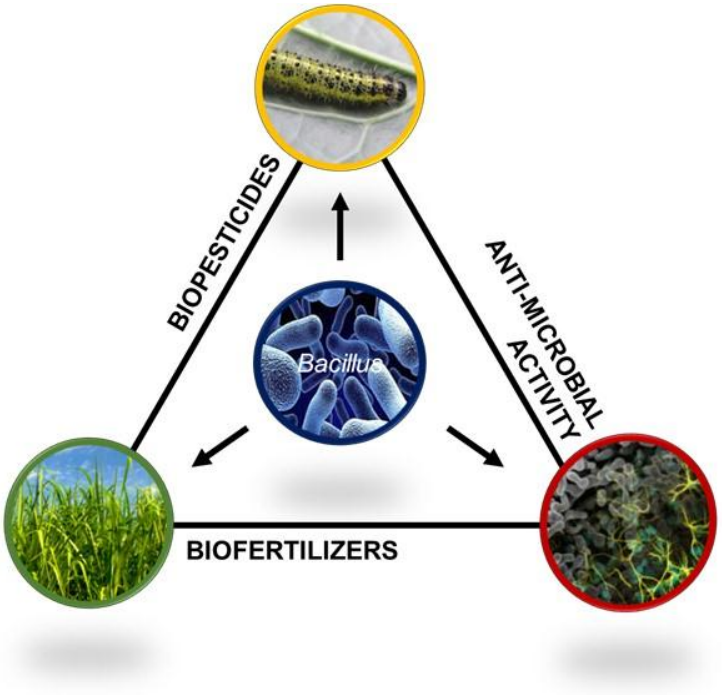


Figure 7 | *Bacillus* target triangle (Adapted from: Pérez-García et al. 2011).

Bacillus subtilis group, due to its importance in biotechnological industry and agriculture, is the most studied bacterial group as it incorporates species that act as BCAs and growth promoters of plants. *B. subtilis, B. amyloliquefaciens, B. licheniformis* and *B. pumilus* are the most important *Bacillus* spp. among others (Pérez-García et al. 2011; Raddadi et al. 2012).

These species have high anti-microbial activity, both anti-fungal and anti-bacterial. Several *Bacillus*-based commercial products have been established to counteract different plant pathogens (Tab. 2).

The *Bacillus* spp. formulation is more readily achieved compared with other BCAs due to endospore-forming that allows these microorganisms to have a resistant life stage.

These products include different types of formulations: liquids (aqueous suspensions and flowables), wettable powders and dry flakes. Moreover they can contain one or more strains of active ingredients and can be applied to foliage, soil and to transplants mix when seeding (Ardakani et al. 2009).

Bacillius spp. BCAs-products possess all possible biological traits to suppress pathogenic microbes and enhance plants health. These processes are carried out through different activities such as a direct mechanism mediated through trophic and spatial competition (only if applied in advance) and antagonizing plant pathogens through a wide range of self-produced substances (such as extracellular lytic enzymes and secondary metabolites), or carrying out an indirect mechanism inducing host plant resistance by promoting growth and nutrient acquisition capabilities (Rahman 2016).

Table 2 | Commercial use of *Bacillus*-based biocontrol products in agriculture.

Bacillus spp.	Strain	Product name	Company	Target	Crops
B. subtilis	QST 713	Serenade®	AgraQuest Inc., Davis, CA (USA)	Bacterial and fungal diseases including *Erwinia amylovora, Alternaria* spp., *Aspergillus* spp.,*Venturia inaequalis* and *Botrytis cinerea*	Pome and ston fruits, grape and horticultural crops
	GB03	Kodiak™	Bayer Crop Science LP, NC (USA) (former Gustafson, LLC)	Fungal root pathogens, including *Rhizoctonia solani* and *Fusarium* spp.	Wheat, barley, cotton, soybean, corn, seed and pod vegetables
	MBI600	Subtilex®	Beker Underwood, Ames, IA	Fungal root pathogens, including *Rhizoctonia solani*, *Fusarium* spp., *Pythium* spp. and *Botrytis* spp.	Pome and ston fruits, grape and horticultural crops
B. amyloliquefaciens	D747	Amylo-X®	CBC (Europe) S.r.l. Biogard Division, Bergamo (Italy)	Bacterial and fungal diseases including *Erwinia amylovora, Pseudomonas syringae* pv. *actinidiae, Botrytis cinerea* and *Monilia* spp.	Pome and stone fruits, grape, actinidia and horticultural crops
	D747	Doubke Nickel 55™	Cetris, Columbia, MD (USA)	Bacterial and fungal diseases including *Pseudomonas* spp., *Xanthomonas* spp. *Erwinia amylovora, Alternaria* spp., *Sclerotinia* spp. and *Botrytis cinerea*	Pome fruits, grape and horticultural crops
	FZB24	RhizoVital® 42 l.	ABiTEP GmbH, Berlin (Germany)	Bacterial and fungal diseases including *Rhizoctonia solani*	Horticultural crops and ornamental plants
B. pumilus	GB34	Yield Shield®	Bayer CropScience LP, NC (USA) (former Gustafson, LLC)	Fungal root pathogens, including *Rhizoctonia solani* and *Fusarium* spp.	Soybean and legumes vegetables
	QST2808	SONATA®	AgraQuest Inc., Davis, CA (USA)	Fungal diseases including *Peronospora* spp., *Puccinia* spp., Venturia spp., *Uncinula necator, Alternaria solani* and *Phytophthora infestans*	Pome and ston fruits, grape, horticultural crops, legumes vegetables, cereals and citrus
B. licheniformis	SB3086	EcoGuard™	Novozymes Biologicals, Inc., Salem, VA (USA)	Fungal diseases including *Sclerotinia homoeocarpa*, *Colletotrichum graminicola*and *Rhizoctonia solani*	Cereals, horticultural crops and grasses
B. amyloliquefaciens + *B. subtilis*	GB99 + GB122	BioYield™*	Bayer Crop Science LP, NC (USA) (former Gustafson, LLC)	Fungal diseases	Horticultural crops and bedding plants

* BioYield™ combines the strong ISR activity of *B. amyloliquefaciens* GB99 with the phytostimulation of *Bacillus subtilis* GB112.

Plant growth promotion induced by *Bacillus* species can involve the production of several hormones that assist development and plant growth, like: auxins, cytokinins and gibberellins.

The production of indole-3-acetic acid (IAA) has been found in many strains of *Bacillus* spp. The synthesis in these bacterial species is tryptophan (Trp)-dependent and involves indole-3-pyruvic acid (IPA). However, it can have a Trp-independent pathway from different aromatic amino acids (such as praline, cysteine alanine) (Acuña and Jorquera 2011).

Quantitative analysis on cytokinins and gibberellic acid production have been carried out on *B.* subtilis, *B. licheniformis* and *B. pumilus*, by gas chromatography and mass spectrometry (Gutiérrez-Mañero et al. 2001; Arkhipova et al. 2005; Hussain and Hasnain 2009).

Plant growth is also promoted improving nutrient uptake of low-soluble minerals, such as phosphorus, that can be found in soils as phosphate and phytate. It can be solubilized by biofertilizer strains that have extracellular phytase activity, leading plant tissues development even under limited phosphate conditions. This phenomenon was described to be promoted by the use of FZB45 strain of *B. amyloliquefaciens* (Idris et al. 2002).

Bio-control is promoted by *Bacillus* spp. to provide plant protection against microbial pathogens. This activity involves antibiotics and extracellular lytic enzymes productions, nutrients and niche competitions and induced defense response in host plants.

Synthesis of antibiotic compounds in *Bacillus* spp. occurs during sporulation and steady growth phase (Alina et al. 2015). Among these zwitteriomicin A, kanosamine and peptides are the most important ones.

The former is a linear aminopolyol with a broad spectrum of inhibitory action against bacteria and eukaryotic organisms, including Oomycetes (Van Straaten et al. 2013).

B. subtilis, *B. pumilus* and *B. cereus* UW85 are Kanosamine (3-amino-3deoxy-D-glucose) producers. This antibiotic has an inhibiting action especially against *Oomycetes* (*Phytophthora* spp.), but also, a moderate activity against *Ascomycetes* (*Botryotinia fuckeliana, Venturia* spp. *Sclerotinia* spp.), *Basidiomycetes* (*Rhizoctonia solani*), mitosporic fungi (*Alternaria* spp. *Fusarium* spp. and *Verticilium* spp.) and some bacteria (Milner et al. 1996; Pal and Mc Spadden Gardener 2006; Van Straaten et al. 2013).

Among the antibiotic compounds produced by *Bacillus* spp., lipopeptides (LPs) are the most extensively studied (Alina et al. 2015).

They are amphiphilic compounds having a common structure consisting of a lipid tail linked to a short linear or cyclic oligopeptide. Depending on their amino acid sequence, cLPs, are classified into three families: iturins, fengycins and surfactins (Ongena and Jacques 2008).

Iturin is reported to have a strong antibiotic effect. Indeed, the one produced by *B. amyloliquefaciens* strains A_1Z (isolated from soybean rhizosphere), successfully inhibited *F. oxysporum, Sclerotinia sclerotiorum* and *Macrophomina phaseolina,* three taxonomically diverse fungal pathogens (Kumar et al. 2008). *B. subtilis* has also proved to be a iturin producer. Specifically the bacillomycin D (Iturin A group) produced by AU195 strain is the compound responsible for antagonism against *Aspergillus flavus* and other plant pathogenic fungi (Moyne et al. 2001). Other four strains of *B. subtilis*, UMAF6614, UMAF6616, UMAF6639 and UMAF8561, suppressed powdery mildew of cucurbits caused by *Podosphaera fusca* thanks to the inhibitory activity of iturin and fengycin (Romero et al. 2007).

In addition to antifungal activity, lipopeptides have antibacterial activity. Surfactin produced by *B. subtilis* provides protection against *Pesudomonas syringae* in *Arabidopsis thaliana* (Bais et al. 2004).

Polyketides are other secondary metabolites that are involved in the antagonism processes against phytopathogenic bacteria. Difficidin and bacilysin, produced by BCAs strains FZB4 of *B. amyloliquefaciens*, are the active compounds against *Erwinia amylovora*, causal agent of fire blight disease in orchard trees.

The antifungal activity of *Bacillus* sp. is also carried out by enzymes, such as chitinase, β-1,3-glucanase and protease. The former acts inhibiting fungal spore germination and germ-tube elongation. β-1,3-glucanase lyses the cell walls of *M. phaseolina*, *F. oxysporum*, *F. solani*, *S. sclerotiorum*, *Colletotricum* spp. and *R. solani*. Protease is able to hydrolyze cell wall, inhibiting fungal growth (Chen et al. 2009c, 2004; Kumar et al. 2012).

Besides antimicrobial activity against several plant pathogens, a particular *Bacillus* species named *B. thuringensi*s is used to control pests important in agriculture, forestry and medicine. This activity is excreted by the bacterium inside the digestive tract of insects, where it is able to synthesize, during sporulation, a crystalline inclusion. It contains proteins known as δ-endotoxins or Cry proteins, which have insecticidal properties (Schallmey et al. 2004; Pérez-García et al. 2011). *B. thuringensi*s has become the leading biological insecticide employed worldwide in agriculture, representing nearly 70% of the global biopesticides market. Moreover it is considered completely safe for non-target organism, including humans and animals, and for the environments (Raddadi et al. 2012).

ISR is another way of action enhanced by *Bacillus* species for promoting plant health (Choudhary and Johri 2009). Specific strains of different *Bacillus*

spp. have been shown to induce systemic resistance, eliciting a significant reduction in the incidence or severity of various diseases in several crops (Kloepper et al. 2004).

The extraordinary competence to survive in extreme environments, the spore-forming ability and versatility make *Bacillus* spp. highly effective as biopesticides and biofertilizers, and stimulate their continuous study and research for their unlimited application in agriculture.

4.4 *Bacillus amyloliquefaciens*

Bacillus amyloliquefaciens was isolated from soil and was described for the first time in 1943 by Fukumoto. The name derives from the ability to produce (faciens) amylase (amylo), an enzyme capable of liquefying (lique) or better degrading complex sugars into simple sugars. Until a few years ago it was considered a subspecies (subsp.) of *Bacillus subtilis*, in fact, only since 1987 it has been recognized as a new species (Logan and De Vos 2009)

As phenotypic and biochemical differentiation between closely related species is difficult to achieve, only in recent years, with new molecular tools, has it been possible to reclassify several strains from *B. subtilis* species to *B. amyloliquefaciens*. The only traits that allow for discrimination between this two *Bacillus* species are that *B. amyloliquefaciens* produces acid from inulin, its cells tend to be enchained and has central-located cell endospores (Alina et al. 2015).

B. amyloliquefaciens is a ubiquitous microorganism, it is commonly found in soil and vegetation all over the world.

There are a multitude of enzymes produced by this species, but α-amylase and protease production make *B. amyloliquefaciens* the largest producer among bacterial species. These enzymes are used in various industrial fields: from food to biotechnology.

Moreover, it does not produce toxins harmful to human health and for this reason granted the QPS status from EFSA.

Nowadays, *B. amyloliquefaciens* is self-standing species with two approved varieties, subsp. *amyloliquefaciens* and subsp. *plantarum* (Alina et al. 2015).

B. amyloliquefaciens subsp. *plantarum* strain FZB42 is the model bacterium of this species.

The entire genome of *B. amyloliquefaciens* was recently available (Chen et al. 2007). Its core-genome contains 3,151 genes while the pan-genome contains more than 6,000 genes. This suggests a high degree of flexibility in the genome of *B. amyloliquefaciens* strains (Chowdhury et al. 2015). Furthermore, fifty-four genes belonging exclusively to the subsp. *plantarum* have been identified, as they were absent in the genome of the free-living bacterium *B. amyloliquefaciens* subsp. *amyloliquefaciens* (such as gene cluster involved in carbon metabolism and that of polyketides synthesis) (Qiao et al. 2014).

B. amyloliquefaciens stimulates plant growth and has a high antimicrobial activity, being able to control several plant diseases. Indeed, the type strain FZB42 is used as BCA, resulting efficient against many fungal and bacterial phytopathogens (Borriss 2011). There are three strains of *B. amyloliquefaciens* constituting four different bioformulates used in agriculture (Tab. 2). These products exploit the potentiality of these strains both as BCAs and as PGPR.

B. amyloliquefaciens ability to promote plant growth and suppress diseases was documented in many crops (Guel et al. 2008 Wang et al. 2009; Chowdhury et al. 2013).

Furthermore, it has been demonstrated that radical colonization processes of this bacteria, are promoted by radical exudates, which stimulate the expression of genes involved in plant-bacteria interactions (Fan et al. 2012, 2015; Borriss 2015; Kierul et al. 2015).

Its exceptional rhizospheric competence and the high capacity to produce antibiotic compounds could create problems for the microbial communities

present in nature (Chowdhury et al. 2015). However, regardless its mode of application *B. amiloliquefaciens* does not alter the composition of the bacterial community of the rhizosphere to a measurable extent (Correa et al. 2009). This happens with the inoculation of pathogens, which change the structure of rhizospheric microbial communities (Erlacher et al. 2014; Borriss 2017).

The ability of this *Bacillus* sp. to support the growth of many crops is due to several factors that involve the complex interplay between root-colonizing bacteria and plants.

These skills are listed below:

- ability to colonize and persist in plant roots (as describe above);
- production of phytohormones (IAA) and volatiles compound (2,3-butanediol and acetoin) that enhance plant growth (Ryu et al. 2003; Idris et al. 2007);
- improvement of nutritional availability (phosphorus solubilization) (Acuña and Jorquera 2011);
- antibacterial and antifungal activity (Borriss 2011).

The analysis of the entire genome of *B. amyloliquefaciens* has shown the remarkable ability of this species to produce a wide spectrum of different secondary metabolites, able to counteract many plants pathogenic microorganisms (Chen et al. 2007).

The synthesis of AMCs, produced by *B. amyloliquefaciens* involves 11 gene clusters, representing more than 9% of the entire genome (Chowdhury et al. 2015). Nine of them are involved in the non-ribosomal synthesis of lipopeptides and polyketides, while two are involved in the synthesis of bacteriocins (Tab. 3).

Table 3 | Genes and gene cluster encoding for biocontrol metabolites in *B. amyloliquefaciens plantarum* FZB42 (Chowdhury et al. 2015).

Metabolite	Genes and gene cluster	Size	Function	Expression *in situ*	Effect against
Sfp-dependent non-ribosomal synthesis of lipopeptides					
Surfactin	*srfABCD*	32.0 kb	Biofilm, ISR	Strong, during root colonization	Virus
Bacillomycin D	*bmyCBAD*	39.7 kb	Direct suppression, ISR	Weak, during root colonization	Fungi
Fengycin	*fenABCDE*	38.2 kb	Direct suppression, ISR	Weak, during root colonization	Fungi
Bacillibactin	*dhbABCDEF*	12.8 kb	Siderophore	During iron deficiency in soil	Microbial competitors
Unknown	*nrsABCDEF*	17.5 kb	Unknown	Unknown	Unknown
Sfp-dependent non-ribosomal synthesis of polyketides					
Macrolactin	*mlnABCDEFGHI*	53.9 kb	Direct suppression	Not shown	Bacteria
Bacillaene	*baeBCDE,acpK, baeGHIJLMNRS*	74.3 kb	Direct suppression	Not shown	Bacteria
Difficidin	*dfnAYXBCDEFGHIJKLM*	71.1 kb	Direct suppression	Not shown	Bacteria
Sfp-independent non-ribosomal synthesis					
Bacilysin	*bacABCDE,ywfG*	6.9 kb	Direct suppression	Not shown	Bacteria, cyanobacteria
Ribosomal synthesis of processed and modified peptides (bacteriocins)					
Plantazolicin	*pznFKGHIAJC DBEL*	9.96 kb	Direct suppression	Unknown	*B. anthrax*, nematodes
Amylocyclicin	*acnBACDEF*	4.49 kb	Direct suppression	Unknown	Closely related bacteria
Synthesis of volatiles					
Acetoin/2,3-butandiol	*bdh,alsDRS*	3.6 kb	ISR	During root colonization	Plant pathogens

In contrast, other *Bacillus* spp. dedicate only around 5%, or less, of their capacity to synthesize antimicrobial compounds (Fig. 8).

As demonstrated in previous studies it is widely assumed that antibacterial activity is carried out primarily by ribosomally synthesized bacteriocins (Scholz et al. 2014), non-ribosomally synthesized poliketides (Chen et al. 2006) and bacilysin (Chen et al. 2009c); whereas, antifungal activity is due to non-ribosomal peptides (NRPs) (Koumoutsi et al. 2004), the cyclic lipopeptides (cLPs).

This arsenal of antimicrobial compounds makes *B. amyloliquefaciens* an efficient biopesticide to be used in plant disease control (Borriss 2011).

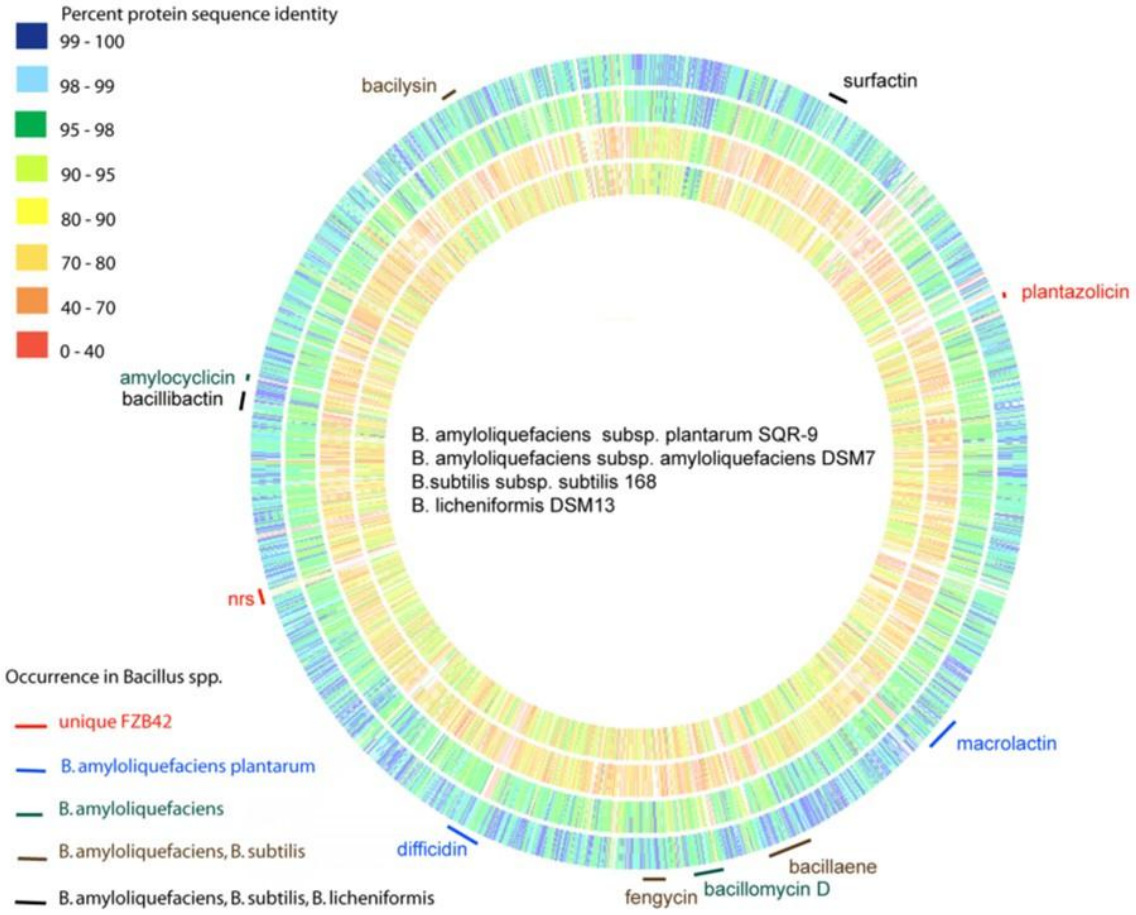


Figure 8 | Genome comparison of *B. amyloliquefaciens, B. subtilis* and *B. licheniformis* type strains. The whole genomes of *B. amyloliquefaciens plantarum* SQR-9 (outside circle), *B. amyloliquefaciens amyloliquefaciens* DSM7T (second circle), *B. subtilis subtilis* 168T (third circle), and *B. licheniformis* DSM13T (inner circle) were aligned with FZB42T. The color code indicates % similarity of single gene products (Chowdhury et al. 2015).

4.5 Cyclic Lipopeptides

The main biocontrol mechanism of plant pathogens is antagonism. This activity is exploited by *Bacillus* spp. in the production of enzymes degrading the cell wall, volatile organic compounds and antibiotics. These capacities make *Bacilli* to be considered as microbial factories of a wide range of biologically active molecules, some of which have potentially antimicrobial activity against plant pathogens (Stein 2005; Pérez-García et al. 2011).

Genetic analyses revealed that 5 - 9% of the genomes of respective *B. subtilis* and *B. amyloliquefaciens* are involved with synthesis of several antimicrobial compounds, that are implicated in their fitness as BCAs (Stein 2005; Chen et al. 2009b; Rückert et al. 2011). Antimicrobial Peptides (AMPs) are the predominant compounds produced by *Bacillus* spp. including *B. amyloliquefaciens*, *B. subtilis B. licheniformis*, *B. thuringiensis*, and *B. cereus* (Rahman 2016).

Bacillus LPs are released into the medium during the stationary phase of the culture, near to endogenous spore formation time (Rahman 2016): they are amphiphilic compounds consisting of a common structure made up of a lipid tail linked to short linear or cyclic peptide. They can differ according to length and composition of the fatty acid tail, to the number, to the type and to the configuration of amino acids of the peptide (Stein 2005; Ongena and Jacques 2008). This considerable structural diversity of cLPs is related with their synthesis processes, that are mediated by proteins defined as non-ribosomal peptides synthetases (NRPSs) (Strieker et al. 2010; Deravel et al. 2014). Synthesis occurs by elongation of activated monomers of amino and hydroxyl acid building blocks (Patrick 2012; Mora 2013).

Based on their amino acid sequence, cLPs can be classified into three different families: Surfactins, Iturins and Fengycins (Ongena and Jacques 2008; Guo et al. 2014; Mejri et al. 2017) (Fig. 9).

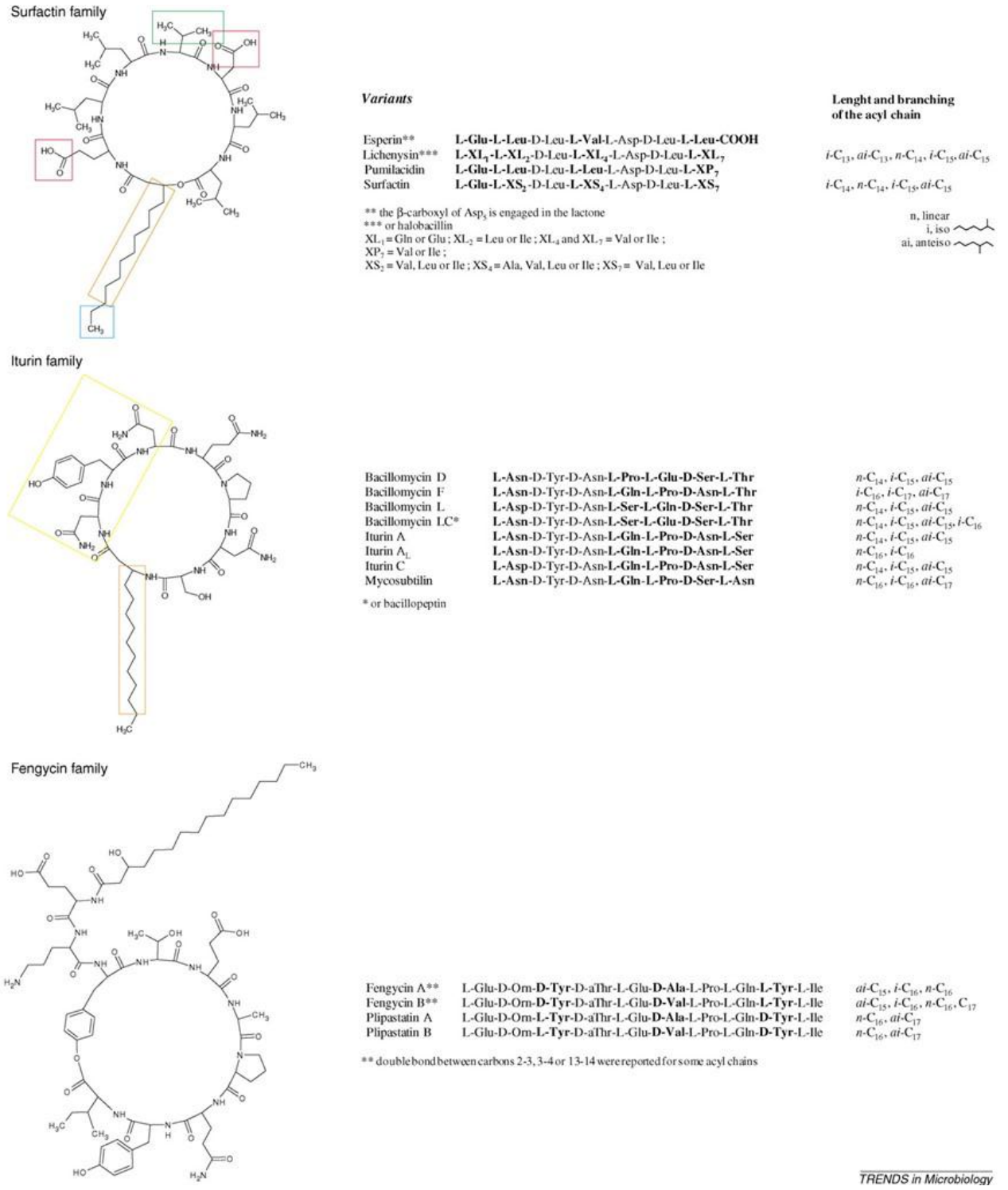


Figure 9 | Structures of representative members and diversity within the three lipopeptide families synthesized by *Bacillus* species (Ongena and Jacques 2008).

Surfactins family includes heptapeptides associated with a β-hydroxy fatty acid, consisting of 13 to 16 carbon atoms. Esperin, lichenysin, pumilacidin and surfactin are members of this cLPs group (Ongena and Jacques 2008; Jacques 2011). Their amphyphilic nature makes them powerful biosurfactants with haemolytic, antiviral, antimycoplasmic and antibacterial activity (interfering with biological membrane), but without a marked fungitoxicity (with some exceptions) (Vollenbroich et al. 1997; Huang et al. 2006, 2011; Tendulkar et al. 2007; Płaza et al. 2013). Because these properties, in addition to being used in biological control of many plant pathogens and pests (Assié et al. 2002; Bais et al. 2004; Toure et al. 2004), surfactins are employed as emulsifying, foaming and oil recovery, having a biotechnological, medical, industrial and environmental applications (Seydlová et al. 2011; Pathak and Keharia 2014).
The synthesizing surfactin complex consists of four enzymatic subunits SrfA, SrfB, SrfC and the acyltransferase SrfD which, during the surfactin initiation process, transfers the β-hydroxy fatty acid substrate from coenzyme A to the first SrfA module (Steller et al. 2004; Shaligram and Singhal 2010).
Surfactin biosynthesis is growth-phase dependent, induced during the transition to the stationary phase (Chen et al. 2009b) and in some cases strictly related with the early stage of sporulation (Nakano et al. 1991). Its regulation is mediated by the complex cellular exchanges network promoted also by the quorum sensing (Nakano et al. 1991; Yazgan et al. 2001).

Iturins is a large family of antimicrobial compounds, which includes bacillomycin D, F and L, iturin A and C, and mycosubtilin (Ongena and Jacques 2008). They are heptapeptides with a β-amino fatty acid, consisting of 14 to 17 carbon atoms (Stein 2005). They have an exceptional antifungal activity against a wide range of fungi and yeasts, a moderate antibacterial and antiviral

activity, a limited surfactant action and enhance swarming motility (Roongsawang et al. 2002; Romero et al. 2007; Jacques 2011).

Iturin antifungal activity is based on their capacity of membrane permeabilization (Bonmatin et al. 2003). This occurs through the formation of ionic pores, which lead to an osmotic perturbation (Aranda et al. 2005). Iturin mechanism of action differs from the one mediated by surfactins, which cause membrane destruction or solubilization (Patel et al. 2011).

Iturins NRPSs gene cluster is composed of four large open reading frames (ORFs) called *bmyD, bmyA, bmyB* and *bmyC* for bacillomycin; *ituD, ituA, ituB* and *ituC* for Iturin; *fenF, mycA, mycB, mycC* for mycosubtilin (Moyne et al. 2004; Duitman et al. 1999; Koumoutsi et al. 2004). The first gene (*bmyD, ItuD* and *fenF*) code for malonyl-CoA transcylase, responsible for the biosynthesis of the fatty acid chains. The last three genes code respectively for the proteic machinery responsible for the incorporation of the first residue (*bmyA, ituA* and *mycA*), the following four residues (*bmyB*, *ituB* and *mycB*) and the two last residues (*bmyC*, *ituC* and *mycC*) of the cyclic peptide in the cLP (Chen et al. 2009b; Ongena and Jacques 2008). Bacillomycin is characterized for the presence of a terminal threonine (Thasana et al. 2010). Instead, iturin A and mycosubtilin present almost the same structure with the exception of the last two amino acids that turn out to be reversed (Ongena and Jacques 2008).

Fengycins are decapeptides containing an internal lactone ring in the peptidic moiety and with a β-hydroxy fatty acid consisting of 14 to 18 carbon atoms. The fengycins, compared to the previous cLPs described above, despite being equally widespread in occurence, have a low structural diversity within the family; including fengycin A and B and plipastatin A and B, closely related (Stein 2005; Romero et al. 2007; Jacques 2011). The fengycin A and the fengycin B differ for a structural variant that corresponds to the amino acid residue in position 6, Ala for the former and Val for the latter (Ongena and

Jacques 2008). These compounds are able to degrade the structure of biological membranes, increasing their permeability with subsequent deformation and collapse (Deleu et al. 2005). This mechanism of action allows the control of many plant pathogens; which also adds the ability to induce systemic resistance in plants (Romero et al. 2007).

If used as biosurfactants, Fengycins can also inhibit the formation of biofilm in some bacteria and degrade polycyclic aromatic hydrocarbons (Das et al. 2008; Rivardo et al. 2009).

The synthesis of fengycins is mediated by NRPSs encoding for an operon with five ORFs *fenA-E*. In *B. subtilis* 168 *fen* operon is related to the *pps* operon and is situated at the same locus as this, instead in *B. amyloliquefaciens* it is about 25 kb distant from the *bmy* gene cluster (Chen et al. 2009a).

Cyclic lipopeptides, as said before, show a wide range of biological activities against many plant pathogens mediated by direct antagonism and/or induction of the plant immune system (Ongena and Jacques 2008; Deravel et al. 2014; Farace et al. 2015). Iturins and fengycins are mainly recognized for their antimicrobial properties, while surfactins are known for their biosurfactant potential, as promoters of many bacterial biological processes and as elicitors of ISR activation in plants (Fig. 10) (Toure et al. 2004; Ongena and Jacques 2008; Pérez-García et al. 2011).

These properties make cLPs a precious resource for modern agriculture, which have to be studied in deep, in order to maximize their value and applications in plant defense and in other research fields.

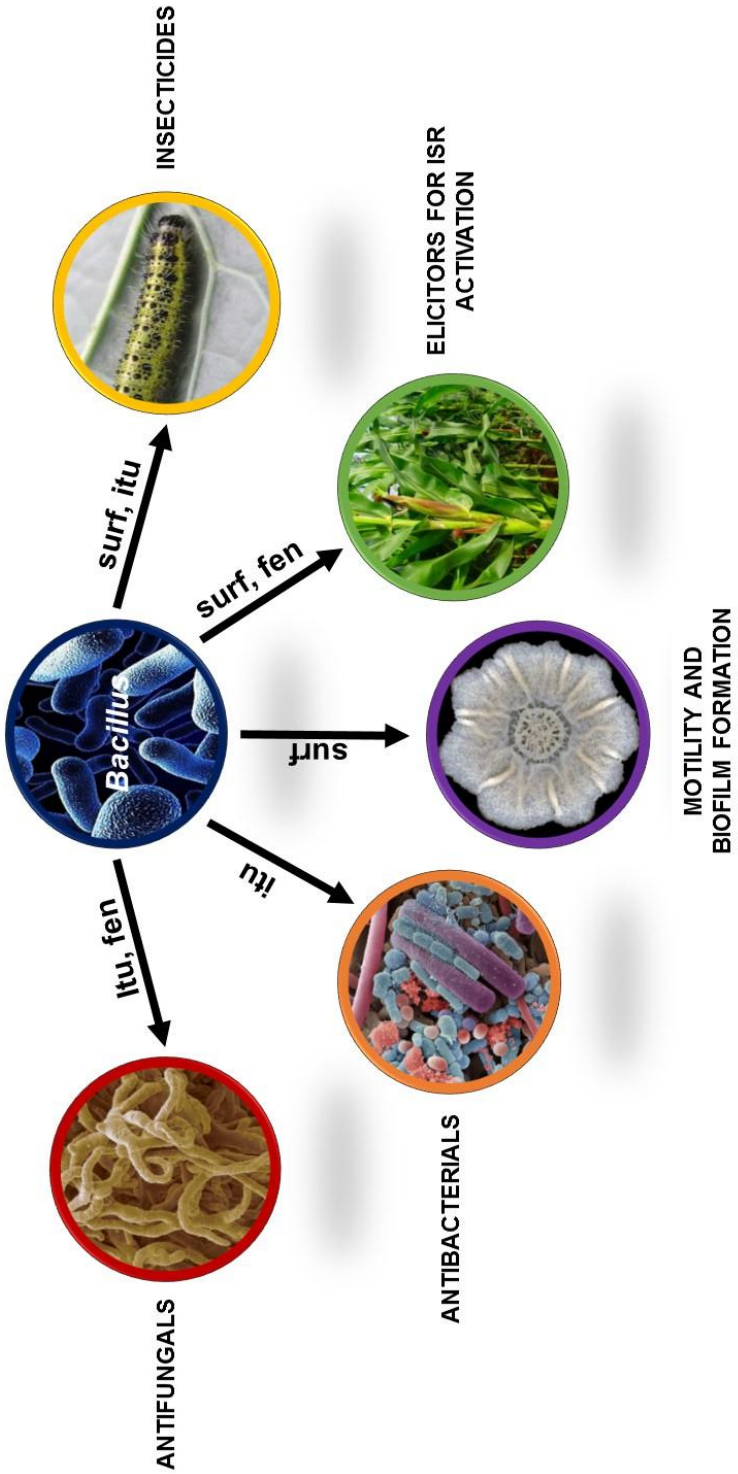


Figure 10 | Main activities of *Bacillus* cycle lipopeptides for biological control of plants. The most important cycle lipopeptides produced by *Bacillus* spp. are: Surfactin (surf), Iturin (itu) and Fengycin (fen) (Adapted from: Pérez-García et al. 2011).

2 AIM OF THE WORK

Wheat, due to its economic importance, is subjected to an intensified crop system, characterized by a massive increase in input such as irrigation, tillage, use of fertilizers and pesticides. This predisposes the environment to be subject to different forms of pollution and the crop to be more vulnerable to the attack of many pathogens.

F. graminearum, F. culmorum, B. sorokiniana and *S. tritici*, represent a steady and a worldwide spread threat for the cultivation of this cereal.

These fungal pathogens are only controlled by the use of chemical compounds. Therefore, it is important to find a viable alternative that allows to control these plant pathogens and in the same time, to be environmentally friend.

In recent years plant defense strategies, proposed by scientific researches, are based on the use of beneficial microorganisms to plants. *B. amyloliquefaciens* represents one of the most important because it combines the BCA and PGPR activities.

As stated, the objectives of this work are to:

Objective 1: Evaluate the *in vitro* antagonistic activity of *B. amyloliquefaciens* against the fungal pathogens of wheat.

Objective 2: Characterize the cLPs through molecular and chemical analysis and evaluate their stability.

Objective 3: Check for *in vivo* wheat growth promotion by *B. amyloliquefaciens* strains and evaluate their *in vivo* antagonistic activity against *B. sorokiniana.*

3 MATERIALS AND METHODS

1 *Bacillus amyloliquefaciens* strains

B. amyloliquefaciens strains used in this work are Ba15, Ba16 and Ba18. They were isolated from tomato spermosphere and were *in-vitro* characterized (Caracciolo 2014) as PGPR and potential BCAs of the most important seed born bacterial diseases of tomato.

B. amyloliquefaciens strains were cultured in Lysogenic broth (LB) agar at 27 °C for 24 h. For long-term preservation, bacteria were stored at -80 °C in LB with 20% glycerol.

B. amyloliquefaciens suspensions were adjusted at 10^8 cfu/ml, using a spectrophotometer at a wavelength of 600 nm, using a standard curve that correlates the optical density (OD) with the cell concentration, obtained by

ten-fold serial dilutions. The OD value corresponding to the proper cell concentration of *B. amyloliquefaciens* strains (10^8 cfu/ml) was 0.4.

2 Fungal strains

The four fungal pathogen of wheat used in this work are listed in Table 4, shown below:

Table 4 | Strains of the fungal pathogen used in this work.

Disease	Fungal Pathogen	Strain
Fusarium head blight	*F. graminearum*	3827
Fusarium crown rot	*F. culmorum*	5761
Leaf spot blotch	*B. sorokiniana*	DSMZ 62608
Septoria tritici blotch	*S. tritici*	MUCL 31967

Fungal strains were cultured in Potato Dextrose Agar (PDA) at 24 °C for 24 h. For long-term preservation, fungal pathogens were stored at 4 °C in vials of PDA slants, and re-cultured every six months.

Fungal cultures of one-month-old PDA Petri dishes were used for conidial production. Conidia suspensions were obtained, for *Fusarium* spp. and *B. sorokiniana*, by flooding the mycelium of pathogens with sterilized distilled water (SDW) added with Tween 80 at 0.05% v/v. *S. tritici* spores, instead, were collected by crushing and washing the picnidia with the same solution described above.

The concentration of conidial suspensions was determined by counting the spore number with Thoma chamber under microscope and subsequently adjusted to 10^6 spores/ml.

For fungal spore germination Potato Dextrose Broth (PDB) was used.

3 *In vitro* assays

3.1 Filtrate assay

Erlenmeyer flask containing 100 ml of LB were inoculated with *B. amyloliquefaciens* strains suspension at 10^8 cfu/ml and incubated at 27 °C in a thermostatic shaking bath for 48 h.

Further, the growth broths were centrifuged (8,000 rpm for 15 min at 4 °C) and the supernatants filtered through 0.21 µm Whatman filters.

One hundred and fifty µl of each filtrate (B) was distributed in well (1.2 cm **Ø**)in the center of LB agar (LBA) Petri dishes.

In the center of the distance between the Petri dish wall and the well, a PDA agar square (5 x 5 mm) containing fungal pathogen mycelium (P) was placed (Fig. 11). The inoculated Petri dishes were incubated at 24 °C. After 72 h, the antagonistic activity was evaluated by measuring the inhibition halo (IH) as the distance between the edge of the well and the edge of the fungal mycelium growth.

Not-inoculated LB and filtered *B. clausii* growth broth, were used as negative controls.

Five replicates for each *B. amyloliquefaciens*-fungal pathogen interaction were carried out.

3.2 Extract assay

Extract of each *B. amyloliquefaciens* strain was obtained through Ethyl Acetate 50% [v/v] by overnight stratification of bacterial filtrates. The next day, after solvent evaporation, the extract obtained was dissolved in 2 ml of 95% Ethanol.

One hundred and fifty µl of each extract (B) were transferred into wells in LBA Petri dishes inoculated with each pathogen (P), as described above (Fig. 11).

Five replicates were provided for each BCA-pathogen interaction.

After 72 h of incubation at 24 °C the IHs were measured.

Ethanol, not-inoculated LB and *B. clausii* extracts were used as negative controls.

3.2 Incorporated filtrate assay

Bacterial filtrates from growth broths were used as dissolving solution for LB components, subsequently agarized and autoclaved at 121 °C for 20 min and finally poured into Petri dishes.

The day after LB Filtrate Agar (LBFA) were inoculated placing a small square of PDA containing the mycelium of each pathogen in the center of each Petri dish. After the incubation period of 72 h at 24 °C, the diameter of fungal growth (Fig. 12) was measured and used to calculate the mycelial growth inhibition of the pathogens according to the following formula by Pandey et al. (1982):

$$\mathbf{MGI}\ (\%) = \frac{\mathbf{d_c} - \mathbf{d_t}}{\mathbf{d_c}} \times \mathbf{100}$$

MGI = Mycelial growth inhibition.

d_c= Growth diameter of pathogen mycelium in the control.

d_t = Growth diameter of pathogen mycelium in LBFA.

Percentage inhibitions obtained were used for statistical analysis.

LBA and LBFA from *B. clausii* filtrate were used as negative control.

This assay provided six replicates for each bacterial-fungus interaction.

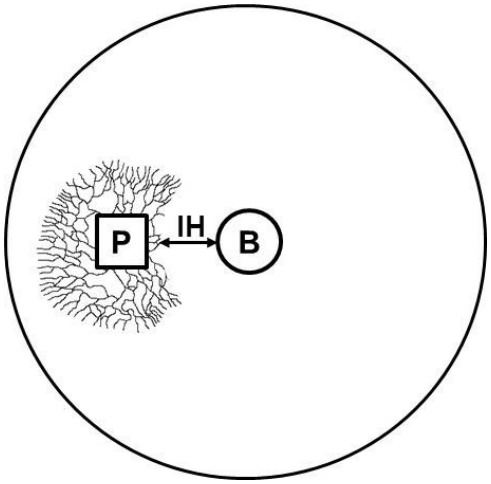


Figure 11 | Antagonistic activity assay performed in PDA Petri dishes inoculate with (B) *B. amyloliquefaciens* filtrate or extract and (P) fungal pathogens mycelium. The distance between the edge of the well and the pathogen mycelium is named as Inhibition halo (IH).

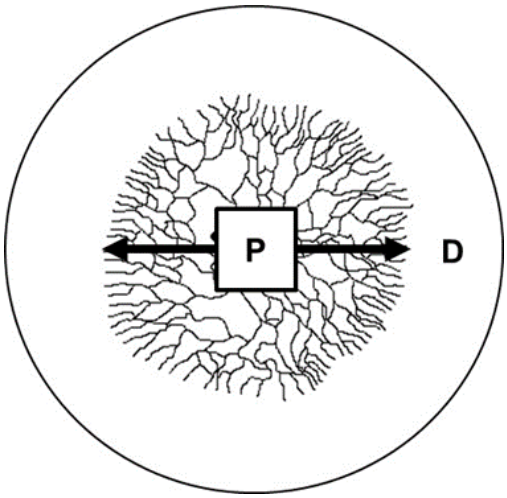


Figure 12 | Incorporated filtrate assay performed in LBFA Petri dishes inoculated with fungal pathogens mycelium (P). Mycelium growth diameter (D) was measured and used to evaluate the antifungal activity of bacterial strains.

3.3 Anti-fungal activity in broth assay

The three strains of *B. amyloliquefaciens* were grown for 72 h at 27 °C in Erlenmeyer flasks containing 100 ml of LB.

At 16, 24, 48 and 72 h, 2 aliquots of 2 ml were taken, from which the filtrates were subsequently obtained. These filtrates were used to verify the anti-fungal activity of bacterial strains in broth.

Filtrate of not-inoculated LB was used as negative control.

The assay was leaded in 96-well microplate, as follows.

Twenty µl of each filtrate, at different hours, were added to 180 µl of fungal spore suspension, forming the contact suspension (CS). The inoculated microplate was stirred at room temperature in an orbital agitator at 100 rpm.

After the incubation period, 20 µl of CS were withdrawn from each well, and added to 180 µl of PDB, into the wells of a new microplate.

In order to allow spore germination, microplates were placed inside a Varioskan® Flash Multimode Reader (Thermo Scientific™) at 23 °C for 72 h.

The absorbance values at 620 nm were recorded at 24, 48 and 72 h after the spore inoculation, which represent the fungal growth monitoring period (day 1, day 2 and day 3).

These values were used to evaluate the percentage of fungal growth inhibition (FGI), according to the following formula:

$$\mathbf{FGI}\ (\%) = \frac{\mathbf{Abs_c} - \mathbf{Abs_t}}{\mathbf{Abs_c}} \times \mathbf{100}$$

Abs_c = Absorbance of not-inoculated LB filtrate (control).

Abs_t = Absorbance of filtrates from bacterial growth broth at 16, 24, 48 and 72 h.

Averages of the percentage values obtained were calculated, then undertaken to statistical analysis.

Each BCA filtrate-pathogen interaction was performed in duplicate in three different microplates.

For every tested bacterial strain non-inoculated broth was used as a negative control.

4 CLPs characterization

4.1 Genetic characterization

Antimicrobial biosynthetic genes were characterized in Ba15, Ba16 and Ba18. Specifically, five sequences were chosen for the coding regions of *srfAA* (surfactin synthetase subunit 1), *bmyB* (bacillomycin L synthetase B), *ituA* (iturin A synthetase A), *mycA* (mycosubtilin synthetase A) and *fenD* (fengycin synthetase) (Tab. 5). For the detection of *srfAA*, *bmyB* and *fenD* primers described by Mora et al. (2011) were used. Instead, for *ituA* and *mycA* new primers have been developed through gene sequences obtained from GeneBank database (NCBI). These were aligned through the MultiAlin software (Corpet 1988), to identify the consensus regions, which were used to design specific primers by means of Primer3 software (version 4.1.0) (Mora et al., 2011). To verify their specificity, the newly designed primers were checked by blasting them through the Basic Local Alignment Search Tool for Nucleotide (NCBI).

Table 5 | Oligonucleotide primers used for cLPs detection.

Expression Product	Gene	Primer	Sequence (5' - 3')	Melting T (°C)	Product size (bp)
Surfactin *	*srfAA*	SRFAF SRFAR	TCGGGACAGGAAGACATCAT CCACTCAAACGGATAATCCTGA	60.4	201
Bacillomycin *	*bmyB*	BMYBF BMYBR	GAATCCCGTTGTTCTCCAAA GCGGGTATTGAATGCTTGTT	59.9	370
Iturin	*ituA*	ITUAF ITUAR	TCGCCCTTCACTTTCAGCAT TTAGCCGTCCTTCGCCATTT	67.0	479
Mycosubtilin	*mycA*	MYCAF MYCAR	GCTGGACAAATTGCGGTTGT TGAGAAACAGCGGGCATCTT	67.1	296
Fengycin *	*fenD*	FENDF FENDR	GGCCCGTTCTCTAAATCCAT GTCATGCTGACGAGAGCAAA	60.1	269

* Primers described by Mora et al. (2011).

DNA extraction

DNA extraction was performed from bacterial suspension of 10^8 cfu/ml using QIAamp DNA Mini Kit (Qiagen, Iberia S.L., Madrid, Spain), a specific kit for Gram+ bacteria. The extraction protocol has been modified in comparison to the original one (Tab. 6). The genomic DNA was quantified by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Barcelona, Spain) and stored at -20 °C in sterile ultrapure water.

PCR

The PCR assay, for each single gene, was performed for each amplification reaction in a final volume of 50 µl consisting of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Invitrogen Technologies), 0.2 µM of each primer, 2.0 U of Taq DNA polymerase (Invitrogen) and 100 ng of genomic DNA (Mora et al., 2015). To amplify all the targets the following cycle conditions have been adopted: 95 ºC for 5 min; 40 cycles of 94 ºC 1 min, annealing temperature for 1 min and 72 ºC for 1 min; final extension step of 5 min at 72 ºC.

The annealing temperature was set to 55 °C for *bmyB* and to 58 °C for all the other genes.

The amplifications were conducted in a T3000 thermocycler (Biometra, Germany). Amplification products were analyzed in a 1.8% agarose gel in 1X Tris-acetate EDTA (TAE) buffer, for 45 min at 90 V. The dimension comparison was estimated by comparison to 1-kb Plus Ladder marker (Invitrogen, CA, USA).

For each PCR assay, *B. amyloliquefaciens* strains EPS2017 and A59 (from CIDSAV collection, University of Girona) were introduced as positive controls, while water and *B. clausii* were used as negative controls.

After permanence in ethidium bromide, gel images were captured with ChemioDoc XRS + System (Bio-Rad, USA).

Table 6 | DNA extraction using QIAamp DNA Mini Kit (QIAGEN).

Step	Description
1	Place 1000 µl of sample into an microcentrifuge tube (1.5 ml) and collect the pellet bacteria by centrifugation for 10 min at 5000 g (7500 rpm)
2	Suspend bacterial pellet in 180 µl of the enzyme solution (20 mg/ml lysozyme; 20 mM Tris-HCl, pH 8.0: 2 mM EDTA; 1.2 % Triton)
3	Incubate for at least 30 min at 37 ºC
4	Add 20 µl proteinase K and 200 µl Buffer AL. Mix by vortexing
5	Incubate at 56 ºC for 30 min and then for a further 15 min at 95 ºC
6	Centrifuge for a few seconds
7	Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 sec., and incubate at 70 ºC for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the lid
8	Add 200 µl ethanol (96-100%) to the sample, and mix by pulse-vortexing for 15 sec. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid
9	Carefully apply the mixture (including the possible precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate
10	Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the collection tube containing the filtrate
11	Carefully open the QIAamp Mini spin column and add 500 l Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed 20000 g (14000 rpm) for 3 min.
12	Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
13	Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 30 l distilled ultrapure water. Incubate at room temperature for 30 min, and then centrifuge at 6000 g (8000 rpm) for 1 min.

4.2 Chemical characterization

The production of cLPs was evaluated in bacterial filtrates at 48 h of growth, obtained as described in § 3.4, through fast protein liquid chromatography (FPLC) analysis. In order to validate the profiles of the chromatograms obtained from the analysis of *Bacillus* filtrates, not-inoculated LB filtrate and three commercial cLPs standards, at the concentration of 100 µg/ml, have been used: iturin A (1 mg/ml), fengycin (5 mg/ml) and surfactin (10 mg/ml) (Sigma-Aldrich, USA). In addition, a solution consisting of their mix was used too.

The FPLC analysis was conducted on Amersham Biosciences ÄKTA FPLC System (GE Healthcare, UK) equipped with a multi wavelenght detector and a fraction collector, using a reverse-phase Kinetex XB-C18 column (column dimension, 75 x 4.5 mm; particle size, 2.6 µm; pore size, 100 A; Phenomenex, Madrid, Spain).

One hundred µl of each sample were analyzed through a flow rate of 1.00 ml/min using an aqueous solution of trifluoracetic acid (TFA) at 0.1% (solvent A) and a solution of TFA 0.085% in acetonitrile (solvent B). The solvent B gradient (%) over time was: 0 min - 5%, 21 min - 75%, 24 min - 100%, 27 min - 100%, 29 min - 5%. The elution was monitored at 220 nm.

4.3 Fraction characterization

In addition to the chromatographic analysis by ÄKTA FPLC, fractions were collected from the run of each filtrate. The eluted collection was performed by ÄKTA Frac-920 Fraction Collector at establish time intervals fixed in individual group of peaks or single peak. In total, 37 fractions were collected for each sample.

In order to remove the solvent from each eluted, the fractions were evaporated under vacuum, by a Scan Speed 40 Teflon (AAPPTEC, LCC, Louisville, USA).

Then they were ten-fold concentrated (compared to the elution volume) rehydrating them in 100 µl of SDW.

Subsequently, the antagonistic activity of the single fractions was tested against *B. sorokiniana*, as described in § 3.4.

The Abs values read by Varioskan® were registered 2 dpi of fungal spores.

Furthermore, fractions from individual cLPs standards, their mix solution and not-inoculated LB filtrate were used as controls.

4.4 Molecule stability

The stability of the molecules responsible for antagonism between bacterial and fungal strains, was evaluated after one year by filtrate assay, as described in § 3.3.

Filtrates were stored in 50 ml falcon at three different temperatures: -20 °C (freezer), 4 °C (fridge) and 23/25 °C (room temperature).

After one year their activity, against the four fungal pathogens of wheat, was evaluated. The IHs were measured and compared, for each bacterial-fungal interaction, with the ones registered the year before.

5 Microbial *in vivo* assays

5.1 Epiphytic survival assay

Ephiphytic survival of Ba15, Ba16 and Ba18 was evaluated on wheat seedlings. Forty-five wheat seeds (*T. durum* cultivar Svevo) were surface sterilized with sodium hypochlorite (0.5%, vol/vol) for 20 min and then rinsed thoroughly in SDW. Then they were seeded in Jyffypots® using commercial Brill substrate (Gebr. Brill Substrate GmbH & Co. KG, Georgsdorf, Germany). Plants were vernalized at 4 °C for 2 weeks and grown in a climatic chamber at 18 to 23 °C with a 14-h photoperiod (300 µE m^{-2} s^{-1}). The growth stages were counted using the Zadoks method (Zadoks et al. 1974).

At second leaf stage the seedlings (15 for each bacterial strains), were inoculated with a 10^8 cfu/ml *B. amyloliquefaciens* suspension, by 180 ml Nalgene® aerosol spray bottle (Merck KGaA, Darmastadt, Germany).

At 0, 16, 24, 48 and 72 h post inoculation 3 seedlings, for each bacterial strain, were cut and taken to the laboratory. Here the samples were washed in tubes containing 5 ml of sterilized Phosphate buffer (PB) (Mariotti 2012) added with Tween 80 at 0.05% v/v. The washing suspensions were later diluted according to ten-fold serial dilutions. One hundred µl from 4^{th} to 6^{th} dilution were plated on LBA Petri dishes and incubated at 27 °C.

After 24 h microbial charge was evaluated counting the number of cfu/Petri dish.

5.2 *In vivo* antagonistic assay

The antifungal activity against *B. sorokiniana* of the three strains of *B. amyloliquefaciens* was verified in wheat seedlings, at the second leaf stage. This assay was performed using both bacterial cells and filtrates obtained from their growth broths at 48 h.

The bacterial inoculation was carried out with 10^8 cfu/ml bacterial suspensions distributed on 10 seedlings (for each bacterial strain) by Nalgene® aerosol spray bottle. Once treated with bacterial strains, seedlings were placed in a vessel inside a climatic chamber at 18 to 23 °C with a 14-h photoperiod (300 µE m^{-2} s^{-1}). After 24 h the spores of *B. sorokiniana* were inoculated on the second leaf of each seedling, distributing a drop (20 µl) of conidial suspension (10^6 conidia/ml) through the use of a small soft brush (Volpi et al. 2011).

Filtrates distribution on seedlings was, instead, carried out both at 24 h before and after the inoculation of the pathogen, through Nalgene® aerosol spray bottles.

Leaves were collected three dpi and the infected leaf areas were analyzed by Assess©: Image Analysis Software for Plant Disease Quantification (American Phytopathological Society - APS).

These values were used for determining the Disease Severity Area (DSA) as follows:

$$\mathbf{DSA}\ (\%) = \frac{\mathbf{area_{lesion}}}{\mathbf{area_{leaf}}} \times \mathbf{100}$$

Finally, the DSA values were used to calculate the IR as follows:

$$\mathbf{IR}(\%) = \frac{\mathbf{C - E}}{\mathbf{C}} \times \mathbf{100}$$

IR= Inhibition ratio;

C = DSA of control leaves;

E = DSA of bacterial cell/filtrate treated leaves.

Infected wheat seedlings with *B. sorokiniana* spores represented negative control.

5.3 Growth promotion assay

The promotion of growth by Ba15, Ba16 and Ba18 was evaluated on wheat seedlings, examining their effectiveness in promoting germination and plant growth and in colonizing the rhizosphere.

5.3.1 Seed Germination

Mangenta™ vessels (77 x 77 mm) (Merck KGaA, Darmastadt, Germany) filled with 30 g of Brill substrate were sterilized and then sowed with sterilized wheat seeds cv. Svevo.

Twenty-five seeds, for each *B. amyloliquefaciens* strain, were watered with 5 ml of bacterial suspension 10^8 cfu/ml and placed in a climatic chamber.

Germination was monitored by recording the seeds germinated daily, up to the total germination of the seeds.

The values were subsequently used to calculate the daily seed germination percentage (DSGP) as follows:

$$\mathbf{DSGP} = \frac{\mathbf{germinated\ seeds_{daily}}}{\mathbf{total\ seeds}} \times \mathbf{100}$$

Twenty-five seeds watered with 5 ml of sterilized water represented the negative control.

5.3.2 Wheat growth promotion

Wheat seeds were germinated in Mangenta™ vessels, after having been watered with *B. amyloliquefaciens* suspensions, as described in § 5.3.1. Twenty-five seeds for each *Bacillus* treatment were used. Watering with sterilized water was the negative control.

Seven dpi the wheat seedlings growth promotion has been evaluated considering the shoot and root length, the root number and the dry weight increase.

The length of the shoots was measured from the collar to the tip of the second leaf, using a ruler.

Instead, the total root length was measured by using the “Root L” function of the ASSESS© - APS program after scanning the root systems of the seedlings.

The number of roots was evaluated by counting the radicles for each wheat seedling.

Finally, the dry weight was measured by precision scale (Sartorius AG, Göttingen, Germany) after drying the seedlings in oven (NKR Continental Pte Ltd©) for 48 h at 80 °C.

5.3.3 Rhizospheric competence

The ability to colonize soil and the wheat root system was evaluated in Ba15, Ba16 and Ba18. Soil and seeds in the 5 Mangenta® vessels (for each bacterial strains) were inoculated with 5 ml of suspensions 10^8 cfu/ml of *B. amyloliquefaciens* strains.

Seven dpi soil and roots of each seedling were washed for 2 min at speed 7, using ULTRA-TURRAX® Tube Drive (IKA®, Werke Staufen, Germany), as follows:

- 1 g of soil in 9 ml of PB added with Tween 80 at 0.05% v/v.;
- 100 mg of rhizosphere (roots and adherent soil) in 10 ml of PB added with Tween 80 at 0.05% v/v.

Then samples were later diluted according to ten-fold serial dilutions. One hundred µl from 4^{th} to 6^{th} dilution were plated on LBA Petri dishes and incubated at 27 °C.

After 24 h colonies for each petri dishes were counted for the quantification of *B. amyloliquefaciens* strains.

The initial bacterial inoculum charge was evaluated in the soil and used as a control, analyzing as previously described 1 g of soil immediately after inoculation with bacterial strains.

6 Statistical analysis

In order to highlight significant differences, data from each assay were analyzed by analysis of variance (ANOVA).

Statistical analysis was performed by the macro DSAASTAT ver. 1.101 (Onofri 2007) of Microsoft Excel 2016. Duncan's multiple range test was performed for $P \leq 0.05$.

4 RESULTS

1 *In vitro* assays

Objective 1: **Evaluation of *in vitro* antagonistic activity of *B. amyloliquefaciens* strains against the fungal pathogen of wheat.**

The antagonistic activity of the three strains of *B. amyloliquefaciens*, Ba15, Ba16 and Ba18, was evaluated through *in vitro* tests against four fungal pathogens of wheat: *F. graminearum*, *F. culmorum*, *B. sorokiniana* and *S. tritici*. Bacterial growth broth filtrates and extracts were used to evaluate the nature of the molecules involved in the antagonism processes.

1.1 Filtrate assay

The antagonism assay performed with filtrates of bacterial growth broths, showed that Ba15, Ba16 and Ba18 are able to inhibit the fungal mycelium growth, of each pathogen tested. The histogram (Fig. 13) shows as the most inhibited fungal pathogen is *B. sorokiniana* with an IH between 1 and 1.2 cm, followed by *S. tritici* (from 0.6 to 0.8 cm) and finally by the two *Fusarium* spp., *F. graminearum* (between 0.3 and 0.5 cm) and *F. culmorum* (from 0.15 to 0.30 cm) respectively. Moreover, from statistical analysis it is clear that the differences among the averages observed, relative to antagonists, pathogens and each antagonist-pathogen interaction, are highly significant.

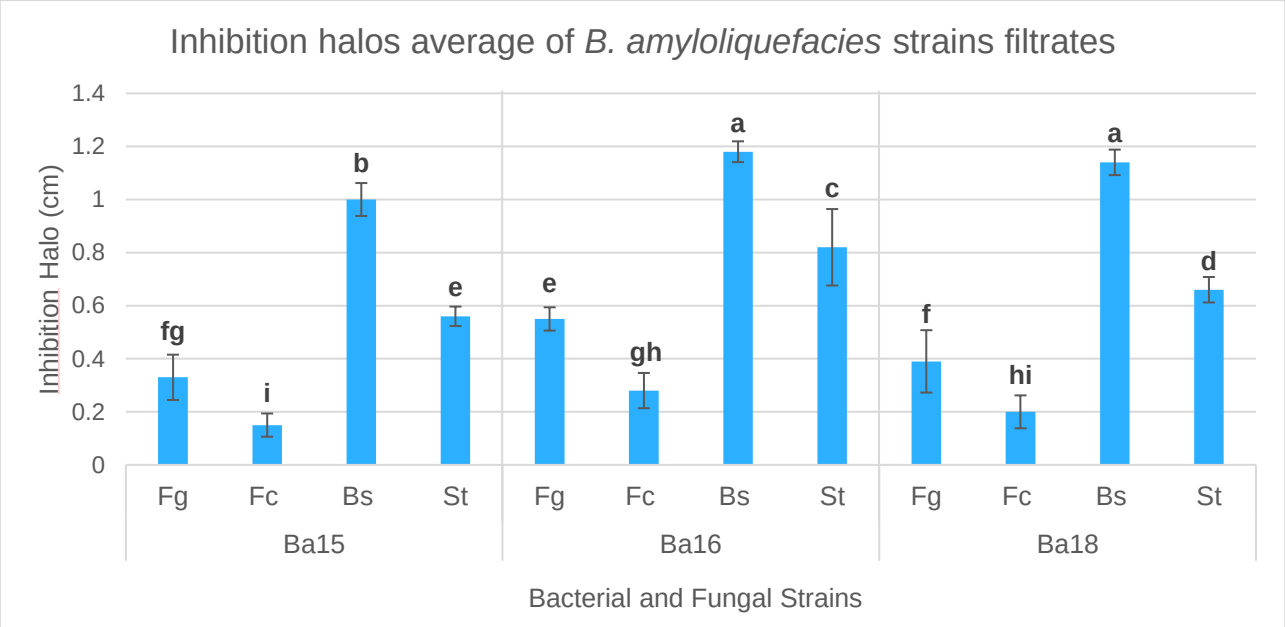


Figure 13 | Filtrate Assay – Average inhibition halos of *B. amyloliquefaciens* strain filtrates against the fungal pathogens of wheat: *F. graminearum* (Fg), *F. culmorum* (Fc), *B. sorokiniana* (Bs) and *S. tritici* (St). Duncan's multiple range test was performed for P≤0.05.

1.2 Extract assay

Fungal growth inhibition, mediated by bacterial extracts, was effective against all pathogens. The differences of the means of the IHs were not very variable, but nevertheless statistically highly significant.

As reported in the histogram (Fig. 14) *B. sorokiniana* proved to be the most inhibited pathogen with an IH of 0.8 cm; while *Fusarium* spp. and S. *tritici* showed a smaller halo, around 0.6 cm.

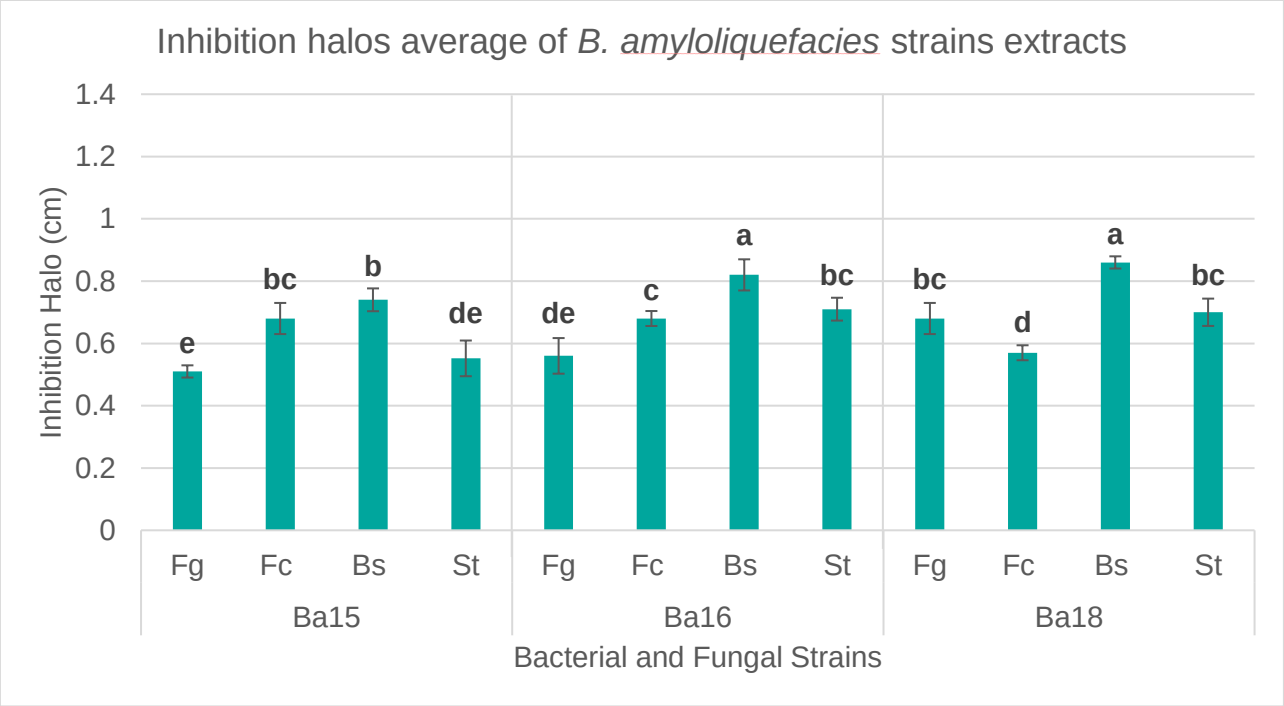


Figure 14 | Extract Assay – Average of the Inhibition halos of *B. amyloliquefaciens* strain extracts against the fungal pathogens of wheat: *F. graminearum* (Fg), *F. culmorum* (Fc), *B. sorokiniana* (Bs) and *S. tritici* (St). Duncan's multiple range test was performed for P≤0.05.

1.3 Incorporated filtrate assay

The IR values obtained from the average diameter of the mycelium growth of each pathogen is reported in histogram below (Fig. 15). *B. amyloliquefaciens* strains can inhibit fungal growth also by incorporation of their growth broth filtrates into LBA.

B. sorokiniana turned out to be, even in this assay, the most inhibited pathogen with almost 100% of MGI. Instead, *S. tritici* and *F. graminearum* growth was inhibited about 65% and 60% respectively. Finally, *F. culmorum* was approximately 40% inhibited.

It is also important to focus the attention on the inhibitory activity of each *B. amyloliquefaciens* strain. If against *B. sorokiniana* the three bacterial strains gave a similar antifungal activity, they behaved dissimilar each other against the other pathogens.

Ba16 appears to be the most inhibitory strain against the two *Fusarium* spp., followed by Ba15, which has greater antagonistic activity against *S. tritici*. In general, Ba15 was the antagonist with less inhibitory activity.

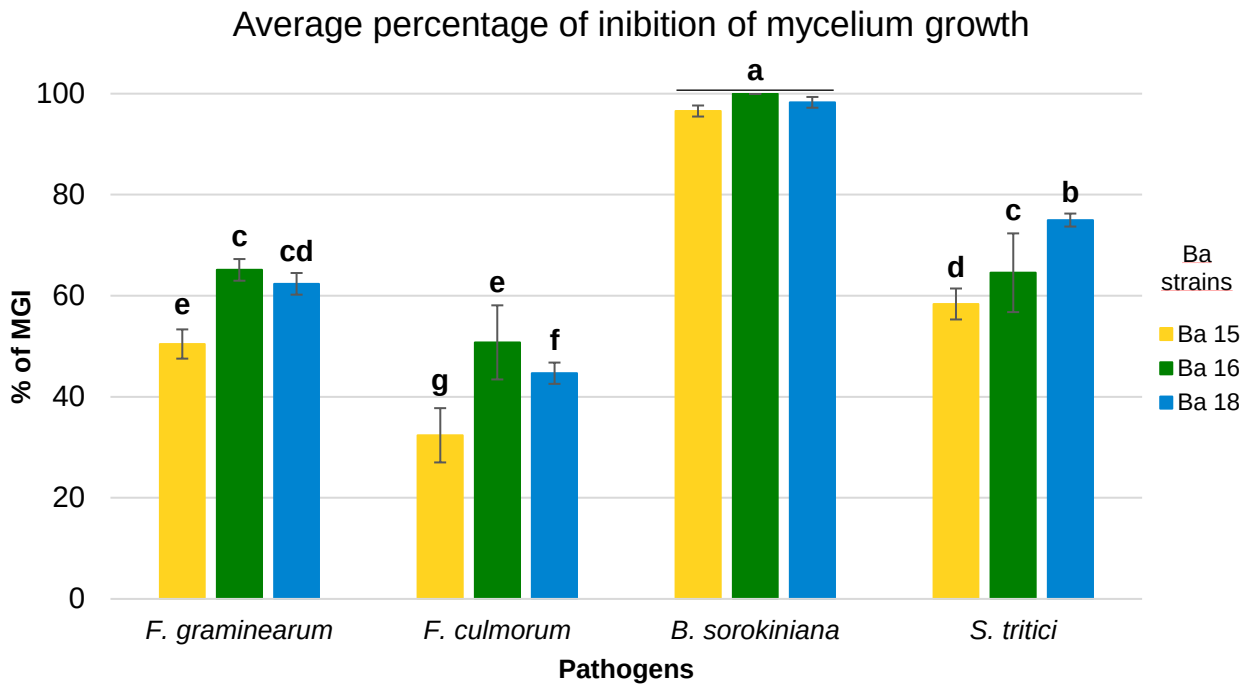


Figure 15 | Incorporated Filtrate Assay – Average percentage inhibition of *B. amyloliquefaciens* strain filtrates against the four fungal pathogens of wheat. The filtrates of each bacterial strain were incorporated in agarized LB. Duncan's multiple range test was performed for P≤0.05.

1.4 Anti-fungal activity in broth assay

The ability of the three *B. amyloliquefacie*ns strains to inhibit fungal growth was evaluated also in liquid. The use of filtrates at different times of bacterial growth, allowed to understand when the BCAs produce the compounds responsible of the antagonistic processes. Moreover, the absorbance readings, in the three days following the microplate inoculation, allowed to control the progress of the microbial interaction over time. The percentage values of FGI are shown in the following histograms (Figs. 16, 17, 18 and 19).

F. graminearum was inhibited by all antagonists on the first day of incubation. The effective filtrates were those at 48 and 72 h, giving a FGI percentage of 20%. Only Ba15 inhibited the pathogen also on the second day, maintaining the same percentage values of the previous day. Moreover, only for this strain, the 16 and 24 h filtrates proved to be effective against the fungal spores (Fig. 16).

F. culmorum was inhibited from the second day onwards, with percentage values of FGI unchanged. All filtrates have proven to be active against the fungal mycelium, especially those at 48 and 72 h. In general, B. amyloliquefaciens strain that inhibited most was Ba16, followed by Ba18 and Ba15, with FGI values around 40, 30 and 20%, respectively (Fig 17).

B. sorokiniana was the most inhibited pathogen even in broth assay. As can be seen from the histograms the levels of inhibition have been increasing day by day. A further singularity, compared to the other pathogens, is that all the bacterial filtrates have proved to be active against the fungal mycelium for all three days of incubation. Indeed, just on the first day, for all the bacterial strains tested and for all their filtrates, there was an antagonistic activity around 40%. This grew on the second day, reaching 60% of FGI, and then remaining stable on the third. Only in the case of Ba18 filtrates the inhibition activity was equal to 80% (Fig. 18).

S. tritici was inhibited between the second and third day. In the first, the values of FGI, were between 5 and 10%. Subsequently, the activity of antagonism for Ba15 was 20%, for all the tested filtrates, except for that at 72 h, which reached 40%, only in the second day.

Ba16 and Ba18 had stable FGI values over the last two days. Especially the filtrates at 48 and 72 h that reached inhibition values of 40% for Ba16 and 50% for Ba18 (Fig.19).

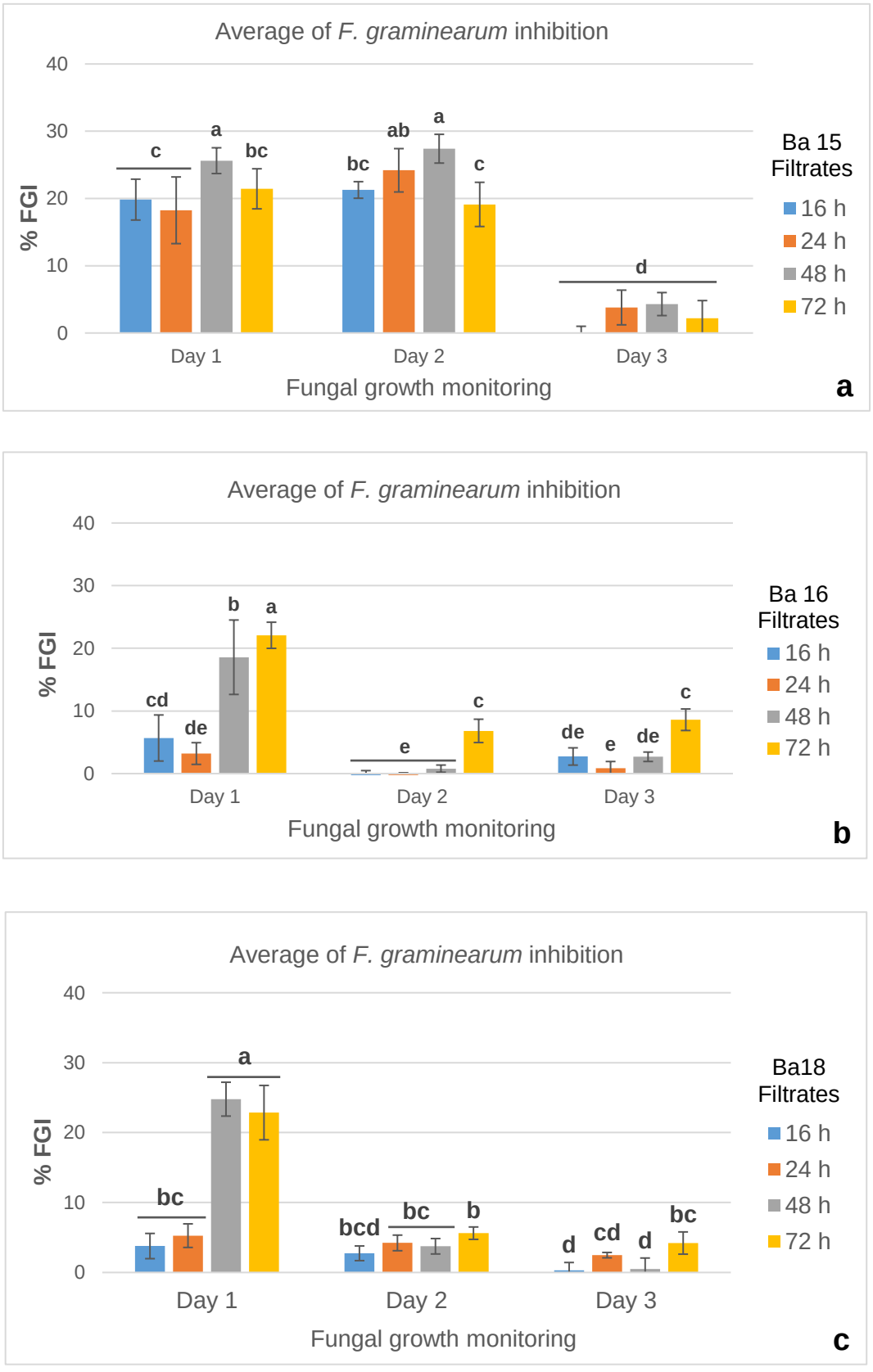


Figure 16 | Average of FGI index of *F. graminearum* inhibited by Ba15 (a), Ba16 (b) and Ba18 (c). Duncan's multiple range test was performed for P≤0.05.

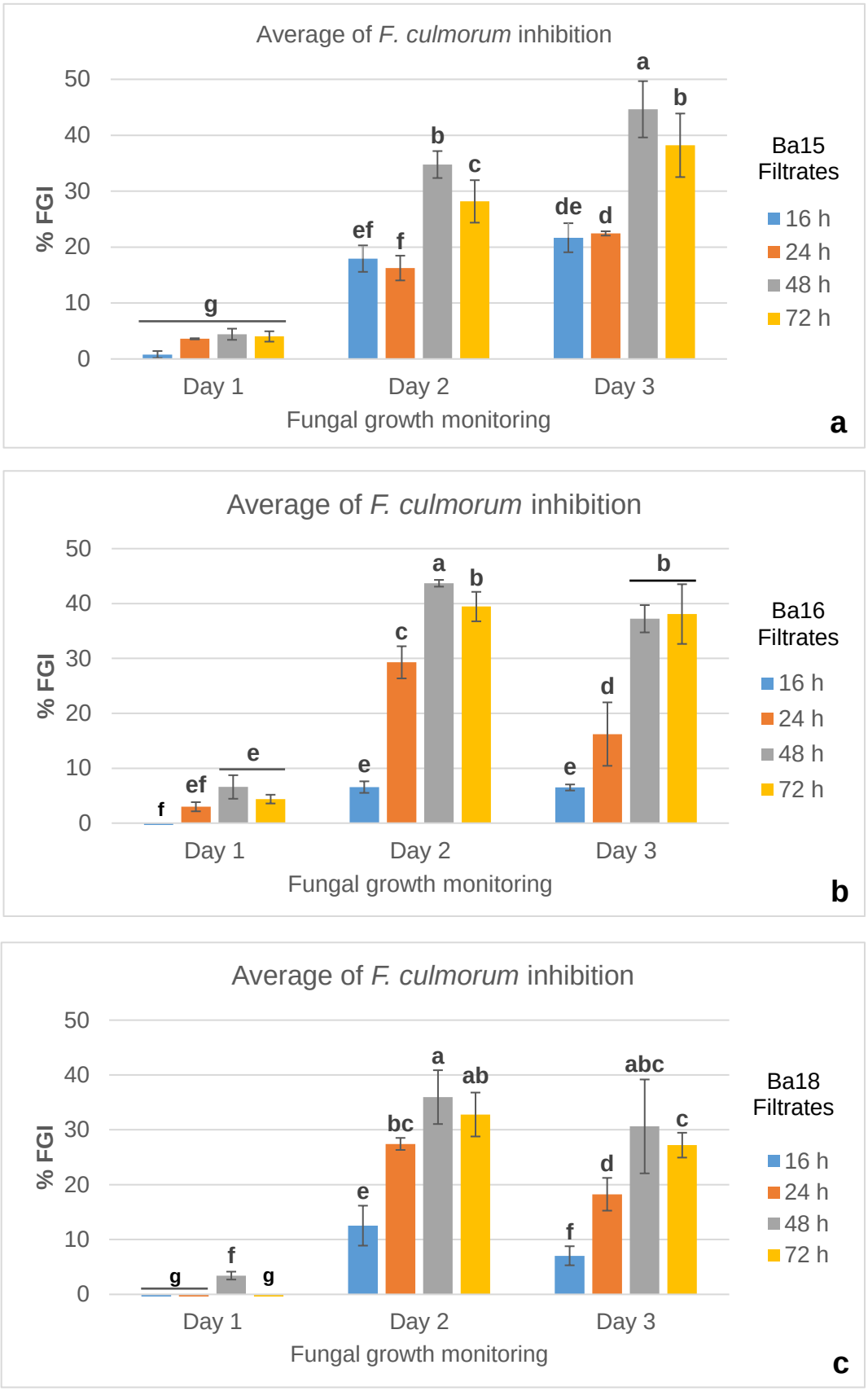


Figure 17 | Average of FGI index of *F. culmorum* inhibited by Ba15 (a), Ba16 (b) and Ba18 (c). Duncan's multiple range test was performed for P≤0.05.

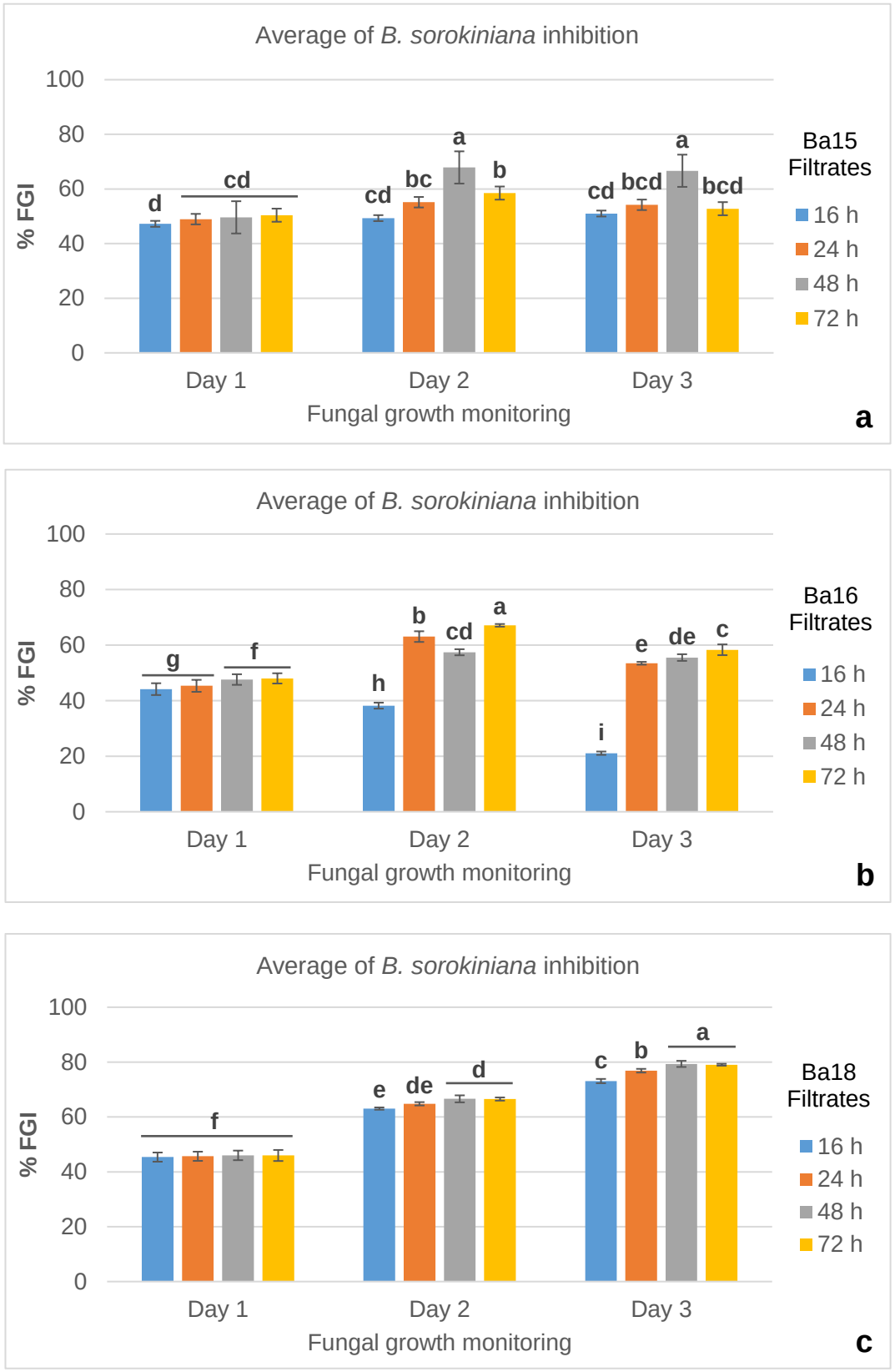


Figure 18 | Average of FGI index of *B. sorokiniana* inhibited by Ba15 (a), Ba16 (b) and Ba18 (c). Duncan's multiple range test was performed for P≤0.05.

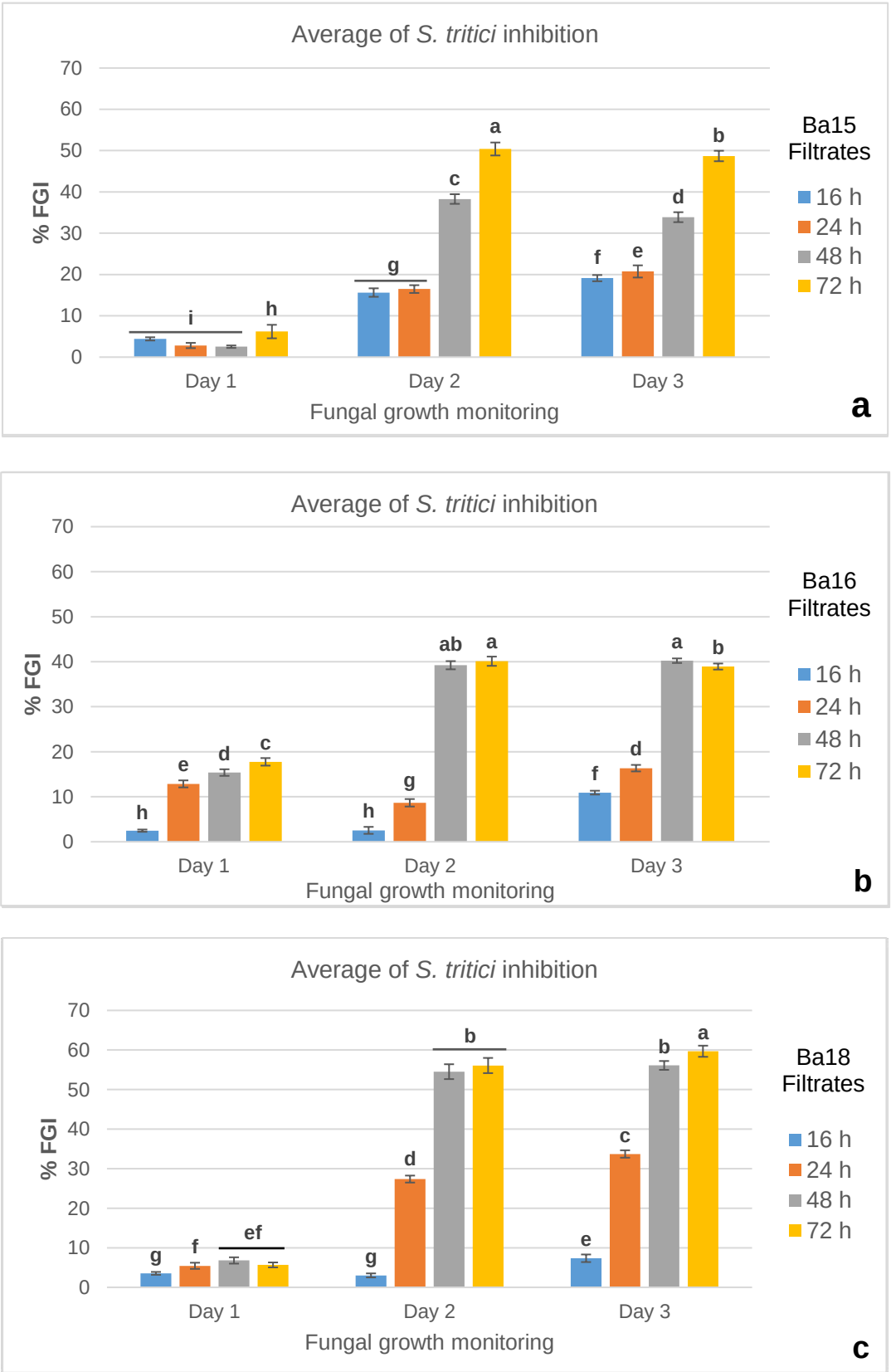


Figure 19 | Average of FGI index of *S. tritici* inhibited by Ba15 (a), Ba16 (b) and Ba18 (c). Duncan's multiple range test was performed for P≤0.05.

1.5 Discussion I

The aim of these assays was to evaluate *in vitro* the antagonistic activity of Ba15, Ba16 and Ba18 against *F. graminearum, F. culmorum, B. sorokiniana* and *S. tritici.*

The assays performed have highlighted the type of interaction between *B. amyloliquefaciens* and the four pathogenic fungi. Specifically, from each assay it was possible to delineate the nature of the antagonism processes.

The filtrates obtained from bacterial growth broths and their extracts (obtained with the addition of ethyl acetate), have proved to be effective against the selected pathogens both in solid and in liquid assays.

The use of filtrates allowed to understand that the compounds involved in the antagonism, mediated by the bacteria, are released in the growth substrate.

On the other hand, the assay carried out with bacterial extracts highlighted the ability of these compounds to be dissolved in apolar solvents. In fact, the molecules involved in the antifungal activity turn out to be liposoluble.

The assay performed incorporating the bacterial filtrates to the growth substrate showed the thermostability of the antifungal compounds produced by *B. amyloliquefaciens* strains. In fact, they have maintained their activity despite the autoclave cycle (121 °C for 20 minutes). This allowed to exclude enzymes from these microbial interactions.

The inhibition of the fungal mycelium growth was verified also in liquid. In fact, mycelium from the germinated spores of each pathogen was inhibited by bacterial filtrates.

Beyond antagonism, two more aspects emerged from this assay, concerning the production time and the persistence of antifungal compounds produced by bacteria.

The bacterial filtrates active against *F. graminearum* are released by *B. amyloliquefaciens* strains already at 16 h, reaching their activity peak between

48 and 72 h. This is in contrast with their persistence, as they are effective only for 24 hours with an inhibition activity of 20%. The antagonism persists even at 48 h only with Ba15. It is also interesting to focus the attention on the difference antagonism response observed on the agarized substrate and the broth. In fact, the pathogen mycelium turns out to be more sensitive in agarized medium than in liquid. This could be related to the ability of the mycelium originated from spore germination to metabolize and to adapt to AMC more easily.

The inhibition of spore-originated-mycelium of *F. culmorum* had a different outcome compared to the previous *Fusarium* species. It started from the second monitoring day, recording double inhibition values (around 40%) compared to those of *F. graminearum*. Despite this fact, active filtrates have proven to be the same for both.

B. sorokiniana was the only pathogen to have a gradual and increasing inhibition trend of the mycelium originated from spores over time. Furthermore, all bacterial filtrates were found to be effective against this fungal pathogen, which probably means that the production of these molecules could be proportional to bacterial growth.

Also for *S. tritici*, as it was for the former pathogen, the trend of mycelium inhibition was gradual throughout the monitoring time. The most active filtrates were those obtained at 48 and 72 h of bacterial growth.

In conclusion, it can be stated that the antagonism operated by *B. amyloliquefaciens* strains against the four pathogenic fungi of wheat is promoted by one or more molecules. It is however certain that they are liposoluble and thermostable compound(s) that are released by the bacteria.

2 CLPs characterization

Objective 2: **To characterize the cLPs through molecular and chemical analysis and evaluate their stability.**

The previously described assays allowed to delineate the intrinsic characteristics of the molecules that promote the antagonistic interaction between *B. amyloliquefaciens* strains and the four fungal pathogens of wheat. This allowed to identify a class of microbial compounds with antifungal activity, the cLPs.

The purpose of the following assays is to verify the production of such compounds by Ba15, Ba16 and Ba18 through molecular and chemical analysis, and to evaluate their stability and activity over time.

2.1 Genetic characterization

The PCR products were successfully obtained for all the genes of interest. Specifically, *surfAA* (surfactin), *bmyB* (bacillomycin), *mycA* (mycosubtilin), *ituA* (iturin) and *fenD* (fengycin) were detected in all the three strains of *Bacillus amyloliquefaciens* (Fig. 20). The two newly designed pairs of primers, *mycA* and *ituA*, gave a product with the expected dimension. In general, non-specific bands were not detected in each reaction.

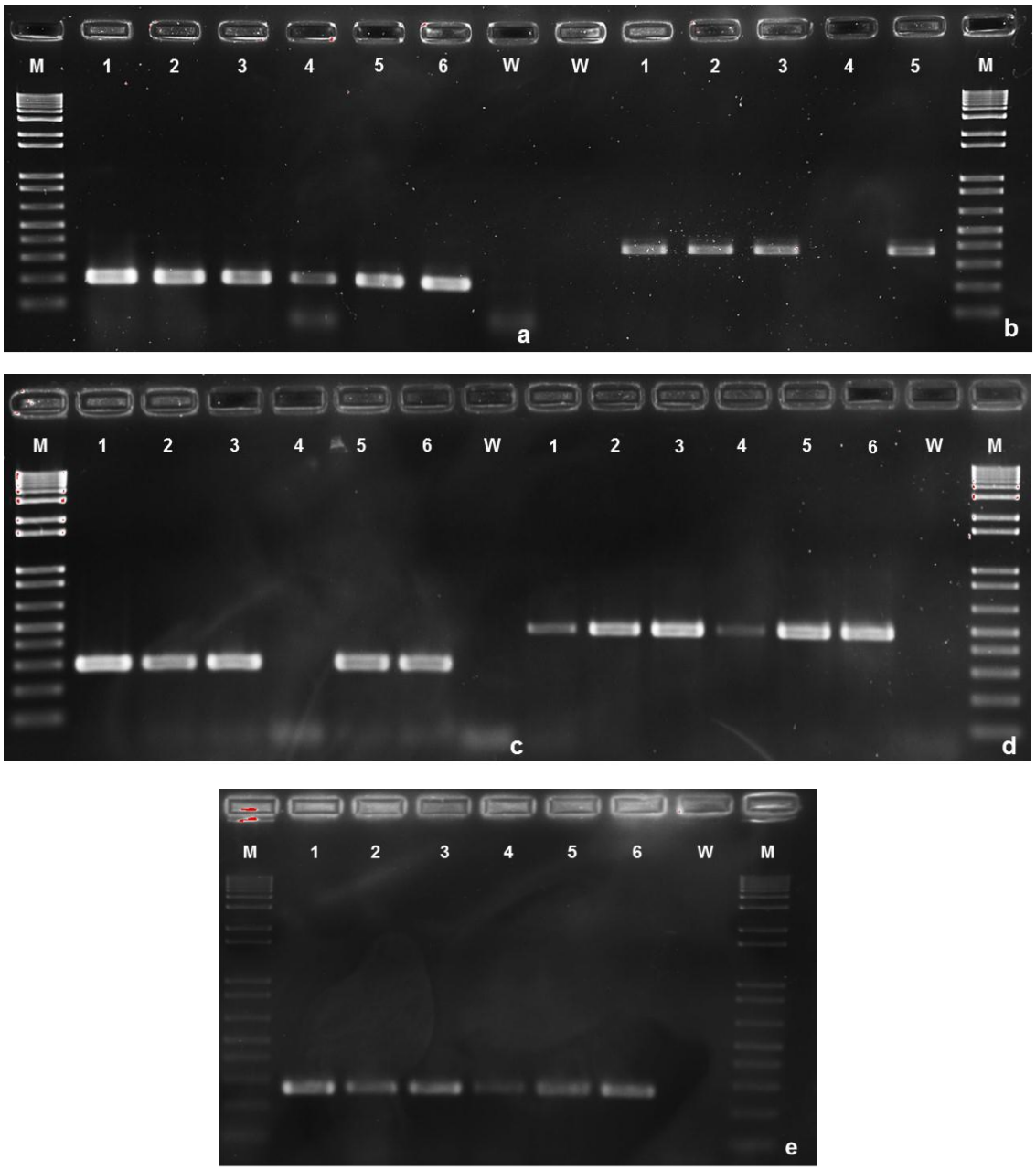


Figure 20 | PCR analysis of *surfAA* (~201 bp) (a), *bmyB* (~370 bp) (b), *mycA* (~296 bp) (c), *ituA* (~479 bp) (d) and *fenD (*~269 bp) (e) genes of *Bacillus* strains: Ba15 (1), Ba16 (2), Ba18 (3), *B. clausii* (4); *B. amyloliquefaciens* strains EPS2017 (5) and A59 (6). M, 1kb Plus Ladder (Invitrogen); W, water.

2.2 Chemical characterization

From FLPC analysis of the 48 h bacterial filtrates, chromatograms were obtained (Fig. 21). It was possible to discriminate the peaks related to LB substrate, located at the beginning of each chromatogram (from minute 3 to minute 12), comparing these with the not-inoculated LB ones.

Moreover, through the chromatograms obtained from standards cLPs and their mix (Fig. 22) it was possible to link the peaks to the respective family of AMCs. Specifically, the peaks detected from minute 13 to minute 22 belong to iturin and fengycin and finally those detected from minute 23 to minute 28 belong to surfactin.

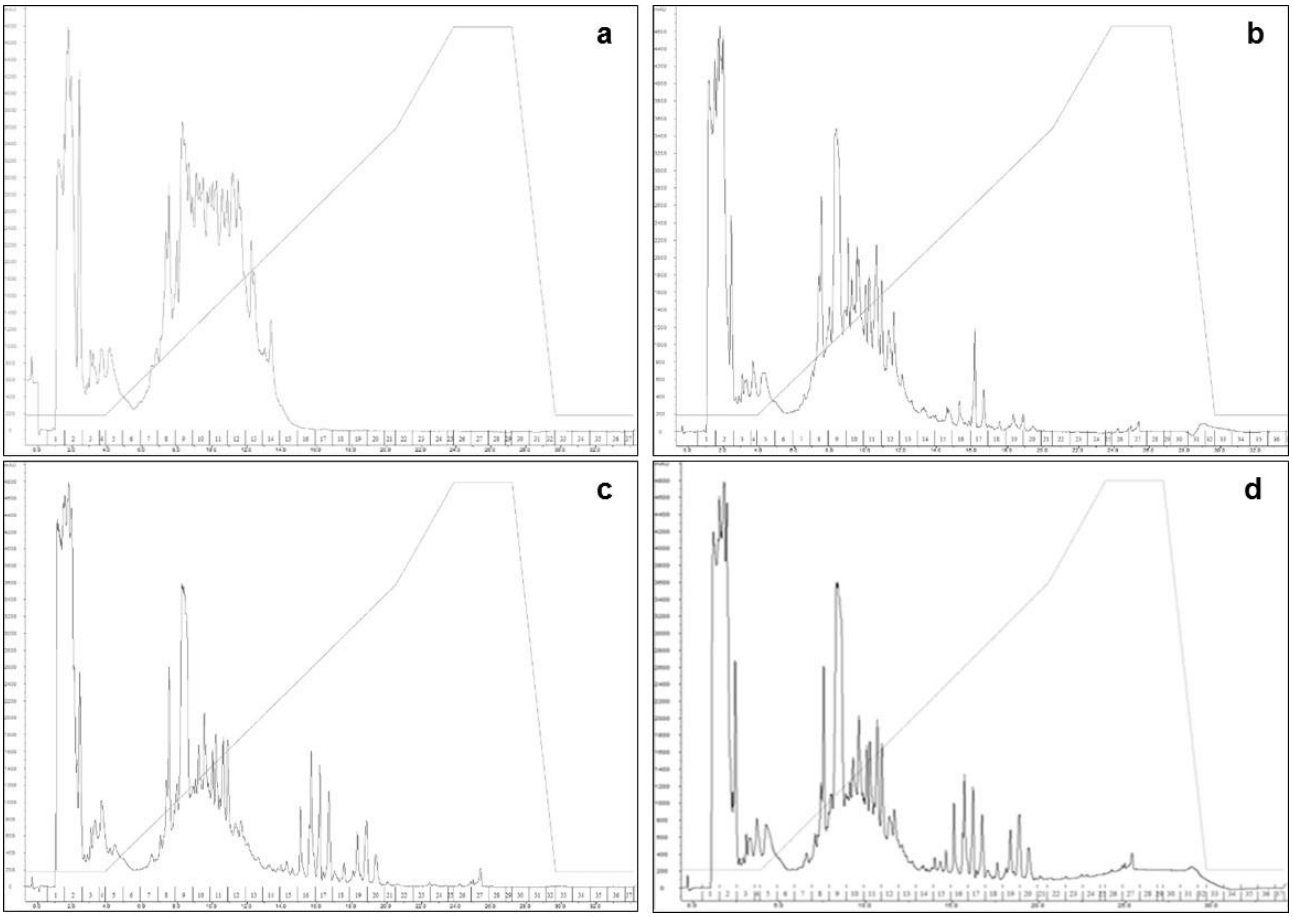


Figure 21 | ÄKTA FPLC chromatograms of non-inoculated LB (a), Ba15 (b), Ba16 (c) and Ba18 (d) filtrates using a reverse-phase Kinetex XB-C18 column.

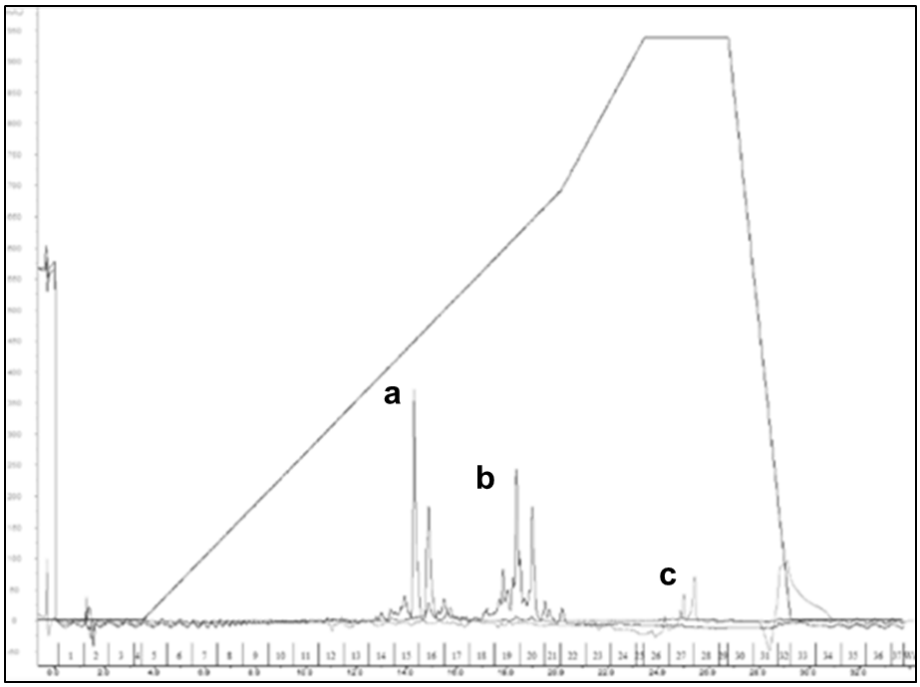


Figure 22 | ÄKTA FPLC chromatograms of commercial cLPs standards iturin (a), fengycin (b) and surfactin (c) using a reverse-phase Kinetex XB-C18 column. Standards has a concentration of 100 µg/ml.

2.3 Fraction characterization

The inhibitory activity of the fractions collected, from each eluted *Bacillus* sample, was evaluated against the germination of *B. sorokiniana* spores.

Almost all fractions had an inhibiting activity against fungal mycelium; however, the most active ones proved to be fractions from 18 to 21, with inhibition values between 50 and 60% (Fig. 23).

The cLP commercial standards, and their mix, inhibited fungal growth of 15% for iturin, from 40 up to 60% for fengycin and 10% for surfactins (Fig. 24).

It is important to highlight that fraction 20 (Fig. 25) was the most active fraction of *Bacillus* strains, fengycin standard and cLP standard mix, with an inhibition value of 60%.

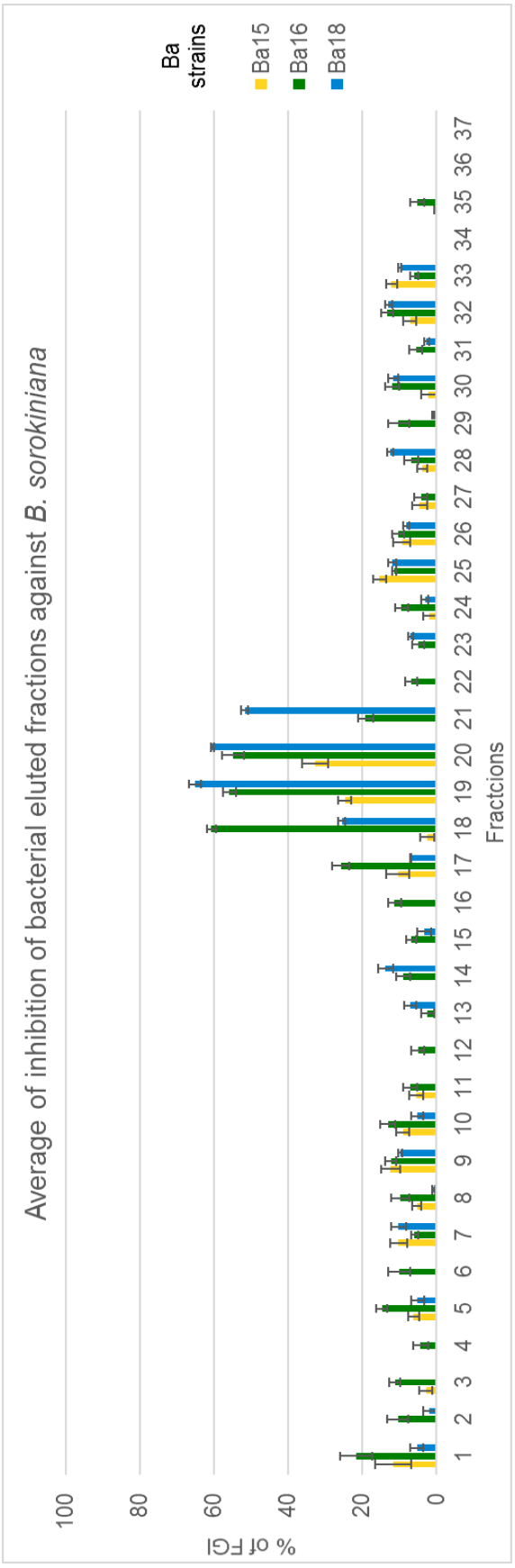


Figure 23 | Average of FGI index of *B. sorokiniana* inhibited by eluted fractions of Ba15 (yellow), Ba16 (green) and Ba18 (blue) filtrates. No statistical analysis was performed because the fractions were not characterized, as their quantity production values were not linked with the inhibition ones.

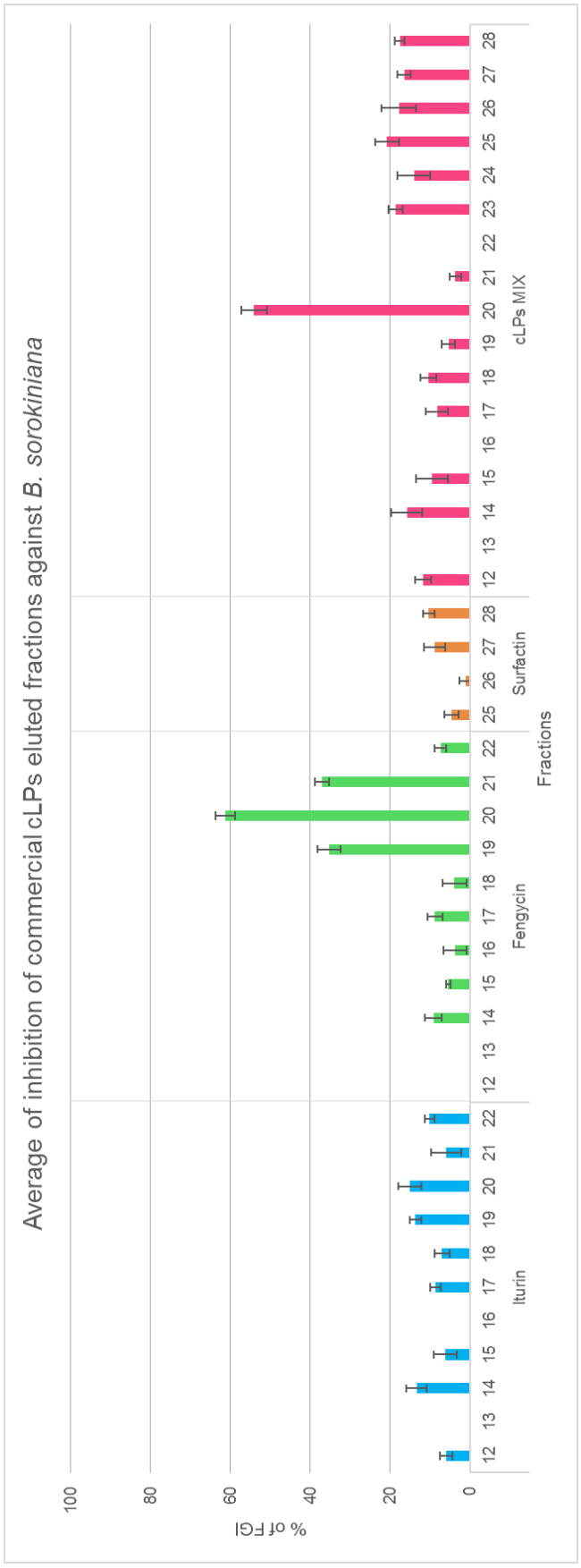


Figure 24 | Average of FGI index of B. sorokiniana inhibited by eluted fractions of commercial cLPs standards: iturin (light blue), fengycin (light green), surfactin (orange) and their mix (pink). The post hoc test, carried out on the averages obtained by ANOVA, is not shown as not decisive for the interpretation of the data obtained. What matters is to highlight the high significant differences ($P < 0.05$) among fraction 20 of fengycin and cLPs Mix and the other fractions.

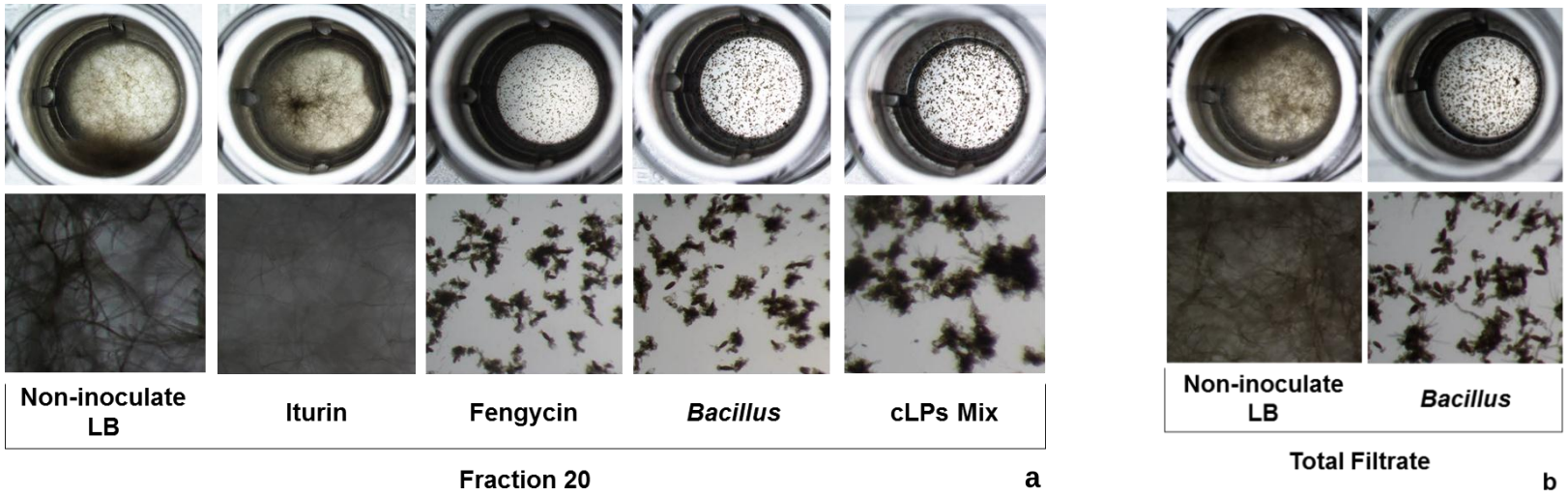


Figure 25 | Pictures of the microplate wells showing the interactions of the different fractions 20 (a) and the filtrates (b) against *B. sorokiniana*. Pictures were taken at the stereomicroscope; with 1x (top - well) and 8x (bottom – mycelium/spores) zoom. The Bacillus strain shown in the photos is Ba18.

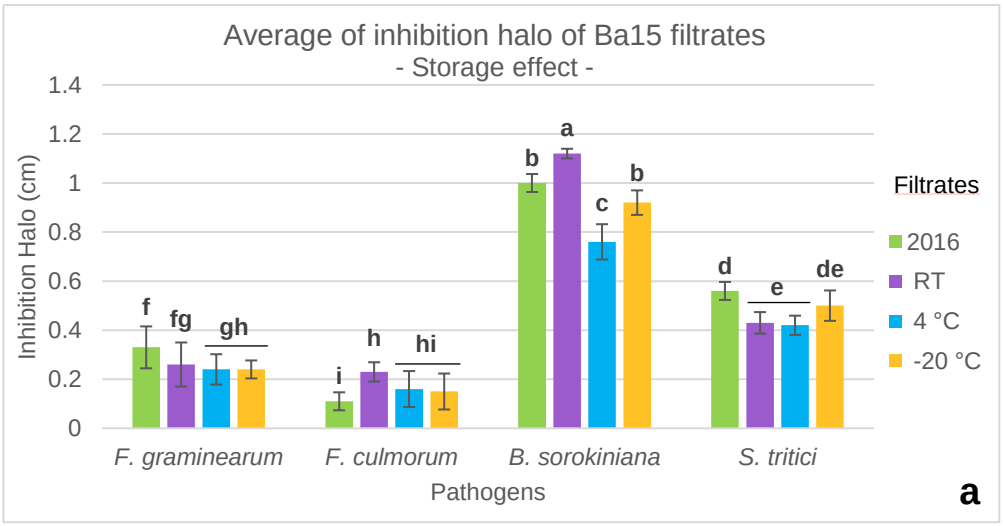
2.4 Molecule stability

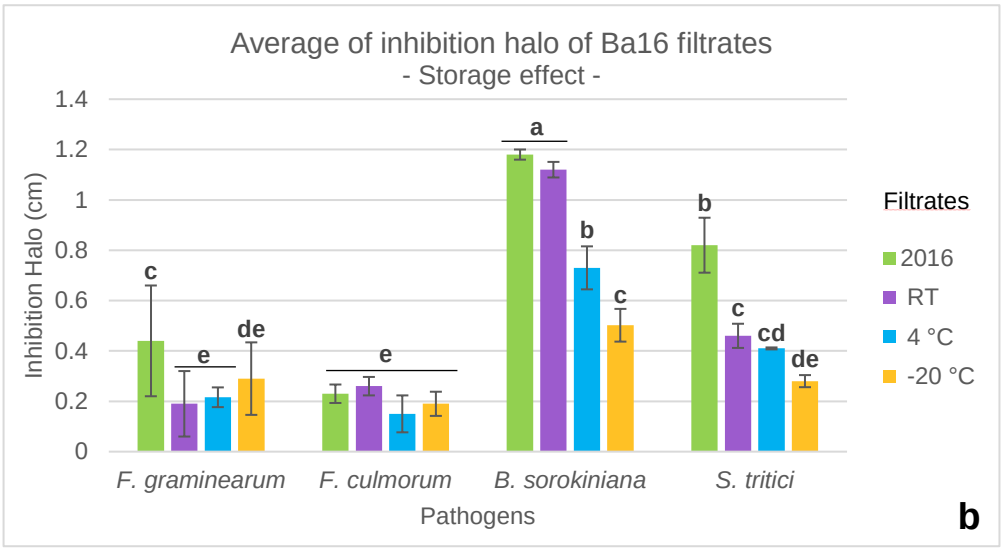
The antifungal activity of bacterial broth filtrates, stored at three different temperatures (RT, 4 and -20 °C), was evaluated after one year (Fig. 26).

Bacterial filtrates after one-year, regardless storage method and *B. amyloliquefaciens* strain, have lost their effectiveness against the causal agent of FHB; instead they maintained in part or slightly increased the antagonistic activity against *F. culmorum*.

The inhibition of *B. sorokiniana*, after one year from the original assay, was different among filtrates of the same bacterial strain. This means that storage temperature influences the effectiveness of the compound(s) responsible for the antagonism. In particular, filtrates stored at room temperature has maintained or increased the inhibition activity against the mycelium of *B. sorokiniana*, compared to those used the year before. In general, the filtrates stored at low temperatures (4 and -20 °C) had drastically reduced, or in some cases halved, their effectiveness against the fungal pathogen.

Also for *S. tritici,* as observed for *F. graminearum*, the stored bacterial filtrates reduced their inhibitory activity compared to the fresh ones used the year before.





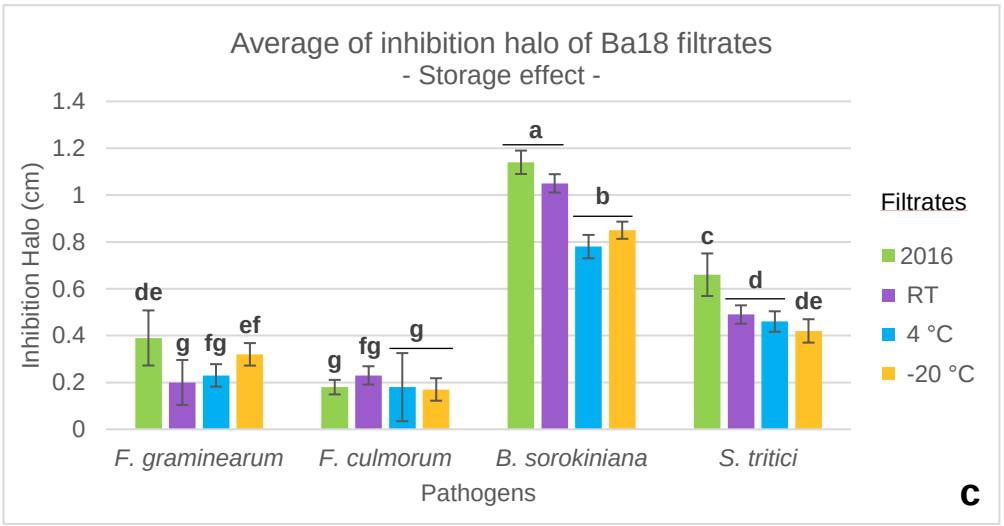


Figure 26 | Average inhibition halos of Ba15 (a), Ba16 (b) and Ba18 (c) filtrates against the four fungal pathogens of wheat. The filtrates were stored at three different temperatures: room temperature (RT), 4 °C, -20 °C. Their effectiveness was evaluated comparing with fresh filtrates of the year before (2016). Duncan's multiple range test was performed for P≤0.05.

2.5 Discussion II

The aim of these assays was to verify the production of cLPs by Ba15, Ba16 and Ba18 and to correlate it with antagonism activity against the four pathogenic fungi of wheat. This was done through genetic and chemical characterization.

Through molecular analysis, all the genes coding for the production of surfactin (*surfAA*), bacillomycin (*bmyB*), mycosubtilin (*mycA*), iturin (*ItuA*) and fengycin (*fenD*) were detected in *B. amyloliquefaciens* strains. Also, Spanish *Bacillus* strains, ESP2017 and A59, confirmed to be valid positive controls for the detection of cLPs genes.

B. clausii, instead, proved to be an excellent negative control for the detection of *bmyB* and *mycA*, but not for *surfAA*, *ituA* and *fenD*. This could be related to the presence of the latter in *B. clausii* genome, possibly as part of a different pathway, not coding for the production of cLPs. It could be said that these genes (*surfAA*, *ituA* and *fenD*) could be important for this genus, as they are found in several *Bacillus* species (Mora et al., 2015).

In general, the detection of these genes cannot be directly correlate with the production of AMPs, because the synthesis pathway of each cLPs involves several genes, so it becomes necessary to compare the results of molecular analysis to those linked to the direct production of cLPs, such as FPLC.

The phenotypic characterization of AMCs was performed, by ÄKTA FPLC, on bacterial growth broth 48 h filtrates, as these showed a high inhibition activity against all the tested pathogens.

Chromatograms have demonstrated the production of iturins, fengycins and surfactins for Ba15, Ba16 and Ba18, thanks to the use of commercial cLPs as standards.

In addition, the eluted fractions peaks were collected and their activity was evaluated against *B. sorokiniana* spores, as it was the most inhibited fungal

pathogen. Through this assay has been possible to identify fraction 20, as the most active fraction, responsible for the antagonism against this pathogen.
Indeed, the eluted 20th fraction of *Bacillus* strains, fengycin standard and cLPs standards mix had the same inhibition value (around 60%) corresponding to the ones obtain with original *Bacillus* 48 h filtrates, at the 2nd dpi (Fig.18).
So probably fengycin should be the AMC involved in this microbial interaction; further analysis of eluted fractions, like HPLC-MS, should be recommended, in order to confirm that evidence.
Furthermore, antifungal *Bacillus* compound stability was assessed verifying filtrates' effectiveness one year after their collection. Moreover, their storage temperature effects were evaluated.
The storage method negatively influenced, apart from the temperature, bacterial filtrate activity against *F. graminearum* and *S. tritici,* which were less effective after one year.
This was not observed in the same filtrates tested against *F. culmorum* and *B. sorokiniana*. In fact, the filtrates stored at RT were as active or more active than fresh ones used the previous year; it did not happen for those stored at low temperature. This phenomenon could be explained by the fact that *B. amyloliquefaciens* could produce different compounds, which are involved depending on the type of interaction.
A further explanation, about the antagonism against *B. sorokiniana*, could be related to the chemical nature of the compound(s) involved. In fact, if we consider fengycin to be responsible for this interaction, it is possible to explain the inhibition gap between the RT filtrates and the other stored at low temperature. One of the amino acids of the cyclic peptide constituting fengycin is Tyrosine. It could be involved in oxidation processes, due to the presence of O_2 inside the falcon, that can lead to the formation of quinones. They, as proved by Bernini et al. (2006), have high antifungal activity. So, the inhibiting gap could be compensated by the activity of these compounds.

3 Microbial *in vivo* assays

Objective 3: **To evaluate *in vivo* the antagonistic activity of *B. amyloliquefaciens* strains against *B. sorokiniana* and to check for their wheat growth promotion activity.**

After *in vitro* characterization of the antifungal activity and cLPs production by the three *B. amyloliquefaciens* strains, it was necessary to evaluate their growth promotion activity on wheat seedlings and to confirm *in vivo* the antagonistic one against *B. sorokiniana*.

3.1 Epiphytic survival assay

In order to verify the ability of Ba15, Ba16 and Ba18 to survive on wheat leaf surface, cv. Svevo seedlings were sprayed with a 10^8 cfu/ml bacterial suspension. The epiphytic survival of *B. amyloliquefaciens* was monitored up to 72 h post inoculation.

The histogram (Fig. 27) shows how *B. amyloliquefaciens* has a very stable growth trend over time. This means that it colonizes the leaves of the seedlings of wheat but it fails to increase its population. This could be explained by the ability of these bacteria to form biofilms (Caracciolo 2014).

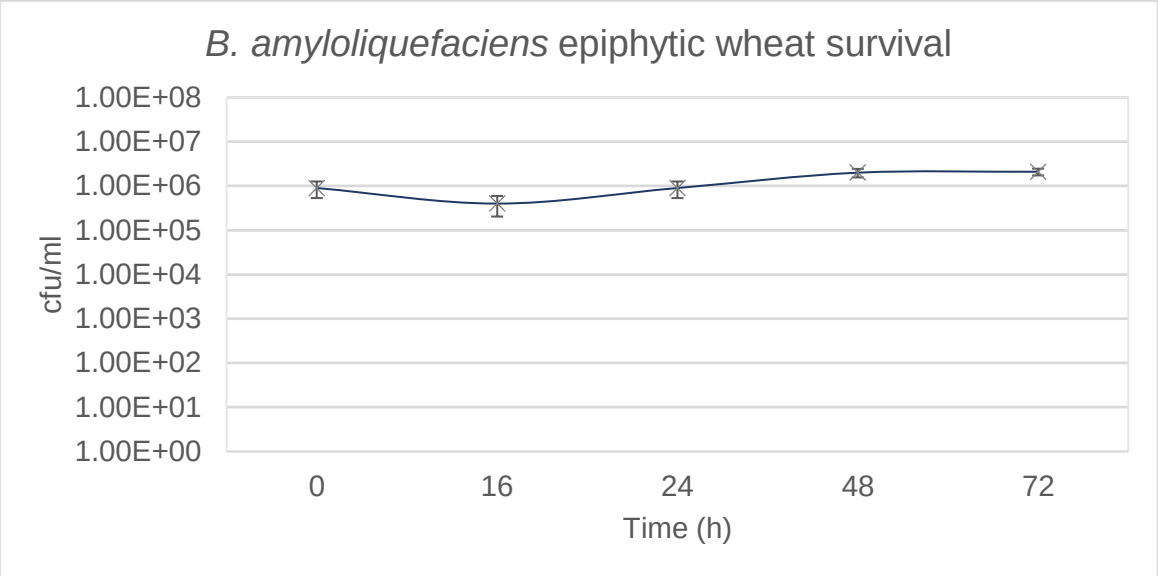


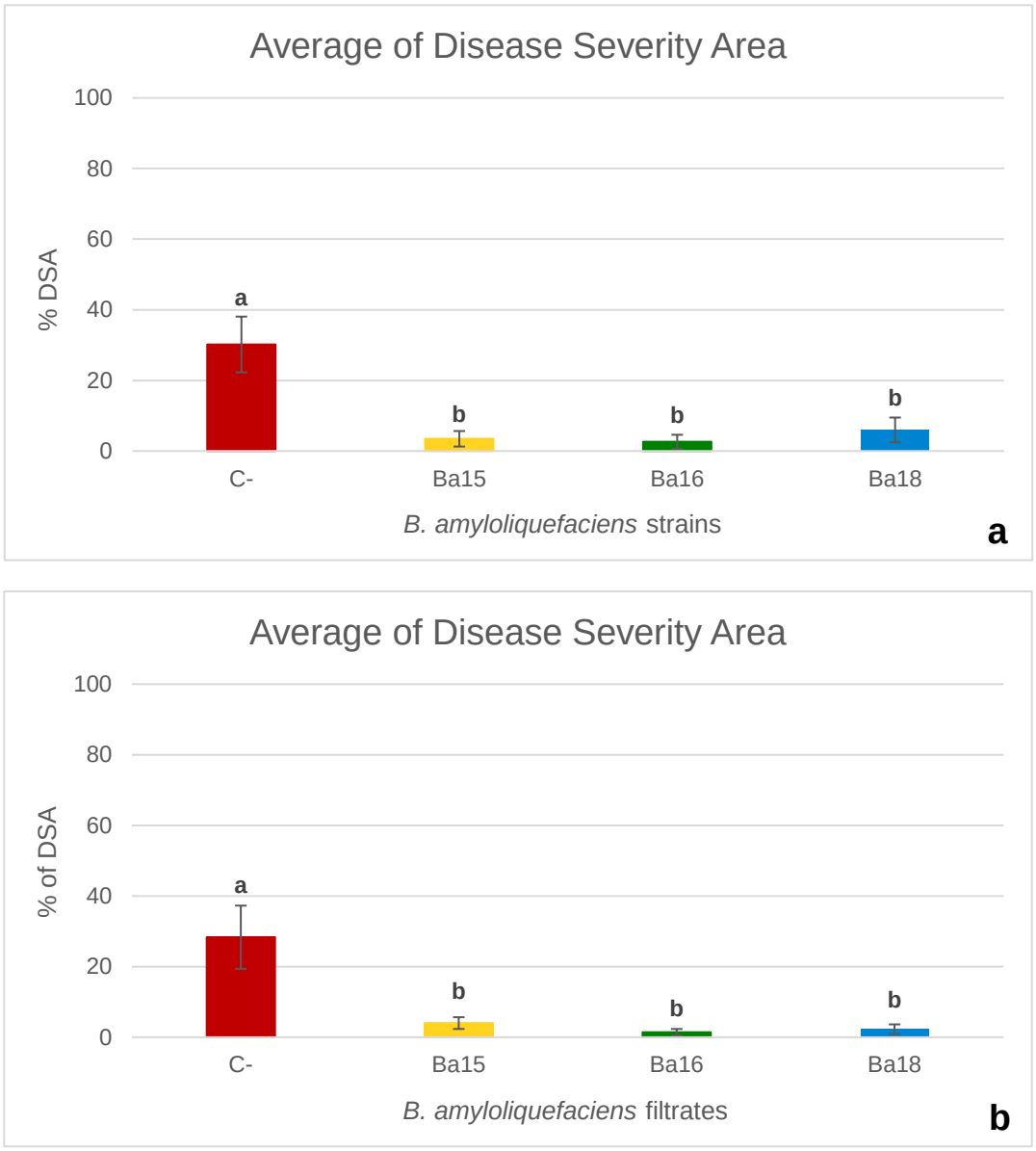
Figure 27 | Epiphytic survival of *B. amyloliquefaciens* on wheat seedlings leaves surface. All the three *B. amyloliquefaciens* strains are reported in this single growth curve, as they had the same growth trend.

3.2 *In vivo* antagonistic assay

Antagonistic activity, performed *in vivo*, was evaluated only for *B. sorokiniana* as it was the most inhibited wheat pathogen in *in vitro* assays.

The antifungal activity has been verified both by the direct use of *B. amyloliquefaciens* strains and their filtrates.

B. amyloliquefaciens strains confirmed their biocontrol action against *B. sorokiniana* (Fig. 28). In fact, the DSA obtain from the seedlings treated with Ba15, Ba16 and Ba18 was significantly lower than the one in the control, with an inhibition percentage around 80% (Table 7). The same result was obtained using the bacterial filtrates, with witch a fungal inhibition around 90% (Tab.7) was reached. It is important to highlight that no significant DSA differences were observed among *B. amyloliquefaciens* strains and among the respective filtrates.



2

Figure 28 | Histograms show the average DSA obtained from the use of *B. amyloliquefaciens* strains (a) and their filtrates (b). Significant differences were found between the seedlings treated with bacteria and their filtrates compared to the control treated with water (C-). Duncan's multiple range test was performed for P≤0.05.

Table 7 | DSA and Inhibition percentage values related to *B. amyloliquefaciens in vivo* antagonism against *B. sorokinianana.*

B. amyloliquefaciens strains	Bacterial cells		Bacterial filtrates	
	% DSA	% IR	% DSA	% IR
Ba15	4.0	86	3.7	87
Ba16	3.3	89	1.5	95
Ba18	5.6	81	2.4	92
Control	29.4		28.3	

3.3 Growth promotion assays

The growth promotion activity of the three *B. amyloliquefaciens* strains was carried out on wheat by evaluating: seed germination time, seedling development and bacterial rhizospheric competence.

3.3.1 Seeds Germination

Wheat seed germination was evaluated after watering seeds with bacterial suspensions (10^8 cfu/ml). The graph (Fig. 29) shows the positive effect of the bacterization with Ba15, Ba16 and Ba18 comparing with the control, watered with SDW.

Specifically, the bacterial strains allowed the germination of about 50%, of the bacterial treated seeds, just from the first day after the inoculation. Moreover, the total germination of the seeds was registered on the third day, two days before the total germination of the seeds of control.

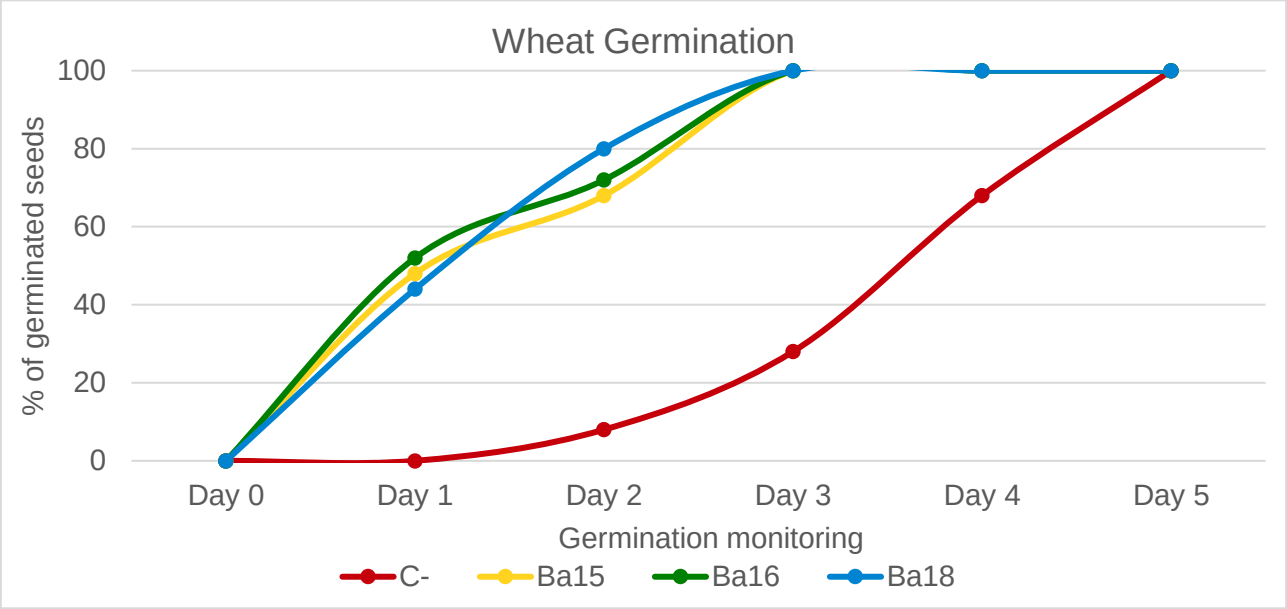


Figure 29 | Effect of *B. amyloliquefaciens* strains on wheat germination. The graph shows the percentage of seeds germinated daily.

3.3.2 Wheat growth promotion

The growth promotion of wheat seedlings by *B. amyloliquefaciens* strains has been evaluated (Fig. 30). The parameters considered were: dry weight, length of the shoot and length and number of roots.

A significant increase, for all the selected parameters, was observed in the seedlings treated with Ba15, Ba16 and Ba18 compared to the control, after seven days (Fig. 31). Significant differences between the strains were observed only for dry weight and total root length.

Figure 30 | Growth promotion of wheat seedlings by *B. amyloliquefaciens* strains. On the left a wheat seedling watered with SDW (C-).

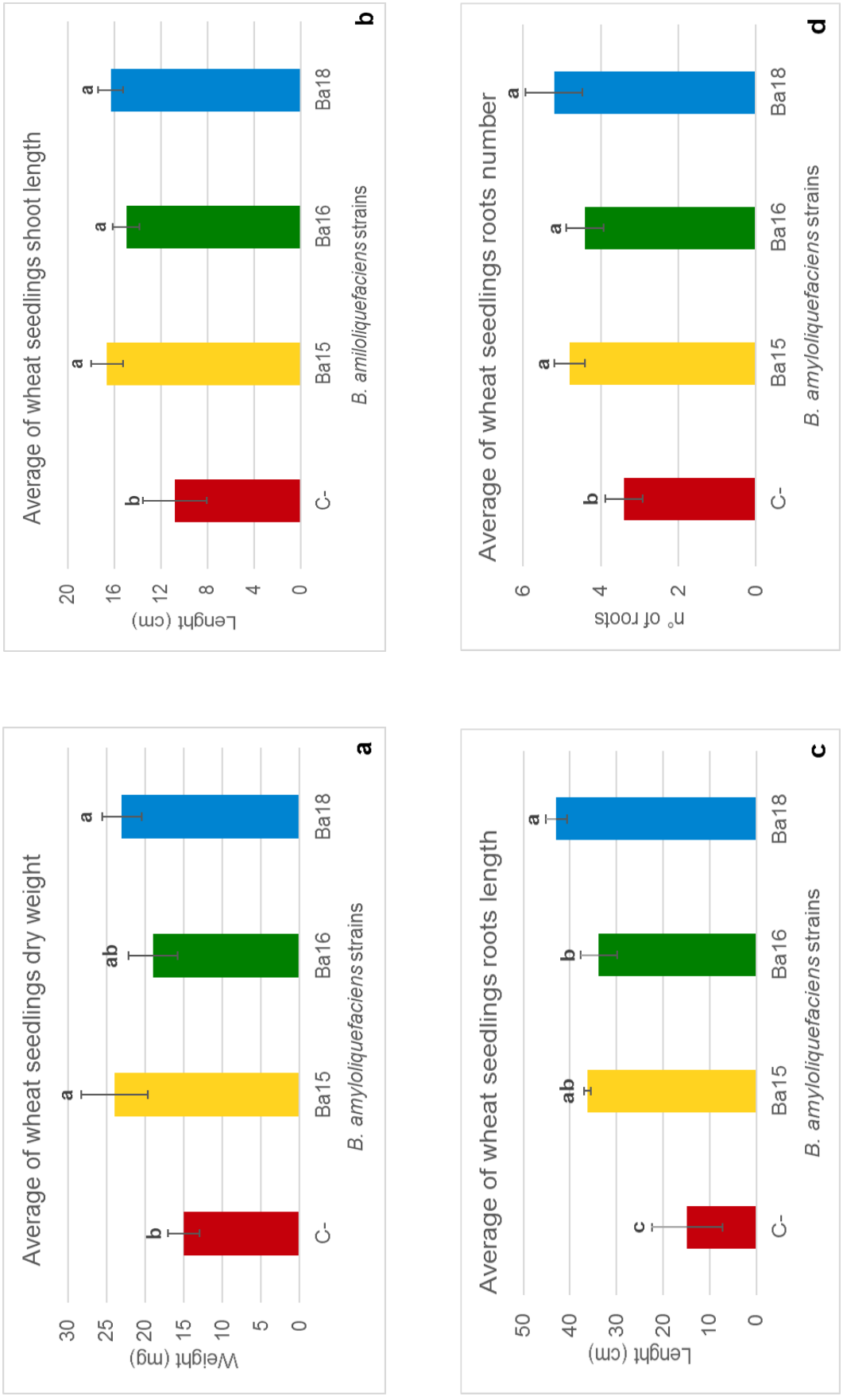


Figure 31 | Wheat growth promoted by *B. amyloliquefaciens* strains. Histograms show the effects of treatment with bacterial suspensions on wheat seedlings in terms of increase: dry weight (a), length of shoots (b), root length (c) and number of roots (d). Duncan's multiple range test was performed for P≤0.05.

3.3.3 Rhizospheric competence

The ability to colonize soil and rhizosphere by Ba15, Ba16 and Ba18 was evaluated in wheat seedlings. To verify these skills, the values of bacterial populations after seven days from the inoculation, were compared with the initial ones.

Bacterial strains have proven to be good colonizers of soil and wheat roots. Indeed, from rhizosphere were isolated little more colonies in comparison of the seedlings' growth substrate (Fig. 32), but these differences seem to be not statistically significant.

Substantial differences were observed in both cases between *B. amyloliquefaciens* strains and the initial bacterial inoculum. While, according to Duncan's multiple range test, a significant difference was observed among the bacterial strains only in rhizosphere as Ba16 developed more than the other two strains.

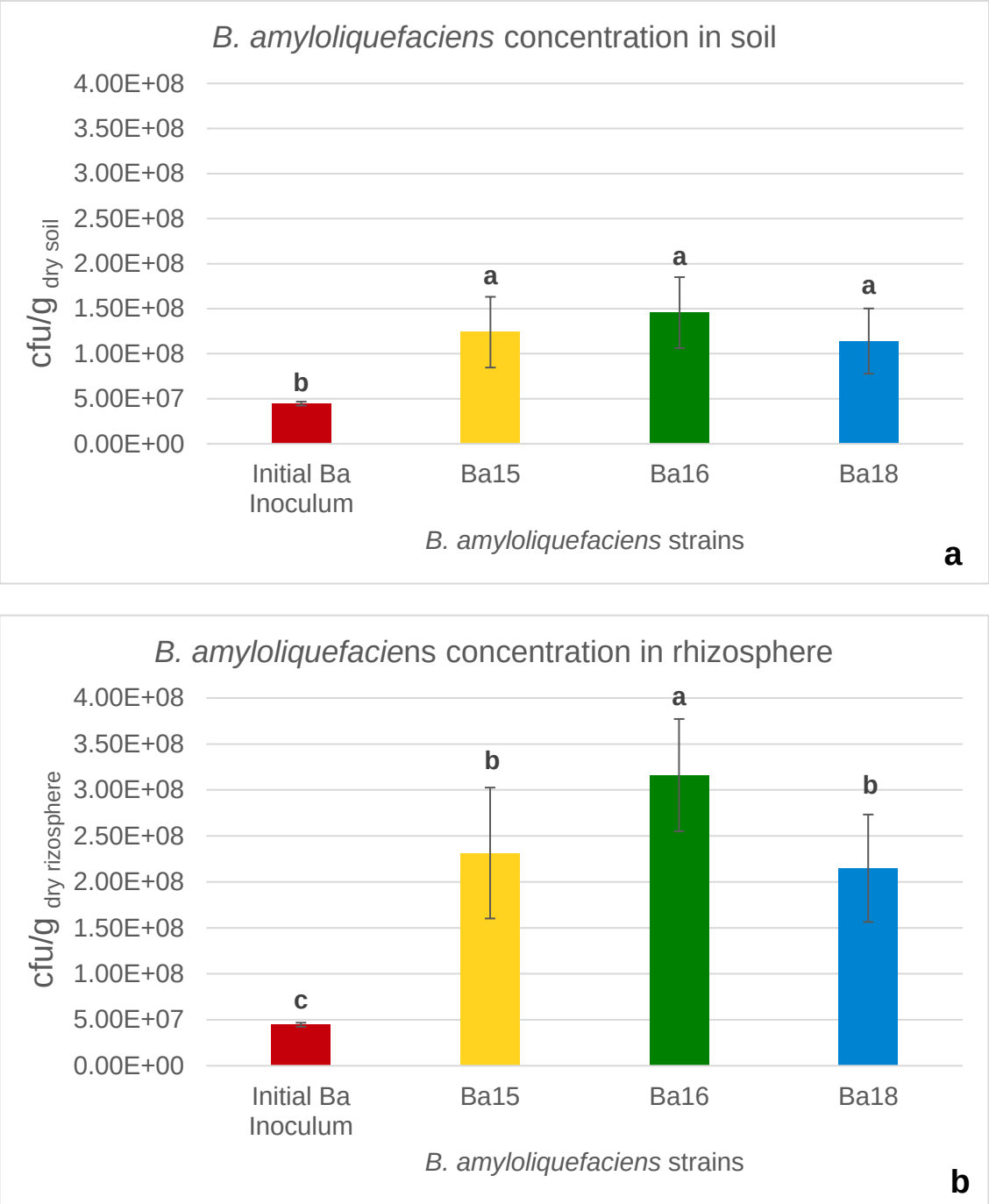


Figure 32 | *B. amyloliquefaciens* ability to colonize soil (a) and rhizosphere (b). Duncan's multiple range test was performed for P≤0.05.

3.4 Discussion III

The *in vivo* assays, carried out on wheat seedlings cv. Svevo, allowed to understand in detail the mechanisms linked to the interactions between *B. amyloliquefaciens* and this cereal.

The three bacterial strains, Ba15, Ba16 and Ba18, have proved to be able to survive on wheat leaf surface, for at least 72 h. Their survival is stationary and is not dependent on their cell growth. This could be due to the ability of these bacteria to form spores. This behavior was observed in *B. pumilus* on tomato leaves (Varvaro and Fabi 2001).

The spores, in addition to allow survival, would also allow *Bacillus* strains to colonize plant niches, promoting an indirect antagonism against other microorganisms.

Direct antagonism was also confirmed through *in vivo* assay, against *B. sorokiniana*, the most *in vitro* inhibited pathogen. The antifungal activity was promoted both by bacterial cells and by their filtrates. However, the latter proved to be slightly more effective than the former. This could mean that *B. amyloliquefaciens* strains would be able to produce cLPs, in particularly fengycin (as demonstrated in the previous chapter), even in natural environments.

In order to confirm the results obtained from *in vitro* assays, further *in vivo* investigations should be performed on the antagonism of the three strains of *B. amyloliquefaciens* against *F. graminearum*, *F. culmorum* and *S. tritici*.

In fact, different strains of *Bacillus* spp. proved to control these pathogens (Baffoni et al. 2015; Zalila-Kolsi et al. 2016; Mejri et al. 2017; Mnasri et al. 2017).

In addition to protect wheat, the three strains of *B. amyloliquefaciens* have proved to be potential growth promoters for this cereal.

In particular, the use of bacterial suspensions in seeds watering during sowing, allowed to anticipate germination of 2 days, in comparison with the usual wheat growth. This stimulation could be linked to the ability of these bacteria to produce α-amylase (Caracciolo 2014) which would make sugars available to seeds (Taiz and Zeiger 2002).

In addition to promoting a faster germination, Ba15, Ba16 and Ba18 allowed a biomass increase of seedlings. In fact, these present epigeal and hypogeal apparatus much more developed than those of the control.

This could also be linked to the ability of *Bacillus* spp. to naturally colonize wheat rhizosphere (Velazquez-Sepulveda et al. 2012).

Moreover, Ba15, Ba16 and Ba18 confirmed their rhizospheric competence (Caracciolo 2014), promoting the establishment of those beneficial ties with wheat seedlings.

In general, all these assays allowed to define the interaction among *B. amyloliquefaciens* strains, wheat seedling and *B. sorokiniana*.

The ability of Ba15, Ba16 and Ba18 to control leaf spot blotch and to promote wheat growth make them to be considered as PGPR and BCAs.

4 CONCLUSION

Wheat is one of the most important crops in the world. Its production is related to the growing global needs. This has led to the intensification of its cropping system, so that it is exposed to the increase of abiotic and biotic stresses affecting it. Among the latter, pathogenic fungi *F. graminearum, F. culmorum*, *B. sorokiniana* and *S. tritici* represent a steady and worldwide spread threat, for this cereal.

The promotion of new defense strategies, whose aim is to reduce the use of chemical products, need valid alternative for controlling plant diseases. The use of beneficial microorganisms represents an innovative solution to this requirement.

Among these microorganisms *Bacillus* spp. are a rich pool where several strains, able to control plant diseases, could be selected from. One of this species is represented by *B. amyloliquefaciens*.

In this research the antifungal activity of three strains of *B. amyloliquefaciens* (Ba15, Ba16 and Ba18) against four fungal pathogens of wheat has been investigated.

The antagonism is promoted by specific molecules, the cLPs. These are liposoluble and heat-resistant compounds. *B. amyloliquefaciens* strains released them during the log exponential phase of bacterial growth and they can reach the maximum production during stationary phase, close to spore formation.

The main cLPs families are: iturins, fengycins and surfactins. They were characterized by molecular and chemical analysis. Their inhibition activity against the phytopathogenic mycelium growth was confirmed *in vitro* on both agarized and liquid media.

Among cLPs, fengycins could be considered as the main compounds responsible for the antagonism against *B. sorokiniana*. Bacterial filtrates, where these molecules can be found, maintained or increased their antifungal activity over time, allowing their conservation at room temperature for at least one year.

The inhibition against *B. sorokiniana* was also confirmed in *in vivo* assay, on wheat seedlings (*Triticum durum*) cv. Svevo. The use of *B. amyloliquefaciens* cells and their filtrates controlled leaf spot blotch.

Furthermore, Ba15, Ba16 and Ba18 proved to be excellent growth promoters, colonizing leaves and roots niches on wheat seedlings. The use of their suspensions on wheat seeds allowed the advance of germination and a considerable increase in seedlings biomass.

The results obtained in this work confirm the potential of the three *B. amyloliquefaciens* strains as a valid plant growth promoters and biocontrol agent against the most important fungal pathogens of wheat, so their use can be recommended in the formulation of commercial bio-fertilizers and bio-pesticides.

ANNEX

BUFFERS AND SOLVENTS

DNA extraction buffer	**[pH 7.5]**	
Tris base [pH 7.5]	24.2	g
NaCl	14.6	g
EDTA	9.3	g
Sodium Dodecyl Sulfate (SDS)	5	g
Polyvinilpyrrolidone (PVP)	20	g
Distilled water	1	l

Sterilization by filtration

(Mora 2013)

Tris-Acetate-EDTA (TAE) buffer 50X	**[pH 8.0]**	
Tris base	242	g
Glacial acetic acid	57.1	ml
EDTA (0.5M) [pH8.0]	100	ml
Volume adjusted with distilled water up to	1	l

(Mora 2013)

Phosphate buffer	**[pH 7.0]**	
K_2HPO_4	1.7	g
KH_2PO_4	1.4	g
Distilled water	1	l

20 min sterilization by autoclaving (121 °C)

(Mariotti 2012)

FPLC Solvent A		
Trifluoracetic Acid (TFA)	1	ml
Distilled water	1	l

Filtered by cellulose nitrate filter (pore 0.45 µm)
30 min homogenization and degasification
(Mora 2013)

FPLC Solvent B		
Trifluoracetic Acid (TFA)	0.85	ml
HPLC acetonitrile	1	l

Filtered by cellulose nitrate filter (pore 0.45 µm)
30 min homogenization and degasification
(Mora 2013)

CULTURE MEDIA

Lysogenic Broth / Agar medium (LB / LBA)	**[pH 7.0]**	
Tryptone	10	g
Yeast extract	5	g
NaCl	10	g
Agar	15	g
Distilled water	1	l

20 min sterilization by autoclaving (121 °C)

Potato Dextrose Broth / Agar medium (PDB / PDA)	**[pH 5.6]**	
Potato-dextrose broth (Merck - P6685)	24	g
Potato-dextrose agar (Merck - 70139)	39	g
Distilled water	1	l

20 min sterilization by autoclaving (121 °C)

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