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Interactions between *Bacillus amyloliquefaciens* Bs006, *Fusarium oxysporum* Map5 and Cape gooseberry (*Physalis peruviana*)

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To my wife Olga and my daughters Mariana and Manuela.

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Moreno-Velandia, C.A., Ongena, M., Franzil, L., Kloepper, J., Cotes, A.M. 2017. Direct effects of CLPs on *Fusarium oxysporum* explain part of the efficacy of *Bacillus amyloliquefaciens* Bs006 against Fusarium-wilt of *Physalis peruviana*. 1st International Symposium on Plant Bioprotection Sciences & Technologies -Plant BioProTech Congress- Reims, France, June 27th – 30th 2017. / Oral presentation.

Moreno-Velandia, C.A., Izquierdo-García, L.F., Ongena, M., Kloepper, J., Cotes, A.M. Soil sterilization, pathogen and antagonist concentration affect biological control of Fusarium wilt of cape gooseberry by *Bacillus amyloliquefaciens* Bs006. Plant and Soil. Submitted.

Abstract

Fusarium oxysporum (FOX) is one of the most difficult plant pathogens to control leading to important economic losses in several crops owing to Fusarium-wilt disease. Biological control through beneficial rhizobacteria has high potential to reduce the devastating effects caused by FOX. *Bacillus amyloliquefaciens* strain Bs006 has potential as plant growth promoter and biocontrol agent against cape gooseberry wilt disease. Although plant growth promoting phenomenon had been consistent in previous experiments, the biocontrol activity by Bs006 was variable, suggesting that biotic and abiotic factors in the rhizosphere affect the efficacy of Bs006. Thus, the main goal of this doctoral research was contribute to knowledge of Bs006-FOX-cape gooseberry-environment interactions. Results show that Bs006 synthesizes cyclic lipopeptides (CLPs) from the iturin, fengycin and surfactin families. However, incubation temperature and culture media affect the production of CLPs. Experimental evidence indicated that iturins have fungistatic effect on FOX, while fengycins have fungicidal effect in a concentration dependent manner. In gnotobiotic cultures, it was determined that Bs006 colonizes the cape gooseberry root surface, forming a biofilm and utilizes root exudates as a nutritional source. In this system, it was found that Bs006 synthesizes compounds of the three families listed above and is associated with the inhibition of FOX growth. *In vivo* studies indicated that biocontrol activity of Bs006 against Fusarium-wilt is influenced by the concentrations of the pathogenic inoculum, Bs006 inoculum and CLPs. These results have provided a framework in which to evaluate the optimal combination of Bs006 cells and supernatant/semi-purified extract or pure CLPs in future studies. Since the production of CLPs by Bs006 is lower in the rhizosphere than in artificial media and that propagules of FOX can be tolerant to CLP action, additional research is needed to design integrated management programs of this disease. These programs should include the development of a biopesticide based on Bs006 and information about compatibility between rhizobacteria, fungicides and another biological control agents.

Keywords: *Fusarium oxysporum*, *Bacillus amyloliquefaciens*, Cape gooseberry, Antibiosis, Lipopeptides.

Resumen

Fusarium oxysporum (FOX) es uno de los fitopatógenos más difíciles de controlar, provocando altas pérdidas agrícolas debido a la enfermedad marchitamiento vascular. El control biológico con rizobacterias promotoras del crecimiento vegetal (PGPR) presenta alto potencial para reducir los devastadores efectos de FOX. Particularmente *Bacillus amyloliquefaciens* cepa Bs006 ha mostrado alto potencial como promotor de crecimiento vegetal y como biocontrolador del marchitamiento vascular de la uchuva. Aunque el fenómeno de promoción del crecimiento vegetal había sido consistente previamente, la eficacia Bs006 contra la enfermedad fue variable, suponiendo que factores bióticos o abióticos de la rizósfera afectan su actividad biocontroladora. En este contexto, el objetivo de la investigación doctoral fue contribuir al conocimiento de las interacciones entre Bs006, FOX, uchuva y el ambiente. Los estudios *in vivo* indicaron que la actividad biocontroladora de Bs006 contra el marchitamiento vascular está influenciado por la concentración de inóculo del patógeno, la dosis de Bs006 y la concentración de lipopéptidos cíclicos (CLPs) de las familias iturinas, fengicinas y surfactinas. La evidencia experimental indicó que las iturinas tienen un efecto fungistático contra FOX, mientras que las fengicinas presentan un efecto fungicida, en una forma dependiente de la concentración. A través de un modelo gnotobiótico se determinó que Bs006 coloniza la superficie de la raíz de uchuva, formando una biopelícula; sintetizó compuestos de las tres clases de CLPs y desde la raíz inhibió el crecimiento de FOX. Adicionalmente, la temperatura de incubación y el medio de crecimiento afectaron la producción de CLPs por Bs006. Globalmente, los resultados de la presente investigación sugieren realizar estudios adicionales para encontrar una óptima combinación entre células de Bs006 y sobrenadante de su cultivo/extracto semi-purificado o una proporción de CLPs puros que no sea contraproducente para la planta. Teniendo en cuenta que en la rizósfera la producción de CLPs por la rizobacteria es mucho más baja que en un medio artificial y dada la tolerancia de FOX a los CLPs, son necesarias nuevas investigaciones para diseñar planes de manejo integrado de la enfermedad, que incluyan la alternativa de control biológico con *B. amyloliquefaciens* Bs006.

Palabras clave: *Fusarium oxysporum*, *Bacillus amyloliquefaciens*, Uchuva, Antibiosis, Lipopéptidos.

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Introduction

Context

Fusarium wilt (FW) of cape gooseberry (*Physalis peruviana*) has caused negative social and economic impacts in Colombia due to important fruit yield losses and the geographical displacement of farmers towards new cropping areas (Fischer *et al*., 2014). However, growing cape gooseberry in new areas has not stopped this phytosanitary problem and the outbreak of disease is frequent in every location where cape gooseberry is grown (Rodríguez *et al*., 2011). Since cape gooseberry is a relatively new crop in Colombia and the area in which this species is planted is comparatively small (743 ha approx., Fischer *et al*., 2014), cape gooseberry has not been an attractive market for commercial brands of chemical pesticides and the registration processes for this crop are barely beginning. For this reason, cape gooseberry growers have adapted application schemes and fungicides from models like potato and cut flowers for FW control. Despite these applications, yield losses due to FW can be up to 50% (Rodríguez, 2010). Although cape gooseberry production (fruit yield and cropping) has slightly decreased in the last few years, this crop is still considered promising for the Colombian export market. This potential is reflected in the rising trend of exports during the past years, mostly to European countries which FOB value is approximately 30 million US dollars (AGRONET, 2013).

FW symptoms in cape gooseberry include loss of turgidity of leaves and stems, lateral chlorosis of leaves, lateral drying of branches, discolored brown areas in cross sections of infected stems, and plant growth stunting. The plant pathogen *Fusarium oxysporum* (FOX) has been isolated from plants with these symptoms and it has been confirmed to cause the same symptoms through artificial inoculations (Estupiñan and Ossa, 2007). Although four additional species from the same genus (*F. sporotrichioides*, *F. solani*, *F. subglutinans*, *F. pseudocircinatum*) have been associated with cape gooseberry wilt (Sánchez and Forero, 2009), FOX has been the most frequently found species in wilted plants (Rodríguez, 2010).

This pathogen causes infection in a specific manner in cape gooseberry, suggesting the presence of a *formae specialis* (Estupiñan and Ossa, 2007; Rodríguez, 2013) designated as *F. oxysporum* f.sp. *physali* (Simbaqueba, 2017).

Nowadays both the international market, and more recently the local market, demands pesticide and fungicide-free food and agronomic activities respectful to the environment. As such, growers have had to reduce the use of fertilizers and chemical pesticides which has led to a growing interest for the implementation of alternative methods for the control of insects, weeds and pathogens. Biological control of vascular wilt caused by several *formae specialis* of FOX is a potential alternative to traditional disease management approaches, due to the ability of biological control agents (BCA) to colonize the rhizoplane and the rhizosphere (Ahmad and Baker, 1987) and interfere in the pathogenesis process (Folman *et al*., 2004; Deacon, 1996). Soil-borne antagonistic microorganisms such as *Trichoderma* spp., bacterial species and non-pathogenic *Fusarium* spp. have shown potential to reduce the effects of disease (Datnoff *et al*., 1995; Larkin and Fravel, 1998; Paulitz *et al.*, 1997; Srivastava *et al*., 2010). Biological control relies on populations of beneficial microorganisms that can prevent fungal diseases through multiple modes of action, there is the potential to prevent the development of fungal resistance to disease management. In practice, it is considered that rhizosphere microorganisms are ideal to be used as BCA against root pathogens (Weller, 1988). In general, it is believed that a BCA will have better performance if applied to the part of the plant from which it was isolated, since it is adapted to those condition (Lucy *et al*., 2004).

Results of biological control reported in scientific literature are variable and sometimes inconsistent (Folman *et al*., 2003). Sometimes the efficacy of BCA in reduction of incidence or severity of the diseases is as high as that obtained by chemical fungicides but other times is significantly lower, which has delayed the application of biopesticides in commercial conditions, especially in high-value crops (Guetsky *et al*., 2001). This fact is reflected in the difference of the size market between biopesticides and chemical pesticides, where the sales of biopesticides represent 2,9% of the total sales of chemical pesticides in the world which stands to 21 thousand million Euro (Blum *et al*., 2011).

Inconsistent results of BCA of soil-borne phytopathogens are due to factors such as deficient colonization of the rhizosphere (Lugtenberg *et al*., 2001), low production of antimicrobials compounds due to low availability of minerals and carbon sources in the soil rhizosphere (Folman *et al*., 2003), sub-optimal temperatures (Pertot *et al*., 2013), or due to the effects of other abiotic factors on biocontrol activity (Burpee, 1989). Additionally, specific interaction between the host plant and the antagonist may affect BCA efficacy (Drogue *et al*., 2012).

To overcome obstacles that delay commercial implementation of biological control, it is necessary to further study the ecology of these microorganisms and their interaction with the pathogen, the host and with the microbial communities in the rhizosphere (Cook, 1993; Handelsman and Stabb, 1996). Ideally BCAs must be adapted to survive and display their biocontrol traits in the specific conditions of the agroecosystem where they will be used. Moreover it must be present at a population density high enough to provide acceptable control of the disease (Larkin and Fravel, 1999).

Plant growth promoting rhizobacteria (PGPR) constitute one of the main group of beneficial microorganisms used to combat diseases caused by soil-borne plant pathogens (Weller, 1988; Pérez-García *et al*., 2011). Currently about 75% of commercial biopesticides are formulated based on bacteria (Lazarovits *et al*., 2014). Many species of genus *Bacillus* are used due to its capacity to form endospores, structures that provide resistance to exposure to chemical compounds, radiation, desiccation and nutritional deficiencies (Emmert and Handelsman, 1999; Ongena and Jacques, 2008; Pérez-García *et al*., 2011). *Bacillus amyloliquefaciens* is well known for its antagonistic activity against plant pathogens based mostly on non-ribosomal compounds such as cyclic lipopeptides (CLPs) and polyketides (PKs) (Argüelles-Arias, *et al.*, 2009; Raaijmakers *et al*., 2010), the induction of systemic resistance (ISR) in the host plant (Kloepper *et al*., 2004; Henry *et al*., 2011; Desoignies *et al*., 2013; Pertot *et al*., 2013; Cawoy *et al*., 2014), and nutrient competition with the pathogen through siderophores (Dunlap *et al*., 2013; Magno-Pérez *et al.,* 2015).

The *Bacillus subtilis* / *amyloliquefaciens* species complex is considered of high importance in biotechnology and agriculture because of its extensive and versatile metabolic activities (Pérez-García *et al*., 2011). For example, the two main biocontrol phenomena observed in *B. amyloliquefaciens* are induced systemic resistance in the host plant and direct

antagonism, through production of biosurfactants such as CLPs (Cawoy *et al*., 2015). Despite the extensive research conducted on the metabolomics, biocontrol and growth-promoting activities, and genomics of *B. amyloliquefaciens* (generated from many strains such as FZB42, FZB24, QST713, GB03, D747, MB1600, GA1, SQR9, NAUB3, YAUB9601 and S499) (Wu *et al*., 2015), it is necessary to further study the *Bacillus*-plant-pathogen-environment interactions, thus improve the consistency of biological control results.

With the aim to contribute to development of control alternatives for FW of cape gooseberry, Caviedes (2010) isolated bacteria from the rhizosphere of healthy cape gooseberry plants, present in a crop seriously affected by FW. From this group of isolates, the strain *Bacillus amyloliquefaciens* Bs006 (Gámez *et al*., 2015) was selected because it showed high antagonistic activity *in vitro* against *F. oxysporum* strain Map5 (one of the most virulent isolate obtained from cape gooseberry (Rodríguez, 2010)). Results from *in vitro* experiments suggested that antifungal compounds produced by Bs006 could be implicated in FOX growth inhibition. However the application of Bs006 to cape gooseberry plants planted in sterile substrate and inoculated artificially with Map5, showed low efficacy on development of FW. However, Bs006 inoculation was shown to promote plant growth on in the absence of pathogenic inoculum (Guacaneme, 2010). Torres (2013) observed that *B. amyloliquefaciens* Bs006 did not reduce stem colonization by Map5 in cape gooseberry plants grown on non-sterile substrate inoculated with Map5. However, plants treated with only Bs006 showed significantly larger growth than untreated plants.

These preliminary studies have shown consistent plant growth promotion activity by *B. amyloliquefaciens* Bs006 but inconsistent biocontrol activity, suggesting that in absence of FOX the bacteria establishes an active population in the rhizosphere and promotes plant growth. However, biofungicidal activity that has been documented *in vitro* is not observed in rhizosphere conditions. Environmental factors of the rhizosphere such as pH, temperature or soil nutritional content, among others, or biotic factors such as rhizosphere competence and FOX presence among others, could potentially affect the expression of Bs006 biocontrol traits. It is also possible that antagonistic modes of action exerted by Bs006 in the rhizosphere are not effective enough to control FOX.

Objectives of this research

The general objective of this doctoral research was to contribute to knowledge about *B. amyloliquefaciens* – plant – pathogen – environment interactions in order to enhance its use as a biopesticide. In order to support directions for use of formulations based on Bs006, the first objective of this study was to determine the effect of population density of both *B. amyloliquefaciens* Bs006 and *F. oxysporum* Map5 on biocontrol efficacy. The second objective was to determine the substances involved in antibiosis showed by *B. amyloliquefaciens* Bs006 against *F. oxysporum* Map5 and to characterize the direct effects on this fungus. Finally, the third objective was to identify the effect of temperature on growth, CLPs production and antagonistic activity of *B. amyloliquefaciens*.

Scientific approach

The *Bacillus* – pathosystem model selected to make the current research was *B. amyloliquefaciens* Bs006 – *F. oxysporum* Map5 / Cape gooseberry. In the present research, important scientific foundations were generated to support the design of directions for use this control alternative and at the same time relevant information about interactions between *Bacillus*, cape gooseberry, FOX and environment also was obtained.

The strategy for this project was to progress from the general to the specific, from the observation of the biological phenomenon to developing a model that allows us to replicate the phenomenon in a controlled way, with respect to the three-trophic levels, host – pathogen – antagonist.

Synopsis of the chapters

In the introductory chapter there is a brief context exposure of the current doctoral research and the objectives are presented.

In chapter one, a scientific literature review is presented about biological control of vascular wilt disease caused by *F. oxysporum* through the use of *Bacillus* species. The most important aspects about biology of both microorganisms, a description of the rhizosphere as the scene where host – pathogen – antagonist interactions occur, and the main factors

related with the pathogen causing variability of biological control, as well as the strategies that have been designed to overcome these difficulties will be covered.

The second chapter includes the first studies to elucidate the *in vivo* interactions between *B. amyloliquefaciens* Bs006 with Cape gooseberry and with *F. oxysporum* Map5. The potential of Bs006 as BCA and plant growth promoter was validated. The effect of sterile and non-sterile soil on biocontrol activity of Bs006 against FW of Cape gooseberry also was validated. Growth promotion activity of Bs006 was evaluated on Cape gooseberry plants inoculated with Map5. The biocontrol activity of Bs006 in response to combinations of different concentrations of pathogen inoculum and rhizobacteria dose also was tested in this first phase of the research.

We observed high biocontrol potential by Bs006, keeping FW incidence significantly lower than in controls for 7 weeks, consistently in three experiments under greenhouse conditions. However, soil sterilization reduced the biocontrol activity of Bs006, and is one potential factor that could explain the variability of the observations in previous works. Even at a low dose (10^7 CFU/ml), Bs006 shows significant effects as both a biocontrol agent and a plant growth promoter. Nevertheless, the presence of Map5 in the soil reduced the plant growth promoting effect by Bs006. Dose response evaluations showed that biocontrol activity of Bs006 was influenced by both inoculum density of Map5 and dose of Bs006. In this first phase we also observed that Bs006 behaves as an endophytic microorganism in Cape gooseberry. Finally, the rhizosphere competence of Bs006 was determined in plants free of pathogenic inoculum as well as plants inoculated with FOX Map5.

Based on knowledge of *B. amyloliquefaciens* as an efficient species producing CLPs, during the next experimental phase, the ability of Bs006 to produce CLPs and the role of these compounds on biocontrol activity against FOX and FW of Cape gooseberry were determined. Thus, the third chapter presents the results of experiments carried out to determine the effect of CLPs produced by Bs006 on Map5. The CLPs-compounds found in the inhibition zones produced by Bs006 against Map5 were characterized in confrontation dual tests. The effect of cell-free culture supernatant of Bs006 and the effect of pure CLPs on growth, conidial germination and morphology of FOX was determined *in vitro*. While the effect of cells suspension, cell-free culture supernatant or their mixture, as well as

application volume of the same treatments on FW incidence was evaluated in *in vivo* experiments.

The results showed that Bs006 has the ability to synthesize compounds from the three families of CLPs like other members of the same species. The profile of CLPs detected on inhibition zone during confrontation dual test in gelified media was different compared to liquid conditions. In gelified media the concentration of iturins was greater than fengycins and surfactins. Direct antagonism exerted by fengycins affected both germination and fungal development. Results depended on the culture medium used for Bs006 growth, which is related to CLPs concentration. There was a differential response of disease development in the presence of Bs006 cells, cell-free culture supernatant and to the cells/supernatant mixture, as well as to the volume of application of these treatments, suggesting an important influence of CLPs concentration on disease control. These results also generated a new hypothesis about this observed phenomenon which requires further research to be confirmed.

We developed a model *in vitro* to analyze the plant – pathogen – antagonist interactions, in which *B. amyloliquefaciens* Bs006 has the ability to colonize the root surface of cape gooseberry and being present, the bacteria reduced FOX growth toward the host. Thus the model suggested that the root exudates from cape gooseberry represents an important food source for Bs006 growth and in turn, Bs006 produces CLPs on the root surface.

In vivo experiments suggested that the presence of *F. oxysporum* in soil affects the performance of Bs006. In this chapter, we also show the potential impact of culture supernatant free of Map5 on bacterial growth and production of CLPs by Bs006. In the same context, the effect of the presence of both microorganisms (Map5 and Bs006) in the same environment (*in vitro* liquid culture) on the viability of each of them and CLPs production by Bs006 was measured. The role of plant pathogens on BCA performance is not frequently studied in developing programs of biopesticides. Phytopathogens have the ability to defend themselves against antagonists and doing so could represent a determinant factor in biological control efficacy.

Next, in the fourth chapter the results on the effect of temperature and culture media on growth, CLPs production, and the antagonistic activity of Bs006 on FOX-Map5 are

presented. The range of selected temperature corresponded to values found in soil where cape gooseberry is grown. The results suggest that production of CLPs by Bs006 in cape gooseberry agroecosystem, characterized by low temperatures, depends on the available nutrients in the rhizosphere, mainly sources of carbon and nitrogen.

Finally, chapter fifth presents general conclusions of this doctoral research which demonstrated that *B. amyloliquefaciens* is an efficient producer of CLPs and has high potential as antagonist against FOX. Such potential observed *in vivo* conditions was supported by the evidence of strong negative effects of the CLPs on the fungus and also by the ability of the Bs006 to colonize cape gooseberry root and to synthesize CLPs on the root surface. Biotic factors such as inoculum concentration of FOX, dose of Bs006 and CLPs concentration are involved in determining the success of the biocontrol activity of *B. amyloliquefaciens* Bs006 on *F. oxysporum* Map5 / FW of cape gooseberry. Additionally, metabolites produced by FOX represent a depressor of the biocontrol activity of Bs006. The interaction between temperature and nutrient availability is a determining factor for Bs006 population growth and CLP production. Application of the mixture of supernatant from liquid culture and cells of *B. amyloliquefaciens* Bs006 can be an alternative for the development of commercial biocontrol product. However, it remains necessary to determine the optimal combination between cells and culture extracts / semi-purified extract / or the proportion of compounds that is not counterproductive for the plant since high doses seem to favor the infections by *F. oxysporum.*

Furthermore, new studies to develop integrated management strategies against FW, which include *B. amyloliquefaciens* Bs006 are necessary. Those strategies should include compatibility tests between rhizobacteria and fungicides used in the crop, whose target is *Fusarium* or other soil-borne phytopathogens. Additionally, the search for other antagonists to complement Bs006 activity is another alternative to develop new proposals for commercial products based on mixtures of compatible beneficial microorganisms. The studies within this doctoral thesis will support future plans for the development of recommendations for use of biopesticides based on *B. amyloliquefaciens* Bs006.

Chapter 1. Review. Biological control of vascular wilts caused by *Fusarium oxysporum* with *Bacillus* spp.

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1.1 Introduction

About 25% of crop yields are lost every year mainly due to disease incidence caused by phytopathogens and the attack of insect pests (Lugtenberg, 2015). These loses necessitate the development of strategies aimed at increasing the effectiveness of existing control measures and the development of effective alternative and sustainable measures, considering the need to generate more food for a growing population, which is estimated to be 8.3 billion people by the year 2025 (Lugtenberg *et al.*, 2013). Even though there are several tools available to prevent or reduce the damage by pests and the diseases in crops, farmers still rely on chemical pesticides despite their high toxicological and polluting risk and their limited effectiveness. The environmental pollution caused by the excessive use of agrochemicals has triggered important changes in public perception related to the use of pesticides in agriculture and today there are strict regulations about the use of pesticides which have led to several withdrawal orders of agrochemicals in the market (Pal and McSpadden, 2006).

The fungus *Fusarium oxysporum* (Sacc.) W.C. Synder and H.N. Hans. causes root rot or vascular wilt in plants and is the fifth most important fungal plant pathogen with scientific-economic importance due to its wide range of hosts and severe losses in crops such as tomato, cotton, banana and melon, among others (Dean *et al.*, 2012). It is also considered one of the most difficult to manage pathogens (Chandel *et al.*, 2010; Alabouvette *et al.*, 2007). Although the reasons are not entirely defined, it has been argued that its strong host-specificity displayed by formae speciales (Bosland, 1988; Ma *et al.*, 2010), ability to form resistance structures, diverse production of mycotoxins (Marasas, 1984; Bacon *et al.*, 1996), detoxifying capacity (Schouten *et al.*, 2004), resistance to fungicides (Brent and Hollomon, 1998) and the ability to manipulate the defense responses in the host (Tatcher *et al.*, 2009) make it a difficult target to eliminate.

The most commonly used method to reduce the inoculum of *F. oxysporum* in soil is fumigants in greenhouse crops such as vegetables and ornamentals (Larkin and Fravel, 1998; Gullino *et al.*, 2015) and fungicides in field crops, while the second most used method has been genetic control through the development of resistant varieties in some crops of high economic value. However, neither of these two methods has eliminated the disease entirely (Garibaldi and Gullino, 2012). The development of resistance to chemical molecules and the overcoming of plant genetic resistance have stimulated the development of alternative methods to control vascular wilt caused by *F. oxysporum*. The main alternative developed is biological control with antagonistic microorganisms, which can be used as a component of integrated disease management programs (Jacobsen *et al.*, 2004).

Biological control of soil-borne plant pathogens began 47 years ago (Alabouvette *et al.,* 2009), at the symposium organized in Berkley in 1965 “Ecology of soil-borne plant pathogens: prelude to biological control”, where the two main approaches of biological control were proposed: Increasing natural populations of antagonists and introducing selected biological control agents. The public concern about the dangers related to agrochemicals encouraged the opening of business enterprises dedicated to the production of biopesticides and led to companies recognized for their agrochemical products in agriculture to develop biological products which are now in their portfolio. In this context for example, Bayer acquired Agraquest; BASF acquired Becker Underwood; Syngenta acquired Pasteuria and Devgen and in 2013 Novozymes and Monsanto established the BioAg alliance, with the aim to discover, develop and offer microbial solutions for agriculture

with a reduced use of agrochemicals (Schäfer and Adams, 2015). Although the difference in the size of markets of biopesticides and synthetic pesticides still is big, where biopesticides represent about 3% of total pesticides sales (Blum *et al.*, 2011), it is estimated that the global market of biopesticides will increase at a rate of 6.9% per year with a market value of US$ 83.7 billion in 2019, according to the study carried out by BCC Research (http://www.bccresearch.com/market-research/chemicals/biopesticides-chm029e.html).
The entry of these big companies into the market of biological control agents is expected to generate a more stable platform of human and financial resources, which should guarantee that products can be marketed on a large scale and have strong support, factors that have been absent to a large extent in the field of biological control (Lazarovits *et al.*, 2014)

Research on biological control of soil-borne plant pathogens with microorganisms, has focused mainly on the genera *Trichoderma*, *Gliocladium*, non-pathogenic *Fusarium*, *Bacillus*, *Pseudomonas* and *Burkholderia* (Cook, 1993; Fravel, 2005), which constitute the active ingredient of the majority of biopesticides registered in Europe (Ravensberg, 2015), USA (Fravel, 2005) and South America (Cotes, 2011) which are the largest markets for biopesticides (Velivelli *et al.*, 2014). All these microorganisms have shown some level of control of vascular wilt caused by *F. oxysporum*, however, the development of products based on *Bacillus* spp. has been preferred due to the advantages it offers in sporulation and formulation which lead to a long shelf-life of the products (Borris, 2015).

Members of the *B. subtilis* species complex, such as *B. amyloliquefaciens* and *B. pumilus* have shown to be effective in the biocontrol of phytopathogens. Several strains of these species have the ability to colonize roots and express beneficial effects to stimulate plant growth and biocontrol (Borris, 2015). Although the number of registered biopesticide products has increased in the last decade, unfortunately, the success of this type of biological product in agriculture is still limited due to their inconsistent efficacy in the field (Borris *et al.*, 2015). As such, it is necessary to continue generating knowledge about the basic mechanisms of interactions between *Bacillus* spp., the plants and the pathogens. In this context, it is also important to know the target pathogen and the environment where the interactions will take place. This review focuses on the role of *Bacillus* spp. in the control of vascular wilt caused by *F. oxysporum*. The first part of the review will briefly describe the traits that characterize *F. oxysporum* as a phytopathogen. In the second part it will describe

the characteristics of the environment where the infection takes place, the rhizosphere. Finally, the third part will describe the characteristics of *Bacillus* that make it a good biocontrol agent and the aspects that affect the antagonistic performance of *Bacillus* and the strategies that are being implemented to increase its effectiveness.

1.2 *F. oxysporum* "The bad"

The *F. oxysporum* species include pathogenic and non - pathogenic strains. In this section we intend to show the arguments that characterize *F. oxysporum* as an aggressive pathogen against plants, but mostly to contextualize the environmental requirements that allow it to express its pathogenic potential and its colonization of the rhizosphere and the host.

Given its importance, *F. oxysporum* has received a great amount of attention from the scientific community which has generated an important amount of knowledge that has allowed us to understand its interactions with economically important hosts and model plants, such as *Arabidopsis thaliana* and *Solanum lycopersicon*. Nevertheless, due to its high genetic variability and the particular relation that it can have with each host, each pathosystem needs to be studied in order to manage the disease effectively. There are currently several reviews about the species in general (Gordon *et al.*, 1997; Di Pietro *et al.*, 2003; Recorbet *et al.*, 2003; Smith, 2007; Chen *et al.*, 2014; Kang *et al.*, 2014) and recently about specific pathosystems (Garibaldi and Gullino, 2012; McGovern, 2015; Elmer, 2015).

1.2.1 High specificity by the host

F. oxysporum is known because it possesses strains within the species that affect a limited range of hosts and strains with similar or identic range of hosts assigned to intraspecific groups called formae speciales (f. sp.) (Armstrong and Armstrong, 1981). Some formae speciales are divided into subgroups called races, based on virulence on specific cultivars of the same plant species (Armstrong and Armstrong, 1981; Roncero *et al.*, 2003). The strains of several formae speciales of *F. oxysporum* have been grouped based on vegetative compatibility, an approach that provides a medium to characterize sub-specific groups based on the genetics of the fungus, rather than the host-pathogen interaction (Correl, 1991). The studies of vegetative compatibility complemented with the use of

molecular tools have helped to understand the pathology, population biology and the relationships of races of this fungus (Correl, 1991).

Nowadays, it is known that the pathogenic character and the host specificity in a species complex of *F. oxysporum* depends on the presence of chromosomes that have been presumably acquired through horizontal transfer. There are about ten virulence genes located on these small chromosomes that encode effector proteins. The comparative study of the genomes of *F. oxysporum* f. sp. *lycopersici* (*Fol*), *Fusarium graminearum* (*Fg*) and *Fusarium verticillioides* (*Fv*) carried out by Ma *et al.* (2010) revealed deep differences between species and within strains of *F. oxysporum* coming from different hosts and suggested that the specificity of the host in strains of *F. oxysporum* lies with the content of the transferred chromosomes mentioned above. The size of the genome of *Fol* is 44% and 65% greater than *Fv* and *Fg,* respectively, which suggests a higher number of coding genes. All three species present a similarity about 85% of the sequence of nucleotides, which corresponds to conserved regions of the genome among the three species. However, the arrangement of genome includes the presence of 15 chromosomes in *Fol*, 11 in *Fv* and 4 in *Fg*. The different portion of the genome of *Fol* is distributed in chromosomes 3, 6, 14 and 15 and portions of chromosomes 1 and 2.

These new regions of the genome of *Fol* were called lineage specific regions of *Fol* (*Fol* LS) (Ma *et al.*, 2010) and are characterized because more than 74% correspond to transposable elements and these genes encode proteins that function as virulence factors, effector proteins, transcription factors and proteins involved in signal transduction. Similar to the LS regions of *Fol*, these were also found in three additional chromosomes to those of the conserved region in the genome of *Fusarium solani* (*Fs*), but these are different to the regions *Fol* LS (Ma *et al.*, 2010). The tests carried out by Ma *et al.* (2010) suggested that the origin of the LS regions of the *Fol* genome were acquired through horizontal transfer of the genetic material.

1.2.2 Pathogenic phase

F. oxysporum is a hemi-biotrophic root pathogen, meaning that it typically acts as a biotroph in early stages of its life cycle, feeding on living cells of the host and establishes the infection before changing to a necrotrophic phase to complete its life cycle (Brown and Ogle, 1997).

It infects about 100 species of cultivated plants (Beckman *et al.*, 1987) and as a soil inhabitant *F. oxysporum* can survive for a long period of time in absence of the host, in the form of chlamydospores (Agrios, 2005).

Root exudates stimulate the germination of chlamydospores and growth towards the host by chemotaxis (Steinkellner *et al.*, 2005). After germination, the infection process is divided into adherence, penetration and colonization. The infective hyphae adhere to the root surface and penetrates directly (Menden *et al.*, 1996). The mycelium then progresses through the cortex intracellularly until it reaches and enters the xylem vessels. Through these conducting vessels the fungus colonizes the host, and can generate microconidia, that can be transported upwards through the sap flow. These microconidia can germinate and colonize the xylem in superior parts of the plant. In severe stages of colonization, the fungus attacks the parenchyma and sporulates on the host surfaces (Di Pietro *et al.*, 2003).

F. oxysporum infects its host plants strictly through the roots (Di Pietro *et al.*, 2003) and during the penetration and colonization of the root secretes a combination of cell wall degrading enzymes such as polygalacturonases, pectate lyases, xylanases, cutinases and lipases, which help to obtain carbon sources, adhere to the root surface, penetrate the host tissues, and also constitute virulence factors (Bravo-Ruiz *et al.*, 2013). The activity of a protein MAP kinase is essential for the pathogenicity of some strains of *F. oxysporum*. Furthermore, fusaric acid is a partial determinant of its pathogenicity (Leslie and Summerel, 2006; Ding *et al.*, 2015).

As a result of severe water stress, mainly due to the obstruction of the xylem vessels by accumulation of fungal mycelia, production of mycotoxins and the defense response of the host, the typical symptoms of vascular wilt appear in the plant (Di Pietro *et al.*, 2003). It is typical to observe external symptoms developing on a unilateral basis from the base of the stem to the top. The symptoms caused by *F. oxysporum* in plants include stunting, wilting (turgidity loss, yellowing, and foliar abscission) and finally the death of the plant. In transverse cuts of the stem near the base of the infected plant, a brown ring is generally seen in the vascular bundle zone (Nogués *et al.*, 2002). Among the responses of the plant to the pathogen infection that obstructs the colonized vessels is the production of gums, gels and tyloses, and the proliferation of adjacent cells of the parenchyma (Beckman, 1987).

1.2.3 Environmental factors affect the development of the disease

Temperature. Both plants and pathogens require a minimum temperature to grow and reproduce. The wilting caused by *F. oxysporum* is benefited at the high temperatures specific to the tropics and sub-tropics, where the disease is more severe (Agrios, 2005). However, the effect of temperature on the development of a particular disease after infection, depends on the specific host – pathogen combination. Usually the disease will have a faster development when the temperature is optimum for the development of the pathogen, but below the optimum for the development of the host (Agrios, 2005). Nonetheless there are cases in which the optimum temperature for the development of the disease is different than the optimum temperature for the growth of both the pathogen and the host, which explains a further weakening of the plants compared to the pathogens, due to unfavorable temperatures (Agrios, 2005).

The pathogenic and non-pathogenic members of *F. oxysporum* are ubiquitous in soil and have been found in diverse ecosystems, including grassland, forests and deserts, from the tropics to the Arctic (Kang *et al.*, 2014), which suggest that this fungus adapts to a wide range of temperatures. It has been described in general terms that the maximum linear growth of *F. oxysporum* is 25 °C (Leslie and Summerel, 2006), and it is clear that temperature in the range of 25 – 35 °C promote the growth and sporulation of *F. oxysporum* (Gupta *et al.*, 2010). However, several studies have shown that pathogenic members of this species can grow *in vitro* in a wide range of temperatures. For example, *F. oxysporum* f. sp. *ciceris* grows in the range of 10 to 35 °C with a strongest growth at 25 to 30 °C and presents a drastic reduction in growth below 15 °C and above 35 °C (Farooq *et al.,* 2005). *F. oxysporum* f.sp. *tracheiphilum* presents a good growth *in vitro* above 18 °C up to 30 °C, with an optimum growth between 24 and 27 °C (Swanson and Van Gundy, 1985); the range for optimum *in vitro* growth temperature of *F. oxysporum* f. sp. *cepae* is also from 24 to 27 °C but this member of the species does not grow below 9 °C or above 36 °C (Abawi and Lorbeer, 1972); the growth of *Fusarium oxysporum* f. sp. *psidii* was maximum to a temperature of 28 °C and presented optimum sporulation at 34 °C, however this fungus grows from 10 °C and still has good growth up to 40 °C (Gupta *et al.*, 2010). Meanwhile *F. oxysporum* f. sp. *radicis-lycopersici* is more susceptible to cold and high temperatures.

Hibar *et al.* (2006) reported that temperatures below 10 and above 40 °C stop the development, while temperatures between 20 and 30 °C promote its growth, with 25 °C being the optimum temperature. Similarly, the growth of *F. oxysporum* f.sp. *lycopersici* has reduced growth in cold temperatures (15 °C) but shows a good growth in the range from 20 to 30 °C, with an optimum of 25 °C (Fayzalla *et al.*, 2008); Scott *et al.* (2010) observed that *F. oxysporum* f. sp. *lactucae* presented a progressive growth from 10 °C to a maximum growth at 25 °C, which was delayed at 30 °C. We have determined that the *in vitro* growth of *F. oxysporum* f. sp. *physalis*, causative agent of vascular wilt in cape gooseberry (*Physalis peruviana*) – a typical crop of moderate cold weather – is delayed for temperatures of 15, 20 and 37 °C but presents an optimum growth between 25 and 30 °C (reference or data not published).

Most studies show that the optimum temperature for the development of the disease matches the optimum temperature for the growth of the pathogen. For example, *F. oxysporum* f. sp. *ciceris* did not cause visible symptoms wilting in chickpea at 10 °C even at high levels of inoculum in soil. The pathogen was able of infect the plant but the most severe symptoms of the disease are present at 25 and 30 °C (Bhatti and Kraft, 1992). In the pathosystem *F. oxysporum* f.sp. *tracheiphilum* / Cowpea the highest levels of severity of vascular wilting are found at 27 °C, coinciding with the highest temperature in which the fungus presented an optimum growth *in vitro* (Swanson and Van Gundy, 1985). The damping-off disease (*F. oxysporum* f. sp. *cepae*) in onion seedlings presented its higher percentage of incidence between 27 and 32 °C (Abawi and Lorbeer, 1972). The development of crown and root rot disease in tomato, caused by *F. oxysporum* f. sp. *radicis-lycopersici* can benefit from fresh temperatures (19 °C) more than high temperatures (29 °C) (Hibar *et al.*, 2006). Ben-Yephet and Shtienberg (1994) described that the relation between the temperature and the severity of vascular wilt in carnation (*F. oxysporum* f. sp. *dianthi*) was explained by a parabolic model, in which symptoms of the disease did not develop at temperature extremes (low and high) but the severity of the disease was the highest at optimum temperature (25 – 26 °C). The virulence of *F. oxysporum* f. sp. *conglutinans* race 2, which causes the wilting of crucifers, showed a linear relation with the increase of the incubation temperature of the substrate, from 10 to 24 °C on susceptible hosts, while its virulence on resistant hosts depended on the temperature, being avirulent at low temperatures (10 to 12 °C) and virulent at temperatures between 22 and 24 °C (Bosland, 1988). The development of the wilting disease of lettuce, caused by *F. oxysporum*

f. sp. *lactucae* was more severe under a warm (33/23 °C) temperature regime and less severe under a cold regime (23/18 °C) in susceptible varieties (Scott *et al.*, 2010).

Soil pH. The pH of soil is important in the occurrence and the severity of the diseases caused by certain soil pathogens. It is clear that the effect of the soil acidity (pH) in several diseases is due to its effect on the pathogen. In some diseases, however, the weakening of the host due to a nutritional imbalance, caused by the soil acidity, can affect the incidence and the severity of diseases (Agrios, 2005). Despite being an important factor, the effect of pH has been studied mainly on the growth of the pathogen but not on the development of the disease. Wilting by *Fusarium* has been commonly associated with acidic and sandy soils rather than heavy soils with high pH (Woltz y Jones, 1981) however there is a gap between the demonstrations *in vitro* and its action *in situ* (Groenewald, 2006).

In this context, Farooq *et al.* (2005) observed that *F. oxysporum* f.sp. *ciceris* grew in the range of pH from 5 to 9 under *in vitro* conditions, however, its growth decreased with the increase or the reduction of pH from the neutral level. Fayzalla *et al.* (2008) reported that *F. oxysporum* f. sp. *lycopersici* presented better growth in a range of initial pH from 7.5 to 10. Gupta *et al.* (2010) reported that the appropriate pH for a maximum growth of *F. oxysporum* f. sp. *psidii* was of 5.5 followed by 5.0. We recently observed that the germination of *F. oxysporum* f. sp. *physalis* is delayed at highly acidic (4.0 and 5.0) and alkaline (8.0 and 9.0) pH but presents the highest percentage of germination to pH 7.0. However, the development of fungus from microconidia is high in the range of pH from 4.0 to 9.0.

Nutrition of the host. Host nutrition affects the growth rate and the ability of plants to defend themselves from the attack of plant pathogens. A balanced nutrition that provides plants with all the required elements, in the appropriate amounts, makes plants more capable of defending themselves against phytopathogens (Agrios, 2005). Despite nutrition being very important, the relation between plant nutrition with the severity of vascular wilt caused by *F. oxysporum* has not been widely studied. The first studies about the relation between the nutrition of the host with the development of a vascular wilt caused by *Fusarium*, were carried out by Neal (1927), Miles (1936), Young and Tharp (1941) in the pathosystem *F. oxysporum* f. sp. *vasinfectum* / cotton and by Stoddard (1942) in *F. oxysporum* f. sp. *melonis* / melon. In these studies the authors confirmed the relation

between potassium deficiencies with the increase of the disease. Subsequent studies corroborated the effect of potassium deficiency and proved the effect of the nitrogenized sources and the effect of low levels of calcium and boron as predisposing factors of the development of vascular wilt caused by *Fusarium* (Groenewald, 2005).

Nitrogen has deep effects on growth, therefore the relation of nitrogenous nutrition with the development of diseases in plants, has been better studied than the relation with other nutrients (Agrios, 2005). It is not just nitrogen excess or deficiency that makes plants more susceptible pathogens, but also the form (ammonium or nitrate) available to the host or the pathogen affects the susceptibility or resistance to the host (Agrios, 2005). The effects of nitrate and ammonia sources on the development of wilting caused by *Fusarium oxysporum*, is apparently related with the effects on soil pH. The application of nitric sources causes an increase in pH while the application of ammonia causes a decrease. High rates of application of ammonium to the soil promote the development of the disease (Woltz and Jones, 1973). Arya and Kuwatsuka (1993) observed that the nitric form of Nitrogen (KNO3) has a suppressive effect on *F. oxysporum* f. sp. *raphani*. This source of nitrogen inhibits the formation of conidia, decreases the viability of conidia and induces the formation of chlamydospores but does not affect vegetative growth.

Phosphorus seems to increase the resistance of the host plant, improving the nutrient balance in plants or accelerating the maturity of tissues (Agrios, 2005). Potassium has been shown to decrease the severity of several diseases but, in excess, seems to increase the severity of others. In addition, the healing effect of potassium as well as its retardant effect on senescence is important in controlling the infections of facultative parasites (Agrios, 2005). Calcium is generally associated with a decrease in the severity of several diseases, because of its effect on the composition of the cell wall, which increases the resistance to the penetration of pathogens. As such, a balanced level of micronutrients is key in plant resistance towards infections by plant pathogens (Agrios, 2005).

1.3 The rhizosphere “The battlefield”

The term rhizosphere was proposed for the first time in 1904 by the German scientist Lorenz Hiltner (Hiltner, 1904). The definition of this term was based on the principle that plant nutrition is influenced by the microbial composition of rhizosphere. Hiltner mentioned that root exudates were responsible of supporting bacterial communities which he called bacteriorhiza in the root zone, in analogy to the symbiotically associated fungi with plant roots. Based on his observations, Hiltner also proposed the hypothesis that the resistance of plants to pathogens and the quality of vegetable products depends on the composition of the rhizosphere microflora (Hartman *et al.,* 2008). After Hiltner, several definitions of the rhizosphere are found in the scientific literature. However, all of them present the elements proposed by this author in 1904: that soil zone closely linked to plant roots has high biological activity, coordinated by the root exudates.

In studies regarding the biological control of plant pathogens in roots, only the biological components (plant and microorganism) are usually analyzed, leaving aside the physical and chemical compounds that affect the interactions of biological components, which could help to explain the variability seen in the efficacy of biological control agents in the field. Below we will construct a definition of the rhizosphere based on proposals by several authors: The rhizosphere is a physical, chemical and biologically complex system, made up by the interface between the plant root and the volume of soil in close contact with it. It is directly affected by the root’s biological activity (Darrah, 1993; Hinsinger, 1998) and at the same time keeps bidirectional communication with it. This zone is characterized for having a high population of microorganisms. The plant roots must compete for space, water and nutrients with the invasive root systems of adjacent plants and with soil microorganisms including bacteria, fungi and insects that feed on an abundant source of organic material (Ryan and Delhaize, 2001). Lynch (1990) proposed the division of the rhizosphere in three parts, the ectorhizosphere including the soil attached to the root, the rhizoplane or root surface and the internal zone of the roots including the rhizodermis and the cortical cells.

1.3.1 Physical-chemical properties of the rhizosphere

The rhizosphere differs from the rest of the soil in a range of biochemical, chemical and physical processes that occur as a consequence of root growth, intake of water and

nutrients, respiration and rhizodeposition. These processes affect the ecology of the microorganisms present and plant physiology in a considerable way. These changes are also derived from the activity of microorganisms that are stimulated in the zone near to the roots as consequence of rhizodeposition (Jones *et al.,* 2004).

The root processes responsible for the main changes in physical and chemical properties of the rhizosphere were reviewed in detail by Hinsinger (1998), subsequently summarized with an emphasis on geometry and heterogeneity of the rhizosphere in space and in time by Hinsinger *et al.* (2005) and in 2006 Hinsinger *et al.* presented a summary of the consequences of basic chemical processes that occur in the rhizosphere on the biochemistry of several elements such as P, K, N, Mg and Ca. Although it is assumed that these changes in physicochemical properties of the rhizosphere caused by the root affect the activities of the microbial communities in the soil, this knowledge is still rarely applied to particular interactions between the species of rhizobacteria and cultivated plants.

Root growth exerts considerable forces that alter the physical properties of soil such as the bulk density, the porosity and soil resistivity. The polysaccharides released by roots in the rhizodeposits (Czarnes *et al.,* 2000), as well as exopolysaccharides produced by the microorganisms play a key role in the aggregation of soil (Amellal *et al.,* 1998). Similarly, water uptake by the root changes the water potential around it, affecting the radial transfer towards its interior and also the activity of microorganisms in the rhizosphere.

The biological functions of plant roots such as absorption of water, respiration and exudation considerably alter several chemical properties of the rhizosphere, which include nutrient concentration, toxic elements such as the aluminum and other contaminants, concentration of chelate-complex compounds, pH, redox potential, and partial pressure of CO_2 and O_2, among others (Hinsinger *et al.,* 2005). Particularly, water absorption and the solute content in the soil solution, cause zones of exhausted ions, which has been observed for P, nitric N and K (Hinsinger *et al.,* 2006). Alternately roots may cause nutrient accumulation next to the root surface, especially in forest ecosystems where the concentration of Ca and Mg in the soil is higher than that required for the plant (Hinsinger *et al.,* 2006).

The exchange of protons, cations and anions between the plant roots and the soil solution is responsible to a large extent for changes of up to two units in the pH of the rhizosphere (Hinsinger *et al.,* 2003). The respiration of the roots and microorganisms of the rhizosphere also causes changes in pH, due to accumulation of CO_2 in the rhizosphere and in the soil. Microbial respiration is known to be an important source of protons (Van Breemen *et al.,* 1984) and the release of acidic compounds from the root can cause the dissolution of calcium carbonate (Hinsinger *et al.,* 2006).

Root exudates into the rhizosphere have a large impact on soil microbiology and on the biogeochemical carbon cycle. Some root exudates and microbial metabolites (phosphatases, proteases, arylsulfatases) have a significant effect on the biogeochemical cycles of P, N and S. Carboxylate compounds such malate, citrate and oxalate exudate by the roots, have biogeochemical effects due to their role in the formation of metal complexes with Al, Ca, Fe and trace elements (Hinsinger, 2001).

1.3.2 Nature and functions of root exudates

It has been estimated that between 5 and 21% of carbon fixed by the plants through photosynthesis is transferred to the rhizosphere as root exudates (Marschner, 1995). The root exudates include the release of ions, oxygen and water but mainly carbon compounds (Bertin *et al*., 2003; Hinsinger *et al*., 2005). These carbon compounds are generally low molecular weight compounds such as amino acids, organic acids, sugars, phenolic compounds among others, or high molecular weight molecules such as polysaccharides and proteins (Walker *et al*., 2003) (Table 1). In the review by Bertin *et al*. (2003) three principal types of release mechanisms are described: transmission, ion channels and transport bladders.

Grayston and Campbell (1996) classified root exudates into five classes: diffusible, secretions, lysates, gases and mucilage. The diffusible compounds are water-soluble compounds of low molecular weight, such as sugars, organic acids or amino acids that are disseminated passively through the cell wall or between the epidermal cells, as a result of concentration gradients between the internal and external part of the root. The secretions are constituted by low molecular weight compounds such as siderophores that are actively secreted by the root in response to electrochemical gradients. Lysates consist of organic

material released in soil by the dead cells, after the autolysis. Released gases are mainly ethylene, carbon dioxide and hydrogen cyanide. Lastly, the mucilage, used to improve the penetration process of the root in soil is composed of polysaccharides and polygalacturonic acids. Mucilage is called mucigel if it contains microbial mucilage. The set of all these compounds associated with the root which accumulate in the rhizosphere also called rhizodeposits and as already described, affect plant growth and the ecology of the rhizosphere (Bertin *et al*., 2003; Faure *et al*., 2009). Root exudation is mainly carried out by the radical hairs and the primary and secondary roots during active growth (Bertin *et al*., 2003), but the apical cells also make a significant contribution (Faure *et al*., 2009).

The composition of rhizodeposits varies according to the species, the variety and the phenological state of the plant, the exposure of plants to stressful conditions and the type of soil, among other factors (Compant *et al*., 2010), resulting in differences in the bacterial community composition associated with rhizosphere (Haichar *et al*., 2008). In fact, root exudates are considered a determining factor in the specific interaction between rhizobacteria and the host and it has been that through the root exudates, plants can select the colonizers of the rhizosphere. For example, some isolates of *Azospirillum* spp. are attracted by the mucilage produced by corn roots but, other isolates of the rice rhizosphere did not respond to corn exudates (Mandimba *et al*., 1986). Humphris (2005) obtained similar results; the exudates of corn perturbed root colonization by *P. fluorescens* SBW25.

The root exudation process is not uniform in space and time since the quantity of rhizodeposits can vary according to the developmental state of the plant (Compant *et al*., 2010). Gamalero *et al*. (2004) described that distribution and density of *P. fluorescens* A6R1 changed according to the root zone of tomato plants which was attributed to the heterogeneity in the root exudation process. Grayston and Campbell (1996) found that in the crown of the root and near the radical hairs there is more exudation, in comparison with distal zones.

Table 1-1: Organic compounds and enzymes released by plants in root exudates and their function in the rhizosphere

Class of compounds[1]	Components	Functions
Sugars	arabinose, desoxyribose, fructose, galactose, glucose, maltose, oligosaccharides, raffinose, rhamnose, ribose, sucrose, xylose, mannitol, complex polysaccharides	lubrication; protection of plants against toxins; chemoattractants; microbial growth stimulation
Amino acids and amides	all 20 proteinogenic amino acids, γ-aminobutyric acid, cystathionine, cystine, homoserine, mugenic acid, ornithine, phytosiderophores, betaine, stachydrine	inhibit nematodes and root growth; microbial growth stimulation; chemoattractants, osmoprotectants; iron scavenger
Aliphatic acids	acetic, acetonic, aconitic, aldonic, butyric, citric, erythronic, formic, fumaric, gluconic, glutaric, glycolic, isocitric, lactic, maleic, malic, malonic, oxalic, oxaloacetic, oxaloglutaric, piscidic, propionic, pyruvic, shikimic, succinic, tartaric, tetronic, valeric acid	plant growth regulation; chemoattractants; microbial growth stimulation
Aromatic acids	*p*-hydroxybenzoic, caffeic, p-coumeric, ferulic, gallic, gentisic, protocatechuic, sinapic, syringic acid	plant growth regulation; chemoattractants
Phenolics	flavanol, flavones, flavanones, anthocyanins, isoflavonoids, acetosyringone	plant growth regulation; allelopathic interactions; plant defence; phytoalexins; chemoattractants; initiate legume-rhizobia, arbuscular mycorrhizal and actinorhizal interactions; microbial growth stimulation; stimulate bacterial xenobiotic degradation
Fatty acids	linoleic, linolenic, oleic, palmitic, stearic acid	plant growth regulation
Vitamins	p-aminobenzoic acid, biotin, choline, n-methionylnicotinic acid, niacin, panthothenate, pyridoxine, riboflavin, thiamine	microbial growth stimulation
Sterols	campestrol, cholesterol, sitosterol, stigmasterol	plant growth regulation
Enzymes and proteins	amylase, invertase, phosphatase, polygalacturonase, protease, hydrolase, lectin	plant defence; Nod factor degradation
Hormones	auxin, ethylene and its precursor 1-aminocyclopropaan-1carboxylic acid (ACC), putrescine, jasmonate, salicylic acid	plant growth regulation
Miscellaneous	unidentified acyl homoserine lactone mimics, saponin, scopoletin, reactive oxygen species, nucleotides, calystegine, trigonelline, xanthone, strigolactones	quorum quenching; plant growth regulation; plant defence; microbial attachment; microbial growth stimulation; initiate arbuscular mycorrhizal interactions

1.3.3 Plant growth-promoting rhizobacteria (PGPRs) – beneficial inhabitants of the rhizosphere

The communities of microorganisms that develop in the rhizosphere use root exudates as source of energy and nutrients (Hartman *et al*., 2008; Smalla *et al*., 2006). This stimulus exerted by root exudates is known as the “rhizospheric effect” (Hinsinger *et al*., 2005; Lugtenberg and Kamilova, 2009). The main groups of microorganisms and other agents

[1] Original source: Faure *et al*. (2009)

found in the rhizosphere include bacteria, fungi, nematodes, protozoa, algae and microarthropods (Raaijmakers *et al*., 2008). Some species of bacteria present in the rhizosphere are naturally beneficial and others are deleterious for plant growth (Raaijmakers *et al*., 2008; Welbaum *et al*., 2004). The species of bacteria that are found in the first group are called plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). Plant growth promoting activity has been described for several bacteria genus, including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* (Kloepper *et al*., 1989; Okon and Labandera-González, 1994; Glick, 1995).

PGPR can stimulate plant growth directly or indirectly. The direct promotion implies the supply of substances synthesized by plant growth promoting bacteria such as plant hormones e.g. auxins (indole-3-acetic acid or IAA) (Loper and Schroth, 1986; Joseph *et al*., 2007), ethylene, abscisic acid (ABA), cytokinins and gibberellic acid (GA) (Faure *et al*., 2009). They may also facilitate nutrient uptake from the environment, for example, through the solubilization of phosphate (Chabot *et al*., 1998). The indirect promotion of plant growth occurs when the PGPR avoid the negative effect of diseases caused by plant pathogens (Kloepper *et al*., 1980 a and b; Lemanceau *et al*., 1992; Neilands, 1995). The interior of plants is colonized by a wide range of endophytic microorganism species including bacteria, most of which come from the rhizosphere and most of them also present functions of plant growth promoting and protection against plant pathogens (Compant *et al*., 2010).

1.3.4 Factors that determine the colonization of the of rhizosphere by PGPRs

The application of PGPRs in agricultural crops is one of the most promising methods to increase the agricultural productivity and the efficiency of biodegradation of pollutants. For a number of years PGPRs have been used as microbial inoculums in the rhizosphere to stimulate plant growth and to control diseases. However, one of the main problems in the introduction of microorganisms, in practice, is that microorganisms do not survive or do not exert the specific expected function. It is known that root colonization by rhizobacteria is an important factor in plant growth promoting and biological control (de Weger *et al*., 1995; Knudsen *et al*., 1997; Lugtenberg *et al*., 2001). Nonetheless it must be taken into account that the successful introduction of a PGPR strain to a determined plant-soil environment,

does not guarantee the success or the survival that was observed in another type of soil or in a different plant genotypes (van Elsas and Heijnen, 1990; Jagnow *et al*., 1991; van Veen *et al*., 1997).

For a PGPR isolate to be an effective biological control agent, after inoculation it must be fixed and distributed in the root, and reproduce and survive for several weeks, competing with the native microflora (Weller, 1988; Benizri *et al*., 2001; Compant *et al*., 2005a). The term rhizosphere competence was used by Ahmad and Baker (1987) to describe the ability that a biological control agent has to grow and exercise its biocontrol activity in the rhizosphere. This concept has also applied to PGPR and it is known that competence varies between species and between strains of the same species.

The dispersal of rhizobacteria from the point of inoculation (usually the seed) to the growing roots is controlled primarily for two mechanisms: active motility of bacteria and passive movement of bacteria through percolation water or through vectors. The importance of both dispersal mechanisms is affected by the type of soil, the host plant, and the characteristics of the bacteria (Benizri *et al*., 2001). However, there are other factors that affect the colonization process of the rhizosphere which may explain why colonization is not uniform such as variable patterns of root exudation, chemotaxis, production of secondary metabolites involved in biocontrol, presence of flagella, quorum sensing, and production of specific compounds such as extracellular polysaccharides and enzymes.

Chemotaxis. The chemoattraction of rhizobacteria towards seed and root exudates is the active bacterial movement regulated by the presence of structures required for movement such as flagella and by chemosensors. In appropriate conditions of matric potential of soil, chemotaxis encourages the colonization process of rhizosphere (Weller *et al*., 1988).

Lugtenberg *et al*., (2001) reviewed several studies which determined that the presence of flagella is an important trait in the process of root colonization. Furthermore, it was shown that the movement of rhizobacterial flagella is not the result of a randomized movement, but rather, is based on the chemoattraction to root exudates. Most of these studies have been carried out with *P. fluorescens* and *P. putida*, genus which is characterized for producing up to nine polar flagella. Most of these studies are based on the generation of

strains of mutant bacteria in the gene *cheA*, which controls the movement of flagella towards attractive compounds (de Weert *et al*., 2002).

The association between host and rhizobacteria involves several interactions and recognition processes. Weert *et al*. (2002) briefly described the process of chemotaxis at a molecular level. That process is mediated by a two compounds regulation system that consists of a kinase sensor, CheA, and a response regulator, CheY. The chemoreceptors, methyl-accepting chemotaxis proteins (MCPs) are transductors of trans-membrane signals located in the cytoplasmic membrane. MCPs monitor the concentration of chemical compounds in the environment. Through methylation of the MCPs, a signal is transduced and CheA is phosphorylated. Subsequently P-CheA donates the phosphate group to CheY and interacts with the flagellar motor.

Whenever the signal falls below a certain threshold, CheY is phosphorylated, and produces clockwise rotation of the flagellum. Subsequently, the bacteria start to spin, which changes the swimming direction. If the signal rises above of the threshold, CheY is dephosphorylated and the rotation will occur in the opposite direction, resulting the directed movement of the bacterial cell (de Weert *et al*., 2002).

Using micro-arrays, Mark *et al*. (2005) examined the influence of the exudates of two varieties of sugar beet on the transcriptome of *P. aeruginosa* PA01. The response to both of the exudates showed only one partial overlap, most of the genes that presented differential expression, were regulated in response to one of the exudates showing that different genes change expression patterns in response to the exudates of the different varieties. The genes involved corresponded to the metabolism of aromatic compounds, chemotaxis and secretion type II. *cheA,* and the *pctA* gene that codifies for a transduced protein involved in chemotaxis, were repressed by the exudates of one sugar beet variety but, were not affected by the exudates of the other.

Siderophores. Siderophores are chelating substances of low molecular weight and with high affinity for Fe^{+3} that transport it towards the interior of the bacterial cell. The work by Kloepper *et al*. (1980b) was the first to demonstrate the importance of the production of siderophores as a mechanism of biological control. Subsequently several studies verified the role of siderophores in controlling several plant pathogens (Weller *et al*. 1988). The

depletion of iron in the rhizosphere as a result of kidnapping by siderophores, limits its' availability for the plant pathogens and in consequence, their development. Two of the best known siderophores are pyoverdine which is produced by fluorescent pseudomonas (Weller *et al*. 1988) and bacillibactin produced by *Bacillus* spp. (Cendrowski *et al*., 2004; Chen *et al*., 2007).

Lytic enzymes. The production of exoenzymes such as pectin-lyase, pectate-lyase, polygalacturonase, cellulase and protease along with the production of antibiotics is in some cases regulated by quorum-sensing. Inter and intra-specific signaling molecules such as N-acyl-homoserine lactone, are synthesized as soon as the bacterial population reaches high densities and play an important role in the regulation of these group of genes. It follows that colonization of a rhizobacteria is positively correlated with a high density of the inoculum (Benizri *et al*. 2001).

Antibiotics. The most well-known examples of antibiotics include 2,4-diacetylphloroglucinol (DAPG), cyanide hydrogen, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, thiotropocin, tropolone, cyclic lipopeptides, ramnolipids, oligomycin A, kanosamine, zwittermicin A and xantobacin (Compant *et al*., 2010). Genomics show that some rhizosphere bacteria have a group of genes responsible for the secretion of antibiotics, siderophores and other detoxifying substances, which are important traits during the colonization process (Compant *et al*., 2010).

The ability of PGPR to synthesize amino acids, vitamin B1, NADH dehydrogenase, lipopolysaccharides (LPS) and pili, or the presence of surface proteins such an outer membrane proteins and agglutinins is also important in the colonization process (Compant *et al*., 2010). PGPR use different mechanisms alone or in combination, to colonize the root.

1.4 *Bacillus* spp. "The good"

Several groups have explored the potential use of bacteria associated with the rhizosphere as biological control agents, of which several species of *Bacillus* and *Pseudomonas* have attracted the most attention. *Bacillus* is a cosmopolitan genus in the soil and it constitutes one of the main groups of beneficial microorganisms used against diseases caused by soil phytopathogens (Weller, 1988; Pérez-García *et al*., 2011). With the exception of some

pathogenic species (*B. cereus* and *B. anthracis*), the genus *Bacillus* includes species with properties generally recognized as safe or with a classification of supposedly safe (GRAS / QPS) (Monaci *et al.,* 2016).

In recent years, the commercial application of PGPR in agriculture has increased to improve the yield of some crops and to reduce the use of agrochemicals. About 75% of the commercial products based on microorganisms are formulated based on bacteria (Lazarovits *et al.,* 2014), among which several species of the genus *Bacillus* spp. are used due to their ability to form endospores, structures that provide them with advantages to resist conditions of chemical exposure, radiation, drying and nutritional deficits (Weller, 1988; Emmert y Handelsman, 1999; Ongena and Jacques, 2008; Pérez-García *et al*., 2011). In fact, the first product based on *B. subtilis* "Alinit" was marketed in 1897 as a bacterial fertilizer for cereals (Borris, 2011).

In particular, members of the *B. subtilis* species complex such as *B. subtilis, B. amyloliquefaciens, B. licheniformis* and *B. pumilus*, have shown to be efficient in promoting plant growth and in the biological control of phytopathogens (Borris, 2015). Members of this *Bacillus* species complex produce a wide range of bioactive compounds, among which cyclic lipopeptides (CLPs) from the surfactin, iturin and fengycin families are recognized for their potential use in biotechnology and biopharmacy (Banat *et al.*, 2000 Singh and Cameotra, 2004, Cochrane and Vederas, 2016). In addition to the antimicrobial properties of these compounds, they have also been shown to be involved in the colonization process and *Bacillus* motility, as well as in the systemic stimulation of plant defense responses (Ongena *et al*., 2010).

These last are the main characteristics that make this genus attractive as biological control agents. However, its variable efficacy, due in part to the gap in the knowledge of biotic and abiotic factors that affect its performance at the application site has limited the use of the formulations. Another problem that makes the use of biopesticides difficult is the high cost of registration and the duration of the process. This situation is more critical in Europe, where dossier evaluation takes more than 70 months, compared to 23 months in the United States (Borris, 2011), mainly due to the fact that the regulatory system for biopesticides has been based on the model used for chemical pesticides (Chandler *et al.,* 2011). In South America, the registration process also encounters difficulties, since in some countries, such

as Argentina, Brazil and Chile, there is no specific regulation for the registration of biopesticides, so they also have to undergo the same procedures necessary for the registration of chemical pesticides (Cotes, 2011). In this region, only Colombia has a specific regulation for the registration, authorization of sale and technical control of biopesticides, which has facilitated the registration of about 48 products. For example, when the proposed microorganism belongs to a species recognized as a biological control agent, only acute toxicity studies are required (Cotes, 2011).

This section, proposes to describe the modes of action used by species of the genus *Bacillus* that have shown biocontrol activity against FOX / vascular wilts, with particular emphasis on *B. amyloliquefaciens*, the environment conditions which affect its biocontrol activity, the colonization processes of the rhizosphere by FOX / *Bacillus,* the strategies used by FOX to defend against the attack of the antagonist rhizobacteria, and the strategies developed to increase the effectiveness of control by *Bacillus*.

1.4.1 Species of *Bacillus* with activity on *F. oxysporum*

Several members of the genus *Bacillus* are considered as microbial factories due to the production of a large number of biologically active molecules, some of which are potential inhibitors of phytopathogen growth suggesting their use in agricultural biotechnology (Pérez- García *et al.,* 2011). In this context, several commercial products have been developed based on several strains of different *Bacillus* species, such as *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. pumilus, B. megaterium, B. mycoides,* and *B. firmus* (Cawoy *et al*., 2011; Pérez-García *et al*., 2011; Borris, 2015).

The species *B. subtilis* and *B. amyloliquefaciens* are among the most commonly used rhizobacteria in agriculture and have been extensively studied. The first being the model organism for Gram positive bacteria, while some representative strains of *B. amyloliquefaciens* v.g. FZB42 have been thoroughly studied for their potential to suppress phytopathogens and to stimulate plant growth. These two species are closely related and share properties such as the production of cyclic lipopeptides with antimicrobial activity, their ability to colonize the rhizosphere and protect plants against phytopathogens. For

example, it has been determined that surfactin production is a determinant of root colonization in both *Bacillus* species (Bais *et al.,* 2004; Chen *et al.,* 2009).

Welker and Campbell (1967) determined that *B. amyloliquefaciens* shows good growth in culture medium supplemented with 10% NaCl whereas *B. subtilis* does not grow at this concentration of NaCl; *B. amyloliquefaciens* has the ability to ferment lactose whereas *B. subtilis* does not; *B. amyloliquefaciens* presents high capacity to hydrolyze starch by producing α-amylase, whereas *B. subtilis* is not typically an α-amylase producer; *B. amyloliquefaciens* shows good growth at temperatures between 30 to 40 °C and does not grow at 50 °C, whereas *B. subtilis* shows growth at 52 °C. Although in the study of Welker and Campbell (1967) it was determined that there is only 15% DNA sequence homology between *B. amyloliquefaciens* and *B. subtilis*, it is now known that their gene sequences are quite similar. Chen *et al.* (2009) compared the genomes of *B. amyloliquefaciens* FZB42 and *B. subtilis* 168 and determined that about 88% of the genes (3271) are conserved between the two species, whereas 310 genes were unique in *B. amyloliquefaciens*.

B. subtilis dedicates between 4% and 5% of its genome for the synthesis of antibiotics, among them, cyclic lipopeptides from the iturins, surfactins and fengycins families (Stein, 2005) and ribosomal peptides such as microcines, lantibiotics and lantipeptides (Scholz *et al.,* 2011). Meanwhile, *B. amyloliquefaciens* dedicates about 8.5% of its genome to the synthesis of non-ribosomal secondary metabolites, such as polyketides (bacillaene, difficidin and macrolactin), cyclic lipopeptides (surfactin, fengycin and bacilomycin D) and siderophore bacillibactin (Chen *et al.,* 2007). The study by Chen *et al.* (2009) described the following genetic differences between these two species: in contrast to the wide potential of *B. amyloliquefaciens* to synthesize lipopeptides and non-ribosomal polyketides, strain FZB42 does not produce most of the ribosomal antibiotic peptides synthesized by *B. subtilis* 168. It also does not possess the clusters of genes for the synthesis of lantibiotics subtilosin and sublacin, and does not produce the antibiotic known as cell killing factor Skf (sporulation killing factor) or sporulation retardant protein SdpC (sporulation delay protein) which are present in *B. subtilis*. Skf is produced by sporulating cells to cause lysis in non-sporulating cells and acts in cooperation with SdpC (Gonzalez-Pastor *et al.,* 2003).

In turn, *B. amyloliquefaciens* species has been divided into two subspecies based on phenotypic and genomic traits. *B. amyloliquefaciens* subsp. *plantarum* associated to plants

and *B. amyloliquefaciens.* subsp. *amyloliquefaciens* not associated to plants (Borris *et al.,* 2011). The characteristics that differentiate the two subspecies are the ability to synthesize enzymes that degrade carbohydrates and some non-ribosomal secondary metabolites. Unlike to *B. amyloliquefaciens* subsp. *amyloliquefaciens, B. amyloliquefaciens* subsp. *plantarum* typically produces macrolactin and difficidin polyketides (Borris *et al.,* 2011; Rueckert *et al.,* 2011; Dunlap *et al.,* 2013) and contains the genes *amyE* (alpha amylase precursor), *BglC* (β-Glucosidase 1,4 B-glucanase), *XynA* (1,4-β-xylanase) and lack the *amyA* gene (Dunlap *et al.,* 2013). Qiao *et al.* (2014) identified 54 unique genes for the subspecies *plantarum*, which are not present in the subspecies *amyloliquefaciens*, among which are genes for polyketide synthesis and carbon metabolism.

1.4.2 Ecology of the antagonists *Bacillus* species

Genus *Bacillus* contains Gram-positive bacteria, with a low proportion of G + C nucleotides in its genome (35% to 46%), are endospore-forming bacilli and are generally aerobic, although some are facultative anaerobes. The endospores confer special properties of dormancy, survival and longevity to the members of this genus in their natural environments since they are a dominant part in their life cycle (Alcaraz *et al.,* 2010). Several species of this genus are used in agriculture as biological control agents and promoters of plant growth, making them economically important. The species of bacteria of this genus are cosmopolitan and are found, and have specialized, in various environments, including soil, alkaline environments, hydrothermal sources, insect guts and seawater (Raddadi *et al.,* 2012; Alcaraz *et al*., 2010). Members of the *B. subtilis-licheniformis-pumilus* species group share traits such as a high number of transport genes and carbohydrate metabolism and a reduced number of genes involved in replication, recombination and repair (Alcaraz *et al.,* 2010) and have antagonistic activity against phytopathogens. Although isolates of *B. subtilis / amyloliquefaciens* from marine environments have been obtained, in general, members of the *B. subtilis* group, with biocontrol activity against phytopathogens, are found in the soil-plant environment. In this habitat these bacteria do not exist simply as donor spores or as free living organisms in their vegetative forms, but are active and dynamic members of the soil microflora, interacting with other organisms such as fungi, plant roots, insects and nematodes (Logan and De Vos, 2011).

Temperature has effects not only on the development of the disease but also on the biological control agents. Larkin and Fravel (2002) observed that the CS-24 and CS-1 non-pathogenic isolates of *Fusarium* spp. reduced the incidence of the disease at 32 °C under greenhouse conditions, but were less effective at 27 °C, optimal temperature for the development of the disease. Landa *et al.* (2001) also showed that chickpea vascular wilt, caused *by F. oxysporum* f. sp. *ciceris* was suppressed by rhizobacteria only at 20 or 30 °C but not at 25 °C, optimal temperature for the development of the disease. In the study done by Priest *et al.* (1987) to differentiate the species *B. amyloliquefaciens* from *B. subtilis* described that the optimal temperature for the growth of *B. amyloliquefaciens* is 30 to 40 °C and that its growth does not occur below 15 °C nor above 50 °C.

1.4.3 CLPs as main responsible of *B. amyloliquefaciens* biocontrol activity

Bacillus amyloliquefaciens subsp. *plantarum* is a rhizobacteria recognized for its antagonistic activity against plant pathogens. This antagonism may be caused through the direct action of antibiotics such as non-ribosomally synthesized cyclic lipopeptides (CLPs) and polyketides (PKs) (Cawoy *et al.,* 2014a; Desoignies *et al.,* 2013; Henry *et al.,* 2011; Pertot *et al.,* 2013; Yamamoto *et al*., 2014; Argüelles-Arias *et al.,* 2009; Chen *et al.,* 2009; Yuan *et al*., 2012; Zhao *et al*., 2014), through induced systemic resistance (ISR) (Kloepper *et al.,* 2004) and via nutrient competition through the production of siderophores (Dunlap *et al.,* 2013; Magno-Pérez *et al.,* 2015).

Compounds of lipopeptide nature such as iturins and fengycins produced by *B. amyloliquefaciens* are known for their direct effect on plant pathogens (Cawoy *et al.,* 2014b; Malfanova *et al.,* 2012), whereas systemic resistance can be induced by surfactins (Cawoy *et al.,* 2014a; Pertot *et al.,* 2013) and fengycins (Ongena *et al.,* 2005; Ongena *et al.,* 2007). Lipopeptides also fulfil an important role in the root colonization process by *Bacillus* (Bais *et al.,* 2004). CLPs are amphiphilic secondary metabolites that consist of a peptide ring with seven (iturins and surfactins) or ten (fengycins) amino acids linked to a *β*-hydroxy (fengycins and surfactins) or *β*-amino (iturins) fatty acid. Each family of lipopeptides has been divided in groups based on its amino acid composition and within each family there are homologous molecules which differ in the carbon number [C_{13} - C_{16} (surfactins), C_{14} -

C_{17} (iturins) and C_{14} - C_{18} (fengycins)], and the branching and saturation of the lipid tails (Akpa *et al.,* 2001; Malfanova *et al.,* 2012; Ongena and Jacques, 2008).

Due to their amphiphilic nature, CLPs can easily associate and be firmly anchored to lipid bilayers (Bonmatin *et al.,* 2003; Carrillo *et al.,* 2003), which allows them to interact with plant cell membranes and induce systemic defense responses in the host (Jourdan *et al.,* 2009), and with fungi membranes causing cell destabilization (Bonmatin *et al.,* 2003; Zhang *et al.,* 2013; Han *et al.,* 2015). The CLPs synthesized by *Bacillus* spp. can interfere with the integrity of the cell membranes in a dose dependent manner but the susceptibility of the membranes varies in a specific manner, which explains why each family of CLPs affects different targets. Surfactins for example, are known mainly for possessing hemolytic, antiviral, antimycoplasm and antibacterial activity, but strangely do not have a typical fungitoxic activity. Meanwhile, the iturins have strong hemolytic and antifungal activities, restricted antibacterial but no antiviral activity. Finally, the fengycins possess a lower hemolytic activity than iturins and surfactins but have shown a strong antifungal activity, especially against filamentous fungi (reviewed by Ongena *et al.,* 2010).

During the interaction with plant cells, the surfactins produced by *B. amyloliquefaciens*, constitute molecular patterns that are recognized by receptors of plant cell membranes, leading this way to the activation of the first line of defense in plants – pathogen-triggered immunity (PTI) – which can extend to all the plant organs – ISR - (Henry *et al.,* 2011). The initial events associated to the defense response of the plant after the recognition of surfactins, include alkalization of the external environment due to exit of ions such as K^+, NO_3^- and Cl^- from the cytoplasm; production of reactive oxygen species; stimulation of activity of phenylalanine ammonia lyase (PAL) enzyme; accumulation of phenolic compounds; and stimulation of lipoxygenase activity (LOX) (Jourdan *et al.,* 2009). However, high concentrations of surfactins can drive to plant cell death (Jourdan *et al.,* 2009) which may be important during the infection process of necrotrophic pathogens. At the same time, research into the molecular dialogue between *B. amyloliquefaciens* and the plant, has revealed that secretion of surfactins takes place during the first hours of interaction between the bacteria and the root, as a result of the perception of polysaccharides present in the cell wall of roots by the bacteria (Debois *et al.,* 2015). In addition, the accumulation of surfactins produced by *B. amyloliquefaciens* on the root surface is encouraged by root exudates

(Nihorimbere *et al.,* 2012) which explains why it is the main CLP detected in the antibiome of *B. amyloliquefaciens* on the root surface (Debois *et al.,* 2015).

Chapter 2. Soil sterilization, pathogen concentration and antagonist dose affect biological control of Fusarium wilt of cape gooseberry by *Bacillus amyloliquefaciens* Bs006

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Abstract

Bacillus amyloliquefaciens strain Bs006 has antagonistic potential against *Fusarium oxysporum* f. sp. *physali* (Foph) which causes vascular wilt of cape gooseberry; however, results of *in vivo tests* have been variable. The aim of the present study was to determine the effect of sterilization of soil and concentration of both antagonist and pathogen on the biocontrol activity of Bs006. Soil sterilization showed to be a source of variability since the efficacy of Bs006 was significantly lower in sterile than non-sterile soil. The evaluation of several doses of Bs006 against some concentrations of Foph strain Map5 (FOX-Map5) in the soil also showed that concentrations of both Bs006 and FOX-Map5 influence biocontrol activity. Diluted liquid culture of Bs006 at $1x10^6$ and $1x10^7$ cfu.mL^{-1} reduced FW (up to 97 %) under low inoculum pressure of FOX-Map5 (10^2 to 10^4 cfu.g^{-1} of soil), but at $1x10^8$ cfu.mL^{-1} significantly reduced FW only when the concentration of FOX-Map5 was $1x10^4$

cfu.g^{-1} (71 %). The evaluation of volumes of Bs006 (1x10^{8} cfu.mL^{-1}) and supernatant (10%) showed a positive correlation between volume of Bs006 and the efficacy in reduction of FW incidence. In contrast, application of high volumes of supernatant favored the development of the disease. Additionally, application of Bs006 at 1x10^{7} cfu.mL^{-1} showed important plant growth promoting effects in soil free of pathogenic inoculum. However, plant growth promoting activity by Bs006 was reduced by the presence of FOX-Map5 in the rhizosphere. Finally, it was determined that Bs006 grows endophytically in cape gooseberry and had high population levels in the rhizosphere inoculated with FOX-Map5.

Key words: Dose-response, biological control, Fusarium wilt, Cape gooseberry.

2.1. Introduction

Colombia was the main cape gooseberry (*Physalis peruviana*) producer and exporter of the 2000-2010 decade (Bonilla *et al*., 2009). This is the second most important fruit crop in Colombia mainly due to exports to European countries but currently its cultivation has expanded to other countries such as Kenya, Zimbabwe, Australia, New Zealand, India and Ecuador (Fisher *et al*., 2014). Cape gooseberry has become an alternative of agricultural production in over 24 countries, due to positive and interesting prospects in the international market, derived from the nutritional characteristics and medicinal properties of this fruit (Fisher *et al*., 2014). However, yield of cape gooseberry in Colombia has decreased from 18 t/ha in 2008 to 14.5 t/ha in 2011 (Legiscomex, 2013) mostly due to the incidence of vascular wilt disease, caused by *Fusarium oxysporum* (FOX) (Estupiñan and Osa, 2007; González and Barrero, 2011; Rodríguez, 2013) (Fusarium wilt (FW) of cape gooseberry).

The pathogenic form of *F. oxysporum* (Sacc.) (W.C. Synder and H.N. Hans.) causes root rot or vascular wilt in host plants and is the fifth most important fungal plant pathogens of scientific-economic importance due to its wide range of hosts (as species complex), and the severe losses in crops of economic importance such as tomato, cotton, banana and melon, among others (Dean *et al.*, 2012). It is also considered one of the most difficult pathogens to manage (Chandel *et al.*, 2010; Alabouvette *et al.*, 2007) because of its host-specificity (as single pathogenic form – *formae specialis*) based on its particular genetics (Bosland, 1988; Ma *et al.*, 2010; van Dam *et al*., 2016), the ability to form resistance

structures, its versatility in producing mycotoxins (Marasas, 1984; Bacon *et al.*, 1996), its detoxifying ability (Schouten *et al.*, 2004), its resistance to fungicides (Brent and Hollomon, 1998) and its ability to manipulate the host defense responses (Tatcher *et al.*, 2009).

Control of FW of cape gooseberry principally depends of use of chemical fungicides, due to the fact that other control measures such as resistant cultivars have not been developed and biological based products have not been registered. Moreover, cultural control practices like soil solarization or removal of infected plant debris are not commonly implemented by growers. Despite the use of fungicides, FW can, in some instances, lead to complete crop loss (Bernal *et al*., 2013). Today consumers increasingly demand food free of agrochemical residues and environmentally friendly production processes, despite the threat posed by FW. Consequently, growers are under pressure to minimize the use of fertilizers and chemical pesticides. For this reason, there is a growing interest in alternative methods to control plant diseases and in this context, microbial biopesticides represent a biotechnological alternative to the use of agrochemicals.

Biological control of vascular wilt of plants caused by FOX is a promising alternative to manage this class of disease, since biological control agents (BCA) may colonize the rhizoplane or the rhizosphere of the plant host (Ahmad and Baker, 1987) and interfere in the pathogenesis process (Folman *et al*., 2004; Deacon, 1996). Biopesticides based on plant growth promoting rhizobacteria (PGPR) are an example of this type of biotechnological development and its use is increasing due to the high potential to control plant pathogens (Borris, 2011). An important group of PGPR are *Bacillus* species which can form spores, making the bacteria more resistant to stress conditions and giving formulations longer shelf-life compared to gram negative biocontrol bacteria (Lazarovitz *et al*., 2014).

Members of the species complex of *B. subtilis* group, such us *Bacillus subtilis*, *Bacillus amyloliquefaciens, Bacillus licheniformis* and *Bacillus pumilus*, have proved to be efficient in plant growth promotion and biological control of plant pathogens (Borris, 2015). This group of bacteria produce a wide range of bioactive compounds, among which cyclic lipopeptides (CLPs) belonging to the surfactin, iturin and fengycin families are recognized for their potential use in biotechnology and biopharmacology (Banat *et al*., 2000; Singh and Cameotra, 2004; Cochrane and Vederas, 2016). In addition to the antimicrobial properties

of CLPs, they have also been shown to be involved in the colonization process and motility of *Bacillus*, as well as in the induction of systemic resistance in the host plant (Ongena *et al*., 2010). These modes of action that protect plants against phytopathogens makes this genus attractive for their use as biological control agents. However, the varying effectiveness observed in pathogen management may be due, in part, to unknown biotic and abiotic factors that affect its performance in the field. Therefore, there is massive room for improvement regarding the formulation of microbial products aimed at curtailing the impacts of plant pathogens.

B. amyloliquefaciens Bs006 (Gámez *et al*., 2015) is a new plant growth promoting and biocontrol agent with potential to minimize the effects of *F. oxysporum* (Caviedes, 2010). However, its variable biocontrol activity against FW of cape gooseberry (Guacaneme, 2010; Zapata and Díaz, 2012; Díaz *et al*., 2012; Torres *et al*., 2012) suggested that biotic and abiotic factors of the rhizosphere might affect the expression of traits related to biocontrol, but not those related with plant growth promoting activity. The aim of this study was to determine the influence of soil sterilization, BCA dose and pathogen concentration on the biocontrol activity of Bs006 against FW of cape gooseberry and to evaluate the effect of FOX-Map5 on the plant growth promotion activity of Bs006.

2.2. Materials and methods

2.2.1. Plant material

Commercial cape gooseberry seeds (Colombia ecotype) were washed with tap water to remove fungicide coating from the surface and then surface disinfected in ethanol 75 % during 1 min, followed by 2.5 % NaOCl for two min and subsequently washed three times in sterile distilled water (SDW). Disinfected seeds were germinated in sterile humidity chambers (Petri dishes with wet filter paper) incubated for 20 days in darkness and 30 °C conditions, to obtain seeds with a 1 cm radicle (approx.). Germinated seeds were planted in plastic trays (72 cells) filled with autoclaved peat (120 °C, 20 PSI, 30 min). They were maintained for 30 - 40 days under greenhouse conditions until they had four fully expanded true leaves. During this time the seedlings were watered daily and a commercial nutritive solution (Tottal®, Colinagro S.A., Bogotá) (N 180 - P 100 - K 40 - Ca 0.27 - Mg 12.5 - S 33

- B 1.35 - Co 0.03 - Cu 2.70 - Fe 0.32 - Mn 2.30 - Mo 0.09 - Zn 7.80 $g.L^{-1}$, respectively) was applied by drench (0.5 – 2 mL/L) once a week. These seedlings were used for biocontrol tests under greenhouse conditions.

2.2.2. Microorganisms and culture conditions

Strain Bs006 was isolated from healthy cape gooseberry plants in a crop strongly affected by vascular wilt disease (Caviedes, 2010) and it was subsequently identified as *B. amyloliquefaciens* (Gámez *et al*., 2015). Bacterial cells cultured in Luria-Bertani agar media were stored in a glycerol (20%) and peptone (1%) solution at –70 °C in the microorganisms collection of Corpoica - Tibaitatá Experimental Station. When required for experimental use bacteria were plated on Luria-Bertani agar media ((LBA) tryptone 10 g (Oxoid ®), yeast extract 5 g (Oxoid ®), NaCl 10 g (Merk ®), bacteriological agar (Oxoid ®) 18 g / 1000 mL). Then it was sub-cultured onto fresh LBA (six plates), incubated during 24 h at 30 °C and then preserved at 4 °C for a maximum of six months. This subculture served as a stock from which a new sub-cultures were prepared as a fresh source of inoculum for liquid cultures of Bs006. FOX-Map5 was isolated from cape gooseberry plants showing vascular wilt symptoms and has been shown to be one of the most virulent isolates found in pathogenicity tests on cape gooseberry in Colombia (Rodríguez, 2010). For its preservation, a monosporic culture was made in PDA and plugs of mycelia/conidia were stored in the cryopreserving solution mentioned above (Rodríguez, 2010). For the experiments in this study, the fungus was plated on PDA (Merk®) and incubated for seven days at 30 °C. Pieces of young mycelia were taken from this culture to inoculate 250 mL Erlenmeyer flask with 50 mL of PDB (Difco®) and incubated for 7 days in shaking conditions (150 rpm, 30 °C). The fermented broth was filtered through two layers of sterile muslin cloth and the recovered suspension was centrifuged (10.000 rpm, 15 min, 6 °C) and the pellet containing the microconidia was suspended in cryopreserving solution and stored in sterile plastic vials at – 20 °C. This preserved collection was used as stock to inoculate PDA or PDB for produce the inoculum of FOX-Map5 for the experiments.

2.2.3. *In vivo* tests of Bs006 against FW of cape gooseberry

The experiments to measure the biocontrol activity of *B. amyloliquefaciens* Bs006 against FW of cape gooseberry were carried out in a glasshouse (56 m^2). The average temperature in the glasshouse is 25 ± 15°C; the average relative humidity is 60 ± 15%. For plant growth a substrate mix of soil (Andosol, pH 5.7, MO 12.90, P 11 $mg.Kg^{-1}$, K 0.69, Ca 4.05, Mg 1.55 $cmol_{(+)}.Kg^{-1}$) and rice husk (3:1 ratio) was prepared (hereafter referred as soil). This substrate was moistened with tap water up to 60 % of moisture retention capacity. 60 day old cape gooseberry seedlings were transplanted and irrigated manually once a day and fertilized (30 mL/plant) at the end of the second and fourth week after transplant with Diammonium Phosphate (DAP Nutrimon®, Barranquilla, Colombia, N 18% - P 46% - K 0%) at dose of 6 $g.L^{-1}$, Potassium chloride (KCl Nutrimon® N 0% - P 0% - K 60%) at dose of 3 $g.L^{-1}$ and minor nutrients (Borozinco 240GR®, Microfertisa, Bogotá) at 0.5 $g.L^{-1}$.

Inoculation of microorganisms. FOX-Map5 inoculum was produced in PDB as described above. The suspension of microconidia in fermented broth obtained after discarding the mycelia was diluted with tap water after being counted using a Neubauer chamber, to obtain the desired concentration which varied as a function of the desired final concentration of inoculum in the soil. The microconidia suspension was added to the soil uniformly. For instance, using a ratio of 100 ml of suspension at $1x10^6$ microconidia.mL^{-1} per 0.9 Kg of wet soil, a concentration of $1x10^4$ cfu.g^{-1} of soil was achieved. Plastic trays of 12 cones (750 cm^3 each) were filled with the inoculated soil (600 g) and one seedling was transplanted per cone. Immediately after transplant cell suspension of *B. amyloliquefaciens* Bs006 obtained from liquid culture (LB, 125 rpm, 30 °C, 48 h) was applied by drench (30 mL/plant). *Bacillus* suspensions were prepared by diluting the fermentation broth with tap water, according to the final concentration growth and the required for each treatment, hence the treatment contained cells as well as metabolites excreted into the medium. Cell density of Bs006 was adjusted measuring the optical density in a spectrophotometer at 600 nm (Optizen®, Mecasys) and using a standard curve ($DO_{600\ nm}$= 0.5 ~ 2.49 x 10^8 ufc/mL).

Evaluation of the effect of autoclaved soil on Bs006 biocontrol efficacy. Biocontrol activity of *B. amyloliquefaciens* Bs006 against FW of cape gooseberry in natural soil was compared with autoclaved soil (120 °C, 20 PSI, 30 min, two cycles). The concentration of inoculated FOX-Map5 in the soil was $1x10^4$ cfu.g^{-1}. Bs006 was applied at a concentration of $1x10^8$ cfu.mL^{-1}. Plants inoculated only with pathogen without treatment to control the

disease and plants without the pathogenic or antagonist were used as negative and absolute controls, respectively.

Analysis of Bs006-dose and FOX-Map5-concentration on biocontrol activity of Bs006. The effect of *B. amyloliquefaciens* Bs006 dose (10^5 to 10^9 cfu.mL^{-1}) on the incidence and severity of FW of cape gooseberry was measured in a first experiment. The concentration of pathogenic inoculum in the soil was 1x10^4 cfu.g^{-1}. In a second experiment the effect of FOX-Map5 concentration (10^2 to 10^6 cfu.g^{-1}) on biocontrol activity of Bs006 (1x10^8 cfu.mL^{-1}) was measured. Autoclaved soil was included in these experiments as a factor to validate the results from the first phase of this research. The relationship between lower doses of Bs006 (10^6 and 10^7 cfu.mL^{-1}) and the above mentioned concentrations of FOX-Map5 also was analyzed in a third experiment in non-sterile soil. A fourth experiment was carried out to determine the individual effects of Bs006 cells and supernatant from the LB-broth fermented by Bs006 (30 °C, 125 rpm, 48 h), as well as the effect of the volume (30, 50, 100, 150 mL/plant) of the treatments on FW development. Inoculum for evaluation of individual effects of Bs006 cells and supernatant was prepared separating the cells from the fermented broth by centrifugation (15000 rpm, 15 min). The obtained biomass was washing twice with SDW and centrifuged. Finally the pellet was resuspended in SDW adjusted to 1x10^8 cfu.mL^{-1}. The supernatant from the fermented broth was passed by 0.22 µm filters (Milllipore®, Ireland) and diluted in SDW to a concentration of 10 % before application. Bs006 cells (10^8 cfu.mL^{-1}) and supernatant (10 %) mix treatment was included and in this case cell suspension was adjusted in the supernatant solution. Controls consisted of plants growing in soil inoculated with pathogen and treated with water (negative control); plants growing in soil free of pathogen and treated with Bs0006 (relative control); and plants growing in soil free of pathogen and antagonist (absolute control).

Effect of Bs006 dose and presence of FOX-Map5 in the soil on plant growth promoting activity of Bs006. Cell suspensions of Bs006 (diluted fermented broth) at 10^5 to 10^9 cfu.mL^{-1} doses were applied to non-sterile soil to measure plant growth variables in nursery and post-transplant conditions in independent experiments. 5 mL of Bs006 suspension were applied to each seedling when they were sown in the nursery while 30 mL were applied to 60 day old plants during transplant for the post-transplant experiment. In a separate experiment growth of plants transplanted in non-sterile and pathogen-

inoculated soil ($1x10^4$ cfu.mL^{-1}) and treated with 30 mL/plant of Bs006 ($1x10^8$ cfu.mL^{-1}) was measured. Controls consisted of non-treated plants with the bacteria.

Measured variables. The incidence and severity of FW of cape gooseberry were recorded weekly, since the appearance of the typical symptoms of the disease in the plants, such as loss of turgidity in young leaves, lateral yellowing in old leaves and plant growth stunting. Incidence is expressed as the proportion of plants with typical symptoms of FW, whereas the severity of the disease expresses the intensity of the symptoms in affected plants. Severity was scored on a scale 0 - 5 degrees, modified from Sánchez and Forero (2009) where 0= no evident symptoms of the disease, expanded and turgid leaves with no foliar bending, green leaves; 1= Bending of young leaves, slight epinasty, mild chlorosis of mature leaves ; 2= Foliar bending, epinasty in 30-50% of the leaves, moderate chlorosis in the oldest leaves and slight chlorosis in leaves from the middle of the plant, clear stunting of the plants; 3= 60-80% of leaves show epinasty, clear loss of turgidity, moderate chlorosis in young leaves, abscission of oldest-chlorotic leaves; 4= All the leaves present epinasty, severe chlorosis, moderate defoliation, the plant is clearly in wilting state; 5= Twisted leaves, severe wilting, severe defoliation, bent stem, dead plant.

Incidence and severity were calculated by follow the formulas described by Zhang *et al*. (1996). Incidence = $[(n_1 + n_2 + n_3 + n_4 + n_5)/n] \times 100$ and Severity = $[(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5)/5n] \times 100$., where 0 – 5: Degrees of disease; $n_0 – n_5$: Number of plants in each degree of the disease; n: Total number of plants in the experimental unit. The area under the progress curve of incidence and severity was calculated using the formula described by Campbell and Madden (1990) and the efficacy of treatments in the reduction of incidence and severity of the disease was estimated with the formula Efficacy= [(A-B)/A]*100. Where A: Value area under the disease curve of the negative control; B: Value area under the disease curve of a given treatment.

Plant growth variables were measured 30 days after they were sown (dps) under nursery conditions and 39 days post-transplant (dpt) under post-transplant phase. Root and shot length and number of secondary roots were measured in nursery plants while shoot length, foliar area and root and shot dry weight were measured in post-transplant plants.

***Evaluation of the endophytic ability of* Bs006.** An indexation procedure was carried out to determine the endophytic ability of *B. amyloliquefaciens* Bs006 and to estimate its population density inside the root and stem tissues. To this end, 39 days after inoculation of the pathogen, samples of tissues were taken from plants that were apparently healthy or with low disease severity (1 or 2 degrees). After removing the rhizospheric soil, the root was washed with a detergent solution and subsequently the entire main root or 15 cm of the stem were surface disinfected, following the procedure from Li *et al*. (2012) to isolate endophytic strains of *Bacillus*. Briefly, tissue segments were immersed in 3 % NaOCl for 10 minutes, soaked in ethanol (70 %) for one minute and finally washed five times with SDW. The sterility of the water from the last root washing was tested by inoculating 50 μL aliquots in Petri dishes with LBA which were incubated to examine the presence or absence of microorganism colonies. Sterilized tissue was subsampled by weighing 0.1 g from the root zone under the crown, from the elongation zone, from the apex and from upper zone of the crown. Each root/stem segment (0.1 g) was macerated with 1 mL of SDW in a sterile mortar. 100 µL of the obtained suspension and decimal dilutions were inoculated on three plates with LBA after being subjected to 90 ºC for 10 minutes in a thermostatic bath. The plates were then incubated at 30 ºC for 48 h and the number of colonies which appeared to be *Bacillus* was registered. Rhizospheric soil (0.1 g) also was sampled and suspended in 9.9 mL sterile Tween 80 solution (0,1 % v/v), stirred in a vortex during two min, then submitted to thermal shock and inoculated in LBA as mentioned above.

2.2.4. Experimental design and data analysis

The experiments to test the effect of Bs006 doses and FOX-Map5 concentrations on biocontrol activity under sterile and non-sterile soil conditions used a randomized complete block (RCB) design with factorial structure of treatments and four replicates. The experiment in which the relationship of low doses of Bs006 were tested against several doses of FOX-Map5 was evaluated using a 3x5 factorial structure. The factors were the microorganisms (Bs006 and FOX-Map5) and the concentrations were used as the levels of the factors (0, 10^6, and 10^7 cfu.mL^{-1} for Bs006 and 10^2, 10^3, 10^4, 10^5, and 10^6 cfu.g^{-1} for FOX-Map5. The experiment used to measure individual effects of Bs006 cells and supernatant had a 3x4 factorial arrangement with 4 replicates. The first factor was the biological treatment (cells, supernatant and the mix of both) and the second factor was the

volume applied (30, 50, 100 and 150 mL/plant). The experiment in which the effect of Bs006 doses on plant growth promotion was measured was carried out under RCB design with 3 replicates. Ten plants were used per experimental unit in these experiments. To measure plant growth variables sample size of three (nursery) and five plants (post-transplant) per experimental unit were used. The experiment used to evaluate the influence of FOX-Map5 on plant growth promoting responses was carried out under a split-plot design with three replicates. The main-plot was the presence of the pathogen and sub-plot was the biological treatment. The sample size for this experiment was three plants per treatment.

The data were submitted to normality according to Shapiro Wilk test ($P > 0.05$), homogeneity of variances with Bartlett and Levene tests ($P > 0.05$). Significance was determined by analysis of variance (ANOVA) using generalized lineal model GLM. Duncan multiple range, Fisher Least Significant Difference (LSD) and Tukey tests ($P = 0.05$) were used to make comparisons between means of the treatments. All analysis were conducted using the Proc GLM of Statistical Analysis Software System (version 9.4; SAS Institute, Cary, NC).

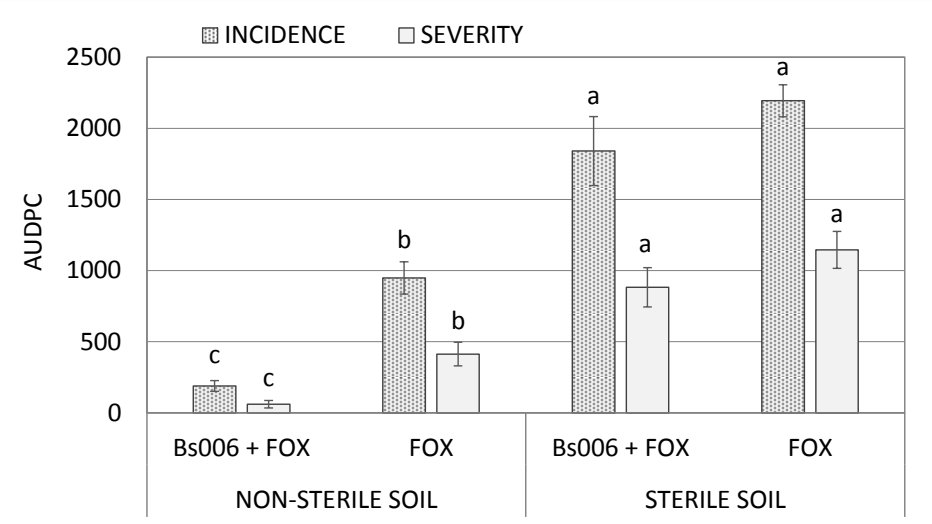
2.3. Results

2.3.1. Effect of soil sterilization on biocontrol activity of Bs006

The effect of autoclaving the soil on the potential of Bs006 to reduce the progress of FW disease was examined, as it was considered one of the factors that contributed to the variability of Bs006 efficacy in previous experiments. Soil sterilization negatively affected the biocontrol activity of *B. amyloliquefaciens* Bs006 against FW of cape gooseberry, since progress of the disease incidence (expressed as the area under disease progress curve - AUDPC) was significantly higher ($P < 0.01$) in plants grown in sterile soil compared with the disease progress of those grown in non-sterile soil (Figure 2-1). The reduction of the disease incidence and severity progress (comparing the AUDPC values) by applying Bs006 to non-sterile soil was 80 and 85 %, respectively, while under sterile-soil conditions the efficacy was 16 and 23 %, respectively.

The incidence of FW in plants grown in non-sterile soil and treated with Bs006 was 23 % at the end of this experiment (43 days post-inoculation – dpi) while in plants grown in FOX inoculated non-sterile soil and without biocontrol treatment (negative control) the incidence was 63 %, which represents 63 % of efficacy in incidence reduction through the application of Bs006. Meanwhile disease incidence was 100 % in plants grown in sterile soil both treated and untreated with Bs006 (data not shown). Observations of disease severity showed a similar behavior to the incidence at this endpoint (data not shown).

Figure 2-1: Effect of Bs006 and soil sterilization on FW development



Note[2]

2.3.2. Effect of Bs006-dose and FOX-Map5-concentration on FW

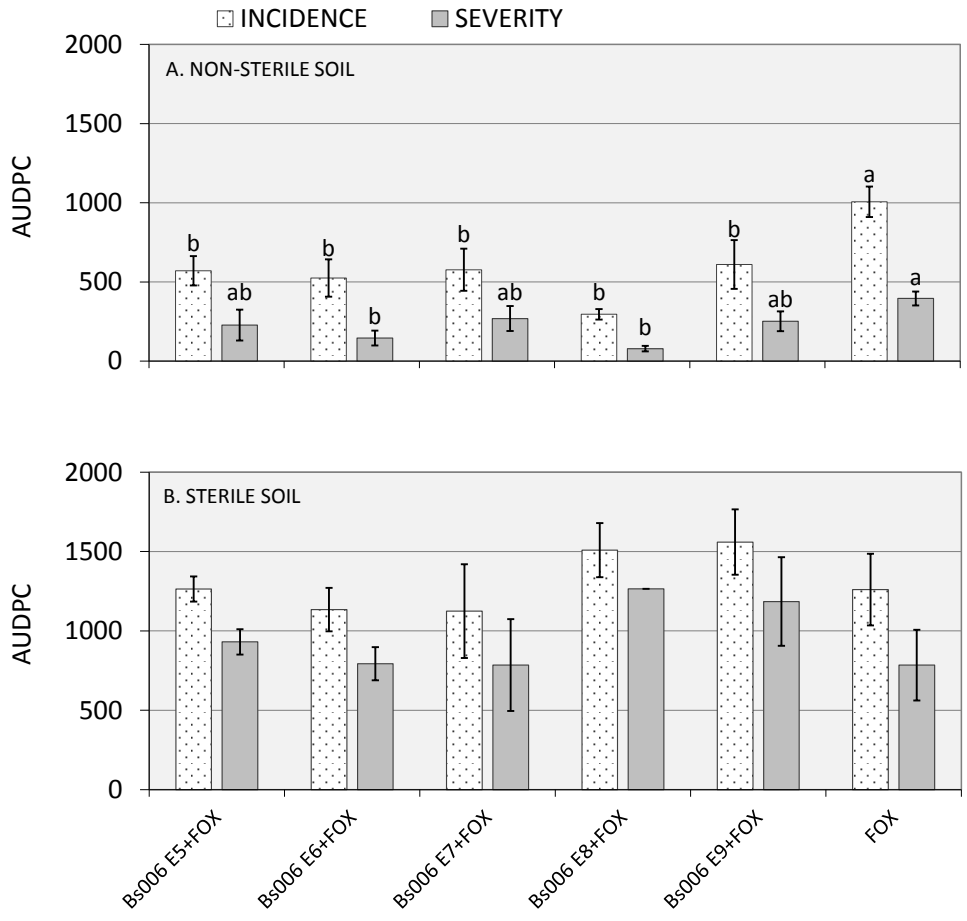
Effect of Bs006-dose against FOX-Map5. Several doses of Bs006 were tested against FOX-Map5 inoculated at a concentration of $1x10^4$ cfu.g^{-1} of soil. The sterility of the soil, as

[2] Area under disease progress curve (AUDPC) of the incidence and severity of FW of cape gooseberry in response to application (30 mL) of *B. amyloliquefaciens* (Bs006) in natural soil (non-sterile soil) or autoclaved soil (sterile soil) at 43 dpi. The concentration of Bs006 was adjusted at $1x10^8$ cfu.mL^{-1}. Columns with same letter inside a variable are not significantly different according to Tukey test (α= 0.05). Bars on the columns represent the standard error of data (n= 3).

possible source of variability was included as additional factor to validate the results detailed above. The application of Bs006 to non-sterile soil showed significant reduction of FW incidence progress ($P < 0.05$) in all tested doses compared to the disease in the negative control (Figure 2-2A). Under these conditions, severity progress was significantly reduced ($P < 0.05$) by Bs006 at $1x10^6$ and $1x10^8$ cfu.mL^{-1} as compared to the negative control. Bs006 ($1x10^8$ cfu.mL^{-1}) reduced the progress of FW incidence and severity by 71 % and 80 % respectively, during 49 dpi under non-sterile soil conditions. The treatments 10^5, 10^6, 10^7, and 10^9 cfu.mL^{-1} showed an efficacy in incidence reduction of 43.3, 47.8, 42.7, and 39.3 %, respectively and a reduction in severity of 42.4, 63.0, 32.0, and 36.4 %, respectively. Surprisingly, plants treated with Bs006 at $1x10^9$ cfu.mL^{-1} showed similar level of the disease to that of the negative control and significantly higher than the rest of dose of Bs006 at 56 dpi (data not shown). In contrast to non-sterile soil, in sterile soil Bs006 did not reduce FW of cape gooseberry (Figure 2-2B). However, the reduction in FW development was due to the delay in the onset of the epidemic in non-sterile soil compared with sterile soil rather than a reduction in the rate of disease progression.

Effect of the concentration of FOX-Map5. In this experiment the pathogen was inoculated at concentration ranging from $1x10^2$ to $1x10^6$ cfu.g^{-1} in both sterile and non-sterile soil. Biocontrol treatment consisted of application of Bs006 at $1x10^8$ cfu.mL^{-1}. Interestingly it was found that under non-sterile soil Bs006 significantly reduced the disease incidence progress ($P < 0.05$) only when the inoculum concentration of FOX-Map5 was $1x10^4$ cfu.g^{-1}. As compared to the respective negative control, no other combination of FOX-Map5 and Bs006 reduced the disease (Figure 2-3 A). The combination of Bs006 ($1x10^8$ cfu.mL^{-1}) and FOX-Map5 ($1x10^4$ cfu.g^{-1}) showed an efficacy of 64 % and 84 % of reduction of the incidence and severity progress of the disease respectively during 49 dpi. Consistent with previous observations in this study, under sterile soil conditions Bs006 did not reduce FW of cape gooseberry (Figure 2-3B).

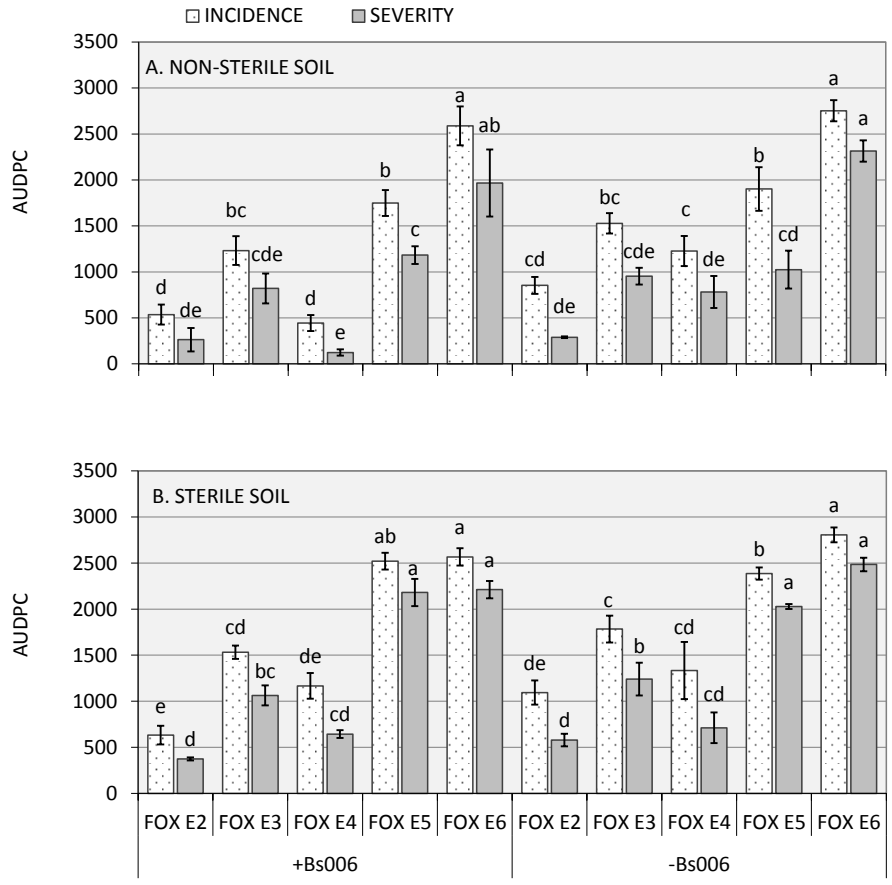
Figure 2-2: Effect of Bs006-doses on FW progress



Note[3]

[3] *B. amyloliquefaciens* Bs006 was applied at $1x10^5$ to $1x10^9$ ufc/mL doses (Bs E5 – Bs E9). FW incidence and severity (AUDPC: Area under disease progress curve) during 49 dpi under non-sterile (A) and 36 dpi in autoclaved soil (B) conditions were measured. *F. oxysporum* Map5 (FOX) was inoculated in the soil at $1x10^4$ cfu.g^{-1}. Columns from the same variable with the same letter are not significantly different according to LSD test (α= 0.05). Bars on the columns represent the standard error of the data (n= 4).

Figure 2-3: Effect of inoculum density of FOX-Map5 on Bs006 efficacy



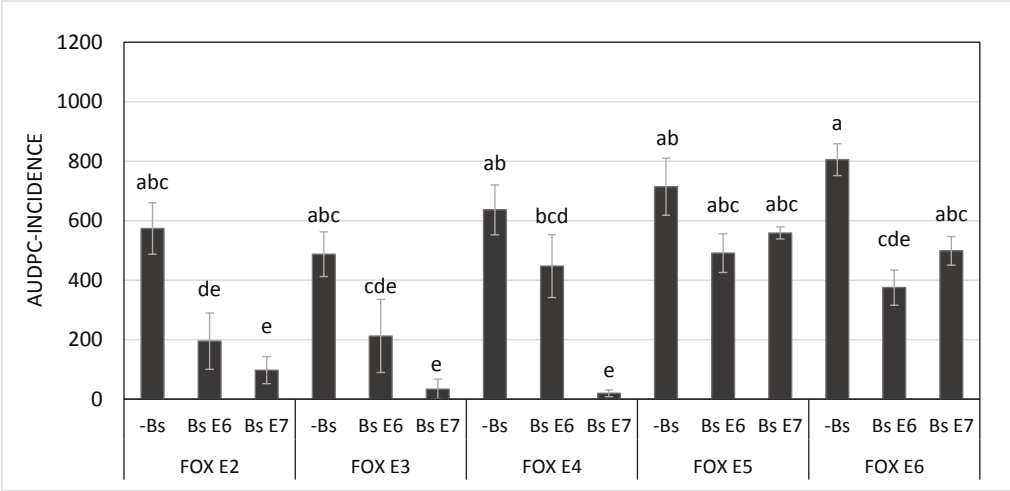
Note[4]

Effect of lower doses of Bs006 on FW progress. Taking into account the results described above, which showed that biocontrol activity of *B. amyloliquefaciens* Bs006 depends on the dose of the bacteria when it is confronted with $1x10^4$ cfu.g^{-1} inoculum density of FOX-Map5, we studied the response of FW disease to the combination of lower doses of Bs006 ($1x10^6$ and $1x10^7$ cfu.mL^{-1}) with several doses of FOX-Map5 ($1x10^2$ to $1x10^6$

[4] Incidence and severity progress of FW of cape gooseberry were measured during 49 dpi under non-sterile (A) and sterilized soil (B) conditions. 30 mL of rhizobacteria suspension was applied to the soil after transplant at $1x10^8$ cfu.mL^{-1} by drench (+Bs006). FOX was inoculated on the soil before transplant in concentrations ranging from $1x10^2$ (FOX E2) to $1x10^6$ cfu.g^{-1} (FOX E6). Inoculated soil with FOX and not treated with Bs006 was used as negative control (-Bs006) Columns from the same variable sharing the same letter are not significantly different according to Tukey test (α= 0.05). Bars on the columns represent the standard error of the data (n= 4).

cfu.g^{-1}). The application of Bs006 at 1x10^6 cfu.mL^{-1} significantly reduced the development of FW incidence when FOX-Map5 concentration was 10^2 and 10^3 in 66 % and 56 % respectively. Whilst Bs006 at 1x10^7 cfu.mL^{-1} showed to reduce more efficiently the progress of FW when FOX-Map5 was inoculated at 10^2, 10^3 and 10^4 cfu.g^{-1} in 83 %, 93 % and 97 % respectively. This experiment showed that low doses of Bs006 such as 1x10^6 and 1x10^7 efficiently reduced FW disease under low inoculum pressure of FOX-Map5, but not under higher concentrations of the pathogen in the soil (Figure 2-4). The interaction of Bs006 x FOX-Map5 concentrations showed significant effects for FW incidence progress (*P*= 0.0210) but not for severity progress (*P* = 0.0935). This result suggests that in this antagonist-pathogen interaction, both the dose of Bs006 and concentration of FOX-Map5 in the soil are determinants in the development of the FW of cape gooseberry.

Figure 2-4: Effect of low doses of Bs006 on FW progress



Note[5]

[5] Area under FW incidence progress curve at 45 dpi in response to low doses of Bs006 1x10^6 cfu.mL^{-1} (Bs E6) and 1x10^7 cfu.mL^{-1} (Bs E7) and inoculum density of FOX-Map5 ranging from 1x10^2 cfu.g^{-1} (FOX E2) to 1x10^6 (FOX E6) in non-sterile soil was calculated. Columns with the same letter are not significantly different according to Tukey test (α= 0.05). Bars on the columns represent the standard error of the data (n= 4).

2.3.3. Individual effect of cells and supernatant of Bs006 culture on FW

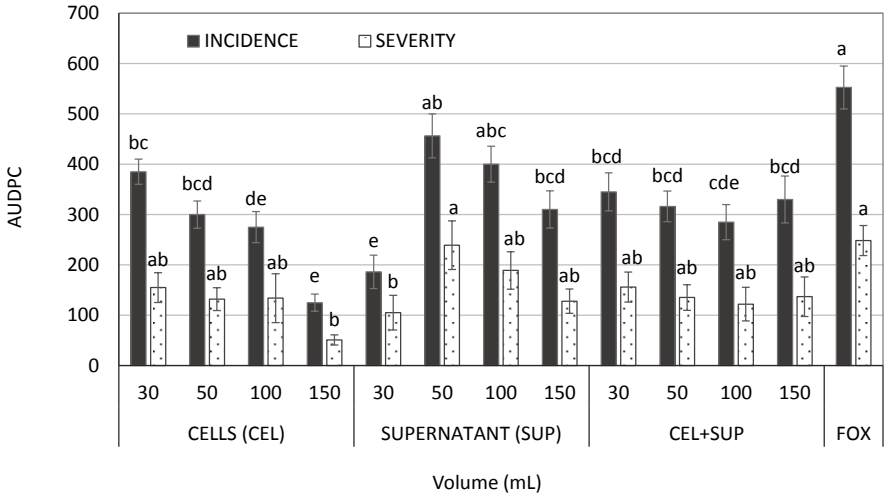
Disease development was significantly different between type of inoculum (Bs006 cells resuspended in water, supernatant diluted in water at 10%, and mix of Bs006 cells resuspended in supernatant solution (10%) ($P < 0.0001$), the volume of application ($P = 0.0089$) and the inoculum x volume interaction was significant ($P < 0.0001$). There was an inverse relationship between volume of Bs006 cells ($1x10^8$ cfu.mL^{-1}) and disease incidence and severity progress, where greater volumes of Bs006 cells led to a lower disease progress. However all volumes of cell suspension significantly reduced the progress of the disease incidence (Figure 2-5). The application of 30 mL of supernatant solution significantly reduced the disease progress (incidence and severity) compared to the negative control but the disease was higher for all other volumes applied (Figure 2-5). The application of the mix of cells and supernatant (mix) at all volumes tested significantly reduced the progress of the disease incidence compared to the negative control. However disease progress (incidence and severity) was similar under all volumes of mix. Moreover application of 30 and 150 mL of mix negatively affected the efficacy of biocontrol since the incidence progress was significantly lower under application of 30 mL of supernatant and 150 mL of cell suspension respectively (Figure 2-5). Application of 150 mL/plant of Bs006 cells suspension at $1x10^8$ cfu.mL^{-1} showed 77 % efficacy in FW incidence reduction while the efficacy of application of 30 mL/plant of supernatant was 66 % and the mix showed 38 to 48 % efficacy.

2.3.4. Plant growth promoting activity by Bs006

Effect of dose of *B. amyloliquefaciens* Bs006 on plant growth. The effect of Bs006 on plant growth was measured in cape gooseberry seedlings and in the early phase of post-transplant, under separate experiments. After 30 days of growth in the nursery Bs006 application did not show a significant effect on the length of the main root or the formation of secondary roots (Table 2-1). However, the application of the bacteria significantly stimulated the height of the plant (length of the main stem), compared to the controls. There were no significant differences among the tested concentrations of Bs006 (Table 2-1A).

In the post-transplant phase, application of Bs006 significantly stimulated plant growth in cape gooseberry at all the doses used, compared to the control. Specifically, Bs006 at $1x10^7$ cfu/mL, significantly increased the leaf area and the shot dry weight compared to the lowest dose of Bs006 evaluated ($1x10^5$ cfu/mL). The shoot dry weight was significantly higher when Bs006 was applied at $1x10^7$ cfu/mL compared to the higher doses (Table 2-1A).

Figure 2-5: Effect of volume of application of Bs006 cells and supernatant on FW progress



Note[6]

[6] Progress of the incidence and severity of FW of cape gooseberry for 41 dpi in response to application of several volumes (30, 50, 100, 150 mL/plant) of *B. amyloliquefaciens* Bs006 cell suspension (CEL), filtered-fermented LB broth by Bs006 (SUP) and the mixture of Bs006 cells with supernatant (CEL+SUP). Bs006 was grown during 48 h in LB broth at 30 °C and 150 rpm. Concentration of Bs006 cells was adjusted to $1x10^8$ cfu.mL^{-1} while supernatant was used at 10 % in tap water. Negative control (FOX) was not treated. Columns with the same letter are not significantly different according to Tukey test (α= 0.05). Bars on the columns represent the standard error of the data (n= 4).

Table 2-1: Growth response of cape gooseberry (*Physalis peruviana*) to application of *B. amyloliquefaciens* Bs006

Experiment

A. Effect of dose[a] of *B. amyloliquefaciens* Bs006

Treatment	Nursery[b] (30 dps)[c]			Post-transplant[d] (25 dpt)[c]		
	Length (cm)		Secondary roots (No.)	Foliar area (cm^2)	Dry weight (g)	
	Root	Stem			Root	Shoot
10^5	53.94 ± 5.65 b	16.11 ± 1.46 a	10.20 ± 0.34 abc	212,17 ± 42.35 b	0,46 ± 0.09 a	1,33 ± 0.34 b
10^6	69.03 ± 14.74 ab	15.93 ± 3.17 a	9.33 ± 0.46 bc	233,09 ± 38.62 ab	0,51 ± 0.16 a	1,38 ± 0.19 ab
10^7	79.27 ± 3.90 a	17.65 ± 1.63 a	9.60 ± 0.40 abc	262,65 ± 34.54 a	0,60 ± 0.21 a	1,61 ± 0.23 a
10^8	60.46 ± 26.13 ab	16.57 ± 0.83 a	8.20 ± 0.40 c	226,21 ± 37.01 ab	0,50 ± 0.21 a	1,26 ± 0.20 b
10^9	72.85 ± 20.42 ab	17.07 ± 0.91 a	11.53 ± 0.61 a	246,93 ± 42.55 ab	0,56 ± 0.07 a	1,31 ± 0.14 b
Control[e]	61.86 ± 12.53 ab	12.34 ± 0.75 b	10.13 ± 0.51 abc	136,96 ± 40.94 c	0,26 ± 0.08 b	0,88 ± 0.20 c
Fertilizer[f]	74.46 ± 2.02 ab	13.03 ± 1.35 b	10.93 ± 0.63 ab	210,15 ± 35.80 b	0,45 ± 0.10 a	1,29 ± 0.28 b

B. Growth of cape gooseberry plants in presence of FOX-Map5[g] – Post-transplant (20 dpt)

Treatment	Shoot height (cm)		Foliar area (cm^2)		Dry weight (g)			
					Root		Shoot	
	-FOX	+FOX	-FOX	+FOX	-FOX	+FOX	-FOX	+FOX
Bs006	20.68 ±1.65 a	14.24 ± 2.02 b	232.54 ± 28.57 a	153.03 ± 34.70 b	0.44 ± 0.11 a	0.26 ± 0.13 b	1.72 ± 0.42 a	0.77 ± 0.21 c
Control	16.11 ±2.95 b	13.49 ± 1.67 b	168.98 ± 43.92 b	140.41 ± 34.42 b	0.33 ± 0.10 ab	0.26 ± 0.09 b	0.98 ± 0.34 bc	0.89 ± 0.24 c

[a] Evaluation of Bs006 concentrations under nursery and post-transplant phases were carried out in independent experiments

[b] Bs006 was applied at sown, one and two weeks after sown. 5 mL of cell suspension were applied to each seedling.

[c] Days post-sown (dps), days post-transplant (dpt)

[d] Bs006 was applied once only immediately after transplant. 30 mL/planta were applied. Bs006 treated plants were fertilized with a half dose of nutrient solution

[e] No treatment were applied to the plants

[f] Commercial product (Tottal®) containing major and minor nutrients at dose of 3 mL/L were used. 5 mL of nutrient solution were applied per plant with same frequency as application of the bacteria

[g] The plants were transplanted in soil free of pathogenic inoculum (-FOX) or artificially inoculated with FOX-Map5 (+FOX) at [$1x10^4$ cfu.g^{-1}]. Bs006 was applied immediately after transplant (30 mL/planta, $1x10^8$ cfu.mL^{-1}). Seedlings for this experiment were rooted in sterile peat during 60 days applying nutrient solution in dose of 1 to 3 mL.L^{-1} once a week from 4th to 8th week after sown and no fertilizer were applied after transplant.

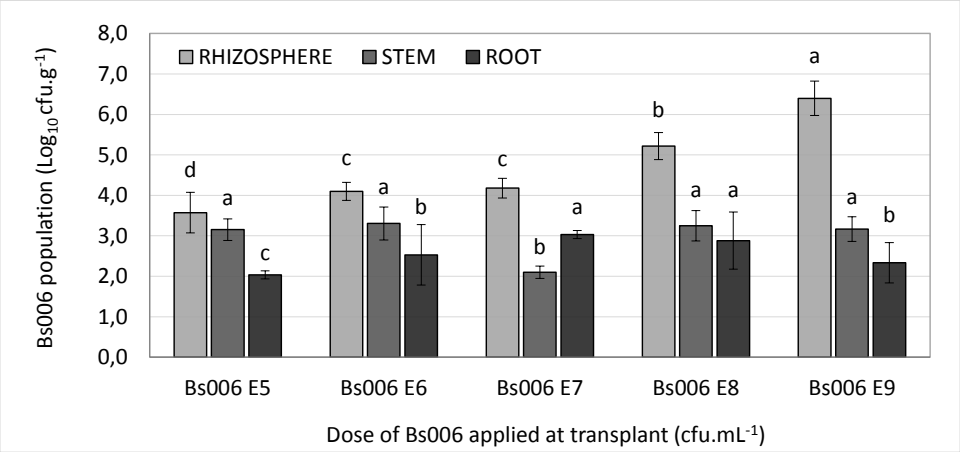
Data correspond to mean ± standard deviation of 15 (A-Nursery) and 9 samples (A-Post-transplant and B). Significant differences between treatments are indicated by different letters in each variable, within each experiment according to Tukey test (α= 0.05).

Plant growth promoting activity by Bs006 in the presence of the pathogen. In soil free of FOX-Map5, Bs006 significantly stimulated shot height, foliar area and shot dry weight by 22.0, 27.3 and 43.1 %, respectively, compared to the control but did not stimulate root growth. However, in soil inoculated with FOX-Map5, growth of plants treated with Bs006 was significantly lower compared to the treated plants grown in soil free of pathogen, but similar to that of the untreated plants in soil free of FOX-Map5 (absolute control) and in pathogen inoculated soil (negative control) (Table 2-1B). Results thus suggested that presence of the pathogen negatively affects plant growth normally promoted by Bs006, nevertheless, it is necessary elucidate if the pathogen affects directly the Bs006 traits expression or the plant responses.

Endophytic ability of Bs006. *B. amyloliquefaciens* Bs006 populations in terms of spores concentration were estimated in the rhizosphere and inside plant tissues of cape gooseberry during growth in nursery and post-transplant phases. The bacterium was recovered from the rhizosphere as well as inside of root and stem tissues, at 2 (seedling) and 5 weeks (post-transplant) after application of Bs006 to the substrate. These results demonstrate that the rhizobacteria acts as endophyte in this plant which can colonize root and stem tissue.

Bs006 populations were more numerous in the rhizosphere than inside plant tissue of seedlings. A positive correlation was identified between the initial concentration of Bs006 cells applied to the substrate and the concentration of bacteria recovered from the rhizosphere (Figure 2-6). The population of Bs006 recovered from the lower section of the stem of the seedling was similar among treatments, except for the treatment $1x10^7$ cfu.mL^{-1} where the population was significantly lower ($P < 0.05$). In the case of seedling roots it was shown that under low and high concentrations of Bs006 ($1x10^5$, $1x10^6$ and $1x10^9$), the endophytic population was significantly lower ($P < 0.05$) than when it was applied the intermediate doses ($1x10^7$ and $1x10^8$ cfu.mL^{-1}) (Figure 2-6).

Figure 2-6: Effect of Bs006 dose on its population density in the rhizosphere and plant tissue

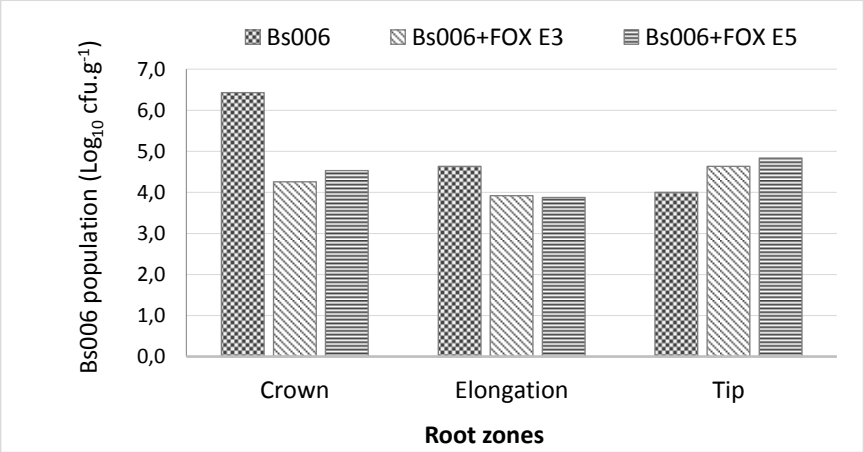


Note[7]

In post-transplant assays the density of endophytic population of Bs006 was negatively affected by the presence of FOX-Map5 in the rhizosphere, since the bacteria reached populations from $1x10^4$ to $2.7x10^6$ cfu.g^{-1} of tissue when it was applied in soil free of FOX-Map5, whereas in the presence of the pathogen the population of Bs006 ranged from $7.5x10^3$ to $6.9x10^4$ cfu.g^{-1}. Under soil free of FOX-Map5 the endophytic population of Bs006 was higher in the root crown ($2.7x10^6$ cfu.g^{-1}), followed by the elongation zone ($4.3x10^4$ cfu.g^{-1}), whereas in the root apex had the lowest density ($1x10^4$ cfu.g^{-1}). Under the presence of FOX the density of the endophytic population of Bs006 was similar in the root crown and root apex ($1.8x10^4$ to $6.9x10^4$ cfu.g^{-1}), whereas in the elongation zone the population was lower ($7.5x10^3$ to $8.3x10^3$ cfu-g^{-1}) (Figure 2-7).

[7] Samples were taken at 30 dpi. Columns from each sampled zone with the same letter are not significantly different according to Tukey test (α= 0.05). Bars on the columns represent standard deviation of the mean (n= 9). Bs006 E5 means bacteria applied at a concentration of $1x10^5$ cfu.mL^{-1} and so on.

Figure 2-7: Bs006 endophytic population in the main root of cape gooseberry at 39 days after transplant



Note[8]

2.4. Discussion

Biological control of plant diseases with antagonistic microorganisms and their bioactive compounds is a recognized tool in plant protection and represents an alternative to conventional chemical methods to control vascular wilt disease caused by *F. oxysporum* in many hosts. Although large scale use of biopesticides has been delayed for a long time due to the low efficacy, inconsistent behavior in the field and high cost, its market has significantly increased over the past years and is estimated to keep rising to an annual rate of 15 % (Köhl and Russ, 2016). Scientific advances related to the biology and ecology of biocontrol agents and technological development have contributed to growth in the market. However, solid demonstrations of the efficacy of biocontrol agents and smart marketing campaigns to increase the reliability of this type of products are needed. Laboratory efficacy

[8] Samples were taken from plants grown in soil inoculated with FOX-Map5 at concentration of 1E3 and 1E5 cfu/g and treated with Bs006 [30 mL, $1x10^8$ cfu/mL] (Bs006+FOX1E3 and Bs+FOX1E5 respectively) and from plants treated only with the rhizobacteria (Bs006) the day of transplant. Data correspond to two biological replicates.

tests and field demonstrations are the most persuasive methods to show the effectiveness of biopesticides and are key for their adoption by farmers (Glare *et al.,* 2012).

In this context, preliminary evaluations of *B. amyloliquefaciens* Bs006 showed high variability among experiments (Caviedes, 2010; Guacaneme, 2010; Díaz *et al*., 2012; Zapata and Díaz, 2012; Torres *et al*., 2012). However, there were methodological differences between studies such as the use of autoclaved soil, doses, times and volumes of application of Bs006, the culture media used for growing Bs006 and FOX-Map5, and the period for disease evaluation. Due to this variability, the potential of Bs006 to control FW of cape gooseberry *in vivo* remained unclear. For those reasons and with the aim of generating a clear perspective about the biocontrol activity of *B. amyloliquefaciens* Bs006, in this study the effect of soil sterility, the dose of Bs006, the inoculum concentration of FOX-Map5 and the supernatant of fermented broth by Bs006 on the efficacy of biocontrol against FW was determined. Taking into account that in the absence of pathogenic inoculum Bs006 has shown consistent plant growth promoting activity, we also tested the influence of the presence of FOX in the soil on plant growth promoting activity of Bs006.

An interesting finding was the negative effect of autoclaved soil on the biocontrol activity of *B. amyloliquefaciens* Bs006. Although we have observed changes in some chemical properties in autoclaved soil (electric conductivity (EC) 4.68 dS/m, S 1.25 mg/Kg, Ca 5.98 $cmol_{(+)}$/Kg, Mg 2.97 $cmol_{(+)}$/Kg, and Mn 79.33 mg/Kg), compared to non-autoclaved soil (EC 2.46 dS/m, S 155.57 mg/Kg, Ca 13.81 $cmol_{(+)}$/Kg, Mg 1.23 $cmol_{(+)}$/Kg, and Mn 2.08 mg/Kg), autoclaved soil did not affect the development of the disease in untreated soil with Bs006, suggesting an important role of the native microflora on the Bs006 - FOX-Map5 interaction in the rhizosphere of cape gooseberry. Sterile soil may provide less competition for the growth of *F. oxysporum* in the rhizosphere and increase contact with the roots of the host. It is also possible that the native microflora plays a synergistic role with Bs006, to reduce the infections of FOX-Map5 in the roots. The findings of the present study agree with Abawi and Lorbeer (1972), who show that biotic factors like the presence of the native microflora and inoculum density of the pathogen determined the potential for the disease development. The authors showed that the germination of conidia and growth of *F. oxysporum* f.sp. *cepae* and the incidence of the disease in onion seedlings were higher under sterile soil conditions compared to non-sterile soil. They also found that low concentration of the pathogen (100 propagules/g of soil) in sterile soil caused high level of

the disease (76 %) whereas a concentration of inoculum 1000 times higher was needed to cause the same level of the disease in non-sterile soil. The population of *F. oxysporum* conidia in natural soil can suffer a significant reduction due to lysis, or to a lesser extent, the formation of chlamydospores.

Since 1977 the risk of using disinfected soil for the production of tomato has been known, owing to strong epidemics caused by recontamination and fast colonization of the soil by aerial inoculum of microconidia of *F. oxysporum* f. sp. *lycopersici* (Rowe *et al.*, 1977). Similarly, the present study showed higher level of the disease in sterile soil inoculated with microconidia of FOX-Map5 than in non-sterile soil. Additionally, we show that the competitive advantages provided to *F. oxysporum* in sterile soil conditions also reduce the opportunities of biocontrol by *B. amyloliquefaciens* Bs006.

Other studies have shown a clear relationship between the efficacy of disease control and the dose of the biological control agent applied to the rhizosphere (Smith *et al*., 1997; Larkin and Fravel, 1999; Bressan and Fontes, 2008). This is a particularly important aspect since it is directly related to the economic viability for implementation of a biopesticide. Nonetheless, few works have studied the development of the disease in response to different combinations between densities of the biocontrol agent and the pathogen. As far as we know, this is the first work studying the dose-response relationship in the system *B. amyloliquefaciens* - Fusarium wilt disease. Our results differ to those documented by Bressan and Fontes (2008) which describe a significant reduction of the disease caused by *F. moniliforme* in corn under high and low doses of *Streptomyces* spp. against both high and low concentrations of the pathogen. In the first stage of the present work, it was surprisingly noticed that Bs006 only reduced disease severity in the combination of Bs006 ($1x10^8$ cfu.mL^{-1}) – FOX-Map5 ($1x10^4$ cfu.g^{-1}). Based on this result, we evaluated the effect of lower doses of Bs006 ($1x10^6$ and $1x10^7$ cfu.mL^{-1}) and observed that Bs006 had no significant effects on the disease under high pressure of FOX-Map5 ($1x10^5$ and $1x10^6$ cfu.g^{-1}) but, under regular density of pathogen population in soil ($1x10^2$ to $1x10^4$ cfu.g^{-1}) (González and Barrero, 2011), low doses of Bs006 (<$1x10^8$ cfu.mL^{-1}) showed significant effects of biocontrol (Figure 2-4).

The biocontrol treatment in these experiments consisted of application of fermented broth by *B. amyloliquefaciens* Bs006 diluted in water, adjusting the cells concentration according

to the required dose. It is well known that *B. amyloliquefaciens* is characterized by synthesizing CLPs and polyketides (PKs) which function as antibiotics with antifungal and antibacterial activity respectively (Chen *et al*., 2007). It has already been show that Bs006 produces compounds of the three families of known CLPs, iturins, surfactins and fengycins as well as PKs in liquid media (Moreno *et al*., 2016). Iturins and fengycins have been shown to have direct negative effects on plant pathogens (Cawoy *et al*., 2015; Malfanova *et al*., 2012) whereas surfactins cause an indirect effect through the stimulation of defense responses in the host plant (Ongena *et al*., 2007; Pertot *et al*., 2013; Cawoy *et al*., 2014). There are several possible reasons why higher doses of biocontrol treatment were not found to be associated with increased disease suppression in this study. Firstly, a high dose of biocontrol treatment (diluted Bs006-liquid culture) could be associated with high concentration of CLPs leading to a secondary effect in the plant, thus facilitating the entry of *F. oxysporum*. Alternately, considering the direct effects of the CLPs and PKs against fungi and bacteria (Ongena y Jacques, 2008; Chen *et al*., 2009), its application could have a soil disinfectant effect whose impact increases with higher doses of biocontrol treatment. In this case, we suppose that FOX-Map5 microconidia that was not affected by the treatment, may have had the opportunity to quickly colonize the rhizosphere and penetrate the host due to low competition. Additionally, if the cells of the BCA have to be close to, or in contact with the pathogen to cause damage, 30 mL of biological treatment may not be enough to uniformly cover all the substrate, where FOX-Map5 was. These hypotheses require further testing.

Our results allow us to discard that high concentrations of bacterial cells (without supernatant) favor the development of vascular wilt of cape gooseberry caused by FOX-Map5. We show that high doses of the fermentation broth supernatant applied to the rhizosphere, was the cause of the mentioned phenomenon. Although the reasons behind the negative effect of the supernatant at high concentrations remain unknown, a possible phytotoxic effect can be discarded given that the highest concentration of Bs006 that has been applied previously (30 mL, $1x10^9$ cfu/mL ~ $3x10^{10}$ cfu/plant), resuspended in the fermented broth which was diluted less than three times (taking into account that the final average concentration in LB broth is $1,6x10^9$ cfu.mL^{-1}), did not cause adverse reactions in plants grown in soil free of *F. oxysporum*, but rather, it has plant growth promoting effects. On the other hand, based on the findings of Du *et* al. (2017) who reported the elicitation by *Paenibacillus polymyxa* NSY50 of resistance genes in cucumber root during the early

stages of interaction with *F. oxysporum* f.sp. *cucumerinum* and reduced expression as the disease developed, thus it is possible to think that the relationship between concentration of elicitors such a CLPs in Bs006-supernatant and the expression of the defense-related genes in the host could have an important role in development of FW of cape gooseberry.

Although it is a poorly explored area, it has already been shown that the effects of beneficial microorganisms on plant growth or against plant pathogens is dependent on the dose of the active substance produced by beneficial microorganisms, and additionally, that the most effective dose may be crop specific (Singh *et al*., 2016). This is an area that requires further study given that it represents one of the possible sources of variation in results of biological control. Since there may be an optimal dose of active compounds produced by microbes, there may also be a dose that elicits adverse results for the crop. This phenomenon was described recently for *Trichoderma asperellum* BHUT8, which when was applied in high doses, it caused growth inhibition of some vegetable species (Singh *et al*., 2016). The secondary metabolites produced by biological control agents can promote plant growth or induce resistance in the host at low concentrations but applied at high doses, may inhibit plant growth (Vinale *et al.*, 2008; Cai *et al.*, 2013; Contreras-Cornejo *et al*., 2016). Recently Shi *et al*. (2016) described that the growth inhibition of *Arabidopsis* roots was induced by Trichokonin VI (peptaibol) produced by *Trichoderma longibrachiatum* SMF2.

In the absence of pathogenic inoculum, this study demonstrates the potential of Bs006 as a plant growth promoter. However, few studies have reported the effect of the presence of plant pathogens on the plant growth promoting activity. The fact that Bs006 has been isolated from endophytic compartments within plant tissue, suggests that the microorganism is competent in the rhizosphere. We observed that the presence of FOX-Map5 influenced the biocontrol activity, the plant growth promoting activity of Bs006 as well as the endophytic population of Bs006, which suggests that this pathogen can affect the expression of biocontrol, plant growth promoting and colonization traits in the bacteria.

The severity of the epidemics of vascular wilt of carnation are governed mainly by the interaction between various factors such as the host (type and degree of resistance of the crop), the pathogen (race, pathogenicity, virulence and initial concentration of inoculum) and the environment (intensity of solar radiation, photoperiod, temperature and growth

substrate) (Ben-Yephet and Shtienberg, 1997). In the current work we have addressed aspects related to the pathogen (inoculum concentration) and the environment (sterility of substrate) that affects the development of the disease but at the same time we could observe how these aspects also affected the biocontrol activity of *B. amyloliquefaciens* Bs006. There is a high interest in determining the factors that affect the biocontrol activity of biological control agents, with the aim to design strategies to increase its activity but, works developed in this topic still are scarce.

To date there are no varieties of cape gooseberry that have been shown to be resistant to FOX, although there is a wide number of accessions of plant germplasm that could be used in plant breeding programs (Osorio-Guarín *et al.,* 2016) and several of these show varying degrees of resistance (Rodríguez, 2013). From an integrated disease management point of view it would be interesting to study the development of the disease in response to the interaction of these accessions with Bs006. Furthermore, there is also a large number of pathotypes of FOX associated to cape gooseberry and it would be valuable to study the interaction of Bs006 with these isolations as well. FOX-Map5 is the most virulent isolate from cape gooseberry which may limit efficacy and increase the variability of the biocontrol activity of Bs006. Applying high volumes of fermented broth supernatant at a concentration of 10 %, the antifungal substances produced by Bs006 did not completely inhibit the ability of *F. oxysporum* to cause disease. As such, a higher concentration of the substances may be necessary to cause significant damage to the pathogen. It is also possible that FOX-Map5 presents tolerance to these compounds, or that these substances caused a fungistatic but not fungicidal effect in FOX-Map5.

2.5. Conclusions

This work aimed to elucidate the role of some biotic factors that affect the biocontrol activity of Bs006 against Fusarium wilt of cape gooseberry. We have shown that the sterilization of soil reduces the efficacy of Bs006 and explains, in part, the variability observed in previous experiments. The densities of the antagonist as well as the pathogen also constitute biotic factors that influence the activity of Bs006 against FW. Doses of Bs006 from 10^7 cfu.mL^{-1} is enough to cause an important reduction of the disease, under inoculum pressure up to $1x10^4$ cfu.g^{-1} of soil. This density dependent effect may be influenced by the concentration of secondary metabolites excreted by the bacteria into the growth media or soil and

suggests that further studies on this interaction are needed. The evaluation of Bs006 populations in the rhizosphere showed that under our experimental conditions is competitive in the rhizosphere, and is able to colonize root and stem tissues of cape gooseberry endophytically.

Our results have several practical implications which are important in the design and implementation of control strategies based on *B. amyloliquefaciens* Bs006. For example, in soils with high pressure of pathogenic inoculum of FOX, Bs006 should be applied at a concentration high enough to reduce the primary inoculum of the disease. After this it should be applied in a way such that the root development zone will be properly covered which could be achieved by applying high volumes of bacterial suspension.

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Chapter 3. Direct effects of cyclic lipopeptides on *Fusarium oxysporum* partially explain the efficacy of *Bacillus amyloliquefaciens* Bs006 against Fusarium wilt of Cape gooseberry

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Abstract

The antagonistic potential of *Bacillus amyloliquefaciens* based on the characterization of its secretome *in vitro* and on the detection of the corresponding genes in genome has been documented in several cases. Some studies have determined the role of cyclic lipopeptides (CLPs) during *in vitro* antagonist-pathogen or antagonist-plant interactions but their role during tritrophic antagonist-plant-pathogen interactions remains largely unknown. The strain *B. amyloliquefaciens* Bs006 has shown antagonistic activity against *Fusarium oxysporum* (FOX) and biocontrol activity against Fusarium wilt (FW) of cape gooseberry (*Physalis peruviana*) but, the modes of action have not been determined. For these reasons, the goal of the current study was to characterize the CLPs profile of Bs006 and to determine its direct effects on FOX and against FW of cape gooseberry. Ultra-high-performance liquid chromatography and electrospray ionization mass spectrometry analysis (UPLC-ESI-MS) revealed that Bs006 has the ability to synthesize homologous compounds of iturin, surfactin and fengycin CLPs families. Bs006 retained its ability to produce the three types of CLPs on the FOX-growth inhibition zone in dual confrontation tests. The supernatant from liquid cultures of Bs006 reduced growth and germination of

FOX and caused swelling and lysis of microconidia and germ tubes in a concentration-dependent manner. Pure fengycins showed fungicidal activity on FOX from 11.25 μg.mL^{-1} while iturins showed only fungistatic action from 15 μg.mL^{-1}. FOX was able to grow under supernatant/CLPs influence indicating tolerance by certain proportion of FOX population. Thus, pathogenic ability of FOX microconidia exposed to supernatant from Bs006 culture was evaluated, showing that tolerant microconidia to the CLPs effects kept the pathogenicity on cape gooseberry. In gnotobiotic systems, Bs006 colonized the root surface of cape gooseberry making a biofilm and inhibiting the growth of FOX which correlated with the production of CLPs on the root surface. However, supernatant containing the highest amount of CLPs applied to the rhizosphere unexpectedly favored the progression of FW disease. These results suggest further studies to select the optimal combination between *B. amyloliquefaciens* and CLPs in the next generation of biopesticides. The results also lead to infer that this tool achieves partial control of the disease being necessary the use of other control measures, under integrated programs to control Fusarium wilt.

Key words
PGPR, Biological Control, Antibiosis, *Physalis peruviana*.

3.1. Introduction

Bacillus amyloliquefaciens subsp. *plantarum* is a rhizobacteria recognized for its antagonistic activity against plant pathogens. This antagonism may be caused through the direct action of antibiotics such as non-ribosomally synthesized cyclic lipopeptides (CLPs) and polyketides (PKs) (Cawoy *et al.,* 2014a; Desoignies *et al.,* 2013; Henry *et al.,* 2011; Pertot *et al.,* 2013; Yamamoto *et al*., 2014 Argüelles-Arias, *et al.,* 2009; Chen *et al.,* 2009; Yuan *et al*., 2012; Zhao *et al*., 2014), through induced systemic resistance (ISR) (Kloepper *et al.,* 2004) and via nutrient competition through the production of siderophores (Dunlap *et al.,* 2013; Magno-Pérez *et al.,* 2015).

Compounds of lipopeptide nature such as iturins (itu) and fengycins (fng) produced by *B. amyloliquefaciens* are known for their direct effect on plant pathogens (Cawoy *et al.,* 2014b; Malfanova *et al.,* 2012), whereas systemic resistance can be induced by surfactins (srf)

(Cawoy *et al.,* 2014a; Pertot *et al.,* 2013) and fengycins (Ongena *et al.,* 2005; Ongena *et al.,* 2007). Lipopeptides also fulfil an important role in the root colonization process by *Bacillus* (Bais *et al.,* 2004). CLPs are amphiphilic secondary metabolites that consist of peptide ring with seven (itu y srf) or ten (fng) amino acids linked to a *β*-hydroxy (fng y srf) or *β*-amino (itu) fatty acid. Each family of lipopeptides has been divided in groups based on its amino acid composition and within each family there are homologous molecules which differ in the carbon number [C_{13} - C_{16} (srf), C_{14} - C_{17} (itu) and C_{14} - C_{18} (fng)], and the branching and saturation of the lipid tails (Akpa *et al.,* 2001; Malfanova *et al.,* 2012; Ongena and Jacques, 2008).

Due to their amphiphilic nature, CLPs can easily associate with and firmly anchor to lipid bilayers (Bonmatin *et al.,* 2003; Carrillo *et al.,* 2003). This property allows them to interact with plant cell membranes and induce systemic defense responses in plant host (Jourdan *et al.,* 2009), while interactions with fungal membranes cause cell destabilization (Bonmatin *et al.,* 2003; Zhang *et al.,* 2013; Han *et al.,* 2015). The CLPs synthesized by *Bacillus* spp. can interfere with the integrity of the cell membranes in a dose dependent manner but the susceptibility of the membranes varies in a specific manner, which explains why each family of CLPs affects different targets. Surfactins for example, are known mainly for possessing hemolytic, antiviral, antimycoplasm and antibacterial activity, but strangely do not have a typical fungitoxic activity. Meanwhile, the iturins have strong hemolytic and antifungal activities, restricted antibacterial but no antiviral activity. Finally, the fengycins possess a lower hemolytic activity than iturins and surfactins but have shown a strong antifungal activity, especially against filamentous fungi (reviewed by Ongena *et al.,* 2010).

During the interaction with plant cells, the surfactins produced by *B. amyloliquefaciens*, constitute molecular patterns that are recognized by receptors of plant cell membranes, leading to the activation of the first line of defense in plants – pathogen-triggered immunity (PTI) – which can, in some instances, extend to all the plant organs in the form of ISR (Henry *et al.,* 2011). The initial events associated with the defense response of the plant after the recognition of surfactins, include alkalization of the external environment due to exit of ions (K^+, NO_3^-, Cl^-) from the cytoplasm; production of reactive oxygen species; stimulation of activity of phenylalanine ammonia lyase (PAL) enzyme; accumulation of phenolic compounds; and stimulation of lipoxygenase activity (LOX) (Jourdan *et al.,* 2009). However, high concentrations of surfactins can provoke plant cell death (Jourdan *et al.,*

2009) which may be important during the infection process of necrotrophic pathogens. At the same time, research into the molecular dialogue between *B. amyloliquefaciens* and the plant, has revealed that secretion of surfactins takes place during the first hours of interaction between the bacteria and the root, as a result of the perception of polysaccharides present in the cell wall of roots by the bacteria (Debois *et al.,* 2015). In addition, the accumulation of surfactins produced by *B. amyloliquefaciens* on the root surface is promoted by root exudates (Nihorimbere *et al.,* 2012) which explains why it is the main CLP detected in the antibiome of *B. amyloliquefaciens* on the root surface (Debois *et al.,* 2015).

Among the mechanisms of action of antimicrobial peptides with antifungal activity, is the change of cell membrane permeation through inhibition of sterol synthesis and the destruction of cell walls (Yeamant and Yount, 2003). Iturins, surfactins and fengycins are known for their ability to form pores and permeate cell membrane (Inès and Dhouha, 2015). Particularly, the antifungal activity of iturins is related to its interaction with the cytoplasmic membrane of the target cells. The active sites of iturins interact strongly with sterols of the membrane, forming complex lipopeptide/sterol in the phospholipid membrane (Maget-Dana and Peypoux, 1994). This complex provokes the permeability of K^+ ions, the formation of small vesicles and the aggregation of membrane compounds that can trigger the loss of electrolytes and products of high molecular weight from the cytoplasm, as well as degradation of phospholipids and lead to the breaking of cell membrane (Kumar and Johri, 2012). That ability of iturins to form pores depends on both the lipid composition of the membrane and the structure of peptide bond of lipopeptide (Maget-Dana and Peypoux, 1994). The cell wall lysis provoked by iturins can occur in a concentration dependent manner (Chitarra *et al*., 2003; Kumar *et al*., 2012). Similarly, surfactins at a low concentration are inserted in the external layer of the membrane and cause only limited membrane disturbances. Intermediate concentrations induce transitional permeability of the membrane, but high doses cause irreversible formation of pores and loss of lipid bilayer integrity (Carrillo *et al*., 2003). Fengycins form aggregations of membrane to a degree that depends on the composition and lipid concentration (Patel *et al*., 2011) causing swelling, waving and cell lysis in fungi (Vanitannakom and Loeffler, 1986; Li *et al*., 2007; Romero *et al*., 2007; Torres *et al*., 2016).

The variation in sterol content in the cytoplasmic membrane of fungi can affects the interaction with CLPs. For example, the low Iturin A activity in *Rhizopus* sp. is attributed to the low ergosterol content in the membrane. Whereas its activity over *Penicillium roqueforti* caused cell lysis, membrane permeation and inhibition of spore germination (Chitarra *et al*., 2003). Additionally, it has been shown that cholesterol can counteract the destabilizing effect of surfactins (Carrillo *et al*., 2003) and many sterols, including ergosterol can limit the fungicidal activity of fengycins in a content dependent manner in the membrane (Vanitannakom and Loeffler, 1986; Wise *et al*., 2014).

Root colonization is an important step in the infectious process of soil-borne phytopathogens and for establishing beneficial associations with microorganisms (Bais *et al.,* 2006). The process of colonization is coordinated by the nutrients contained in root exudates, which are attractive to microorganisms (Hartman *et al*., 2008). During the colonization of the root surface, plant growth promoting rhizobacteria (PGPR) can form biofilms (Lugtenberg, 2015) and it has been shown that the participation of lipopeptides is essential in this process (Bais *et al*., 2004). Other studies have shown that available nutrients in root exudates, mainly organic acids, sugars and amino acids (Kamilova *et al*., 2006) are responsible for the growth of *Bacillus* and influence the pattern of lipopeptide production in biologically relevant amounts (Nihorimbere *et al*., 2012; Cawoy *et al*., 2014b; Debois *et al*., 2014). Nonetheless the production of CLPs is very low in the rhizosphere, compared to what is found under laboratory conditions (Nihorimbere *et al*., 2012). Therefore, the knowledge of the ability to produce CLPs *in planta* has a great relevance in the context of biological control, due to each family of CLPs has proved to have biological functions and specific mechanisms of action in plant pathogen control (Raaijmakers *et al*., 2010).

The participation of each family of CLPs in inhibition of plant pathogens of agricultural importance through *in vitro* dual confrontation tests has been studied. Although some studies have begun to unravel how *B. amyloliquefaciens* colonizes plant roots and produce bioactive compounds such as CLPs on the root surface (Nihorimbere *et al.,* 2009; 2012; Debois *et al.,* 2013, 2014, 2015), the analysis have been made during PGPR-plant interaction but not during tripartite PGPR-plant-pathogen interactions. Furthermore, *in vitro* experiments have shown that the profile of CLPs produced by *Bacillus strains* during the interaction with the pathogen can vary according to the pathogen encountered (Cawoy *et*

al., 2014). However, the multitrophic interactions that occur in the rhizosphere among plant pathogens, PGPRs and plants are complex and in general, still poorly understood. As such, it remains necessary to carry out studies to analyze the relationship between CLPs and biocontrol activity, through detection and quantification of these compounds on the root surface and in the presence of pathogens, while also proving the effectiveness of the pure compounds *in planta*.

B. amyloliquefaciens Bs006 (Gámez *et al.,* 2015) has shown high antagonistic activity under *in vitro* conditions against *F. oxysporum* strain Map5 (FOX-Map5) (Caviedes, 2010) and high biocontrol activity against vascular wilt (Fusarium wilt – FW –) of cape gooseberry caused by Map5 (Moreno *et al.,* 2014). FOX-Map5 is one of the most virulent isolates found in cape gooseberry (Rodríguez, 2010). However, the mechanisms behind Bs006 control against FOX have not been determined. As such, the objective of this study was to characterize the CLPs profile of Bs006 and to determine its effects against FOX and its efficacy against FW of cape gooseberry. We determined the ability of Bs006 to produce compounds of the three CLPs families in liquid culture, and in the inhibition zones against FOX-Map5 in dual confrontation test. The effects of pure CLPs (itu, fng and srf) on germination of microconidia and growth of FOX-Map5 were evaluated along with the ability of Bs006 to colonize roots and its antagonistic potential against FOX-Map5 during the interaction with the plant. Finally, the efficacy of pure CLPs against FW of cape gooseberry was measured.

3.2. Materials y methods

3.2.1. Biological material

Cape gooseberry. Commercial cape gooseberry seeds (Colombia ecotype) were used for gnotobiotic system and greenhouse experiments. The seeds were washed to remove fungicides from the surface, and then surface disinfected in NaOCl (3%) during 20 min and subsequently washed three times in sterile distilled water (SDW). Disinfected seeds were germinated in sterile humidity chambers (Petri dishes with wet filter paper) and incubated for 20 days under darkness and 30 °C, to obtain seeds with a 1 cm radicle, approximately. Germinated seeds were planted in plastic trays (72 cells) filled with autoclaved peat (120 °C, 20 PSI, 30 min). They were maintained for 30 - 40 days in a greenhouse, until the first four true leaves were fully expanded. During this time, the seedlings were watered daily and a commercial nutritive solution (Tottal ®) was applied by drench (0.5 – 2 mL/L) once a week.

***Bacillus amyloliquefaciens*.** The strain Bs006 isolated from the rhizosphere of cape gooseberry (Caviedes, 2010), is stored in glycerol (20%) and peptone (1%) solution at –70 °C at the national germplasm collection in Tibaitatá Research Center of Corpoica (Mosquera, Colombia). Inoculum for the experiments was prepared from the second subculture from the glycerol stock, after reactivation in Luria-Bertani agar (LBA) (tryptone 10 g (Oxoid®), yeast extract 5 g (Oxoid®), NaCl 10 g (Merk ®), bacteriological agar (Oxoid®) 18 g / 1000 mL).

***Fusarium oxysporum*.** Strain Map5 was isolated from cape gooseberry plants showing vascular wilt symptoms and is cryopreserved in a glycerol and peptone solution as mentioned above from a monosporic PDA culture (Rodríguez, 2010). Pre-inoculum stock consisting of a microconidia suspension in the above-mentioned cryopreserving solution, conserved at -20 °C, was prepared for this study. PDA (Merk®) and PDB (Difco®) media were inoculated with the stock suspension to produce pathogenic inoculum for *in vitro* tests of antifungal activity and *in vivo* tests of biocontrol activity respectively (see methodology of antifungal activity for more details about pathogenic inoculum production).

3.2.2. Liquid fermentation of Bs006

Cellular suspension of Bs006 in SDW was prepared from a 24 h old culture in LBA and used as the inoculum added to 30 mL of sterile culture media in 125-mL Erlenmeyer flasks. The initial concentration of bacteria in the medium was adjusted to $5x10^6$ cfu.mL^{-1} by measuring the optical density ($OD_{600\,nm}$) using a spectrophotometer (OPTIZEN, MECASYS) and using a standard curve [$OD_{600\,nm}$= 0.5 ~ 2.49 x 10^8 cfu.mL^{-1}]. Growth of Bs006 was carried out in three media, LB which is commonly used for growing *Bacillus* spp.; Landy which was initially used for growing *B. subtilis* for Bacillomycin production (Landy *et al.,* 1948) and lipopeptides such as Mycosubtilin (Leclère *et al*., 2005; 2006) and polyketides such as Bacillaene and Difficidin (Chen *et al.,* 2006); and another media which we named PZN that has been used for production of Plantazolicin (Scholz *et al*., 2011) and Amylociclicin (Scholz *et al*., 2014). The pH of these three media was adjusted to 7 before autoclaving. Inoculated media were incubated during 48 h with continuous shaking (150 rpm) at 30 °C. There were three inoculated Erlenmeyer for each medium and three flasks inoculated with SDW as control. After the incubation period, 10 mL of fermented media were sampled. One mL was used to measure the growth of Bs006 through the optical density ($OD_{600\,nm}$). The remaining 9 mL of the sample were centrifuged (12000 rpm, 10 min), and the recovered supernatant was filtered using 0.22 µm filters (Millipore®) and then used to CLPs extraction process.

3.2.3. Extraction and characterization of CLPs

CLPs extraction. Supernatant from the filtered cultures was subjected to solid phase extraction in C_{18} cartridges (10 g, Alltech™) according to the methodology described by Nihorimbere *et al*. (2012) with modifications. Briefly, C_{18} column was activated with pure methanol (10 mL) then cleaned with 10 mL of HPLC water grade. 5 mL of supernatant sample were injected to fix non-polar compounds inside the cartridge. The column was then washed with HPLC water (5 mL) to remove salts and hydrophilic compounds and 5 mL of HPLC grade acetonitrile (ACN) solution (10 % in HPLC water grade) were injected to remove low affinity compounds. Finally, 2 mL of pure ACN were injected to remove the CLPs and the resulting solution was collected in glass vials (VWR®).

Analysis through UPLC-ESI-MS. Resulting samples were analyzed in a UPLC (Acquity H-class, Waters S.A., Zellik, Belgium) coupled to a single quadrupole mass spectrometer (Waters SQD mass analyzer) on a ACQUITY UPLC® BEH C18 (Waters) 2.1 x 50 mm, 1.7 µm column. Elution of the sample was performed at 40 °C with a constant flow rate of 0.6 mL/min using a gradient of ACN in water, both acidified with 0.1 % formic acid as follow: two minutes at 15 %, from 15 % to 95 % in 5 min and maintained at 95 % for two min. The compounds were detected through electrospray ionization with the source in positive ion mode, by setting SQD parameters as follows: source temperature 130 °C, desolvation temperature 400 °C, and nitrogen flow of 1000 L/h. For optimal detection, different cone voltages were applied to the sampler cone, depending on the purpose of the analysis: 70 V for detection of compounds in the ranges m/z 200-900 (polyketide) and m/z 1200-2040 (large peptide antibiotics) or 120 V for detection of lipopeptides (m/z range of 900-1550) as previously optimized Nihorimbere *et al*. (2012). CLPs were identified by comparison in the retention times and the molecular mass of known patterns (provided by AIBI asbl, Gembloux, Belgium, consisting in a mixture of homologous compounds).

3.2.4. Antifungal activity against FOX-Map5

Dual confrontation test. The ability of *B. amyloliquefaciens* Bs006 to inhibit the growth of FOX-Map5 was measured through dual confrontation tests in sterile plastic Petri dishes (90 mm diameter) on solid LB, Landy and PZN media. 10 µL of fungal suspension ($1x10^7$ microconidia.mL^{-1}) harvested from two-week-old cultures in PDA were inoculated in the center of a Petri dish. The Bs006 inoculum was prepared pouring SDW on a 24 h old culture in LBA and scraping the cells with a loop. The cell suspension was centrifuged (10000 rpm, 10 min) and the pellet with Bs006 cells was washed three times with SDW and resuspended in SDW. The concentration was adjusted to $1x10^8$ cfu.mL^{-1} by measuring the optical density at λ= 600 nm in spectrophotometer. On two opposite sites, two cm apart from the edge of the Petri dish, 10 µL of Bs006 suspension were inoculated. When the inoculum drops were dried, the dishes were incubated at 30 °C and darkness for seven days and the diameter of the FOX-Map5 colony in direction to the bacterial colonies was measured. Petri dishes with only FOX-Map5 or Bs006 were used as controls. There were five replicates for each treatment. The efficacy of *B. amyloliquefaciens* Bs006 to reduce the growth of FOX-Map5 colony was calculated by following the equation: Growth inhibition = [(C – T)/C] * 100.

Where C= diameter growth of fungus in control, T= diameter growth of fungus in dual culture (Singh *et al*., 2008).

3.2.5. Germination and growth of FOX in liquid medium

Effect of supernatant of Bs006. The effect of several concentrations of filtered supernatant of Bs006 (0.5, 1.0, 2.0, 5.0 and 10 %) on germination of microconidia and growth of FOX-Map5 was tested in sterile PDB. Bs006 was grown in LB, Landy and PZN broth using the procedure previously described. The microconidia of FOX-Map5 were produced growing the fungi for 7 days in 50 mL of PDB in a 250-mL conical flask under continuous shaking (125 rpm, 30 °C, 12L:12D photoperiod). To prepare the microconidial suspension the mycelium was discarded by passing the fermented broth through a double layer of sterile muslin cloth and the obtained suspension was centrifuged (4000 rpm / 12 min). The supernatant was discarded and obtained biomass of microconidia was washed two times with SDW. Finally, the microconidia were resuspended in SDW and concentration was calculated by counting in a Neubauer chamber. The experiments were carried out in a set of two sterile flat-bottom 96-well microtiter plates (FALCON®, CORNING) per media, one to measure the growth of fungi by measuring absorbance at 620 nm with a microplate reader (Beckman Coulter AD 340) and the other to measure the percentage of germinated microconidia by counting in an Axioscop 2 mot microscopy (Carl Zeiss®). In detail, 1 mL of PDB supplemented with supernatant or with non-fermented sterile medium as a control (LB, Landy and PZN in each case) was dispensed in Eppendorf tubes. 250 µL of homogenized broth were poured per well, which served as blanks in each case (PDB + Supernatant or PDB + Medium). FOX-Map5 was inoculated to the remaining 750 µL, adjusting the concentration to $1x10^5$ to measure the growth of fungi and to $1x10^7$ microconidia.mL^{-1} to measure the germination, in independent plates. The inoculated broth was homogenized using a vortex and then 250 µL of the suspensions were dispensed to each of three wells for each treatment (concentration). The plates were sealed with their cap and incubated for 48 h in continuous shaking and darkness (125 rpm, 30 °C).

Effect of pure CLPs. The effect of pure surfactins, iturins and fengycins was tested on the germination and growth of FOX-Map5 in PDB, following the protocol described above for evaluating the supernatant. Purified metabolites (provided by AIBI asbl, Gembloux,

Belgium) consisting in a mixture of homologous compounds were reconstituted in HPLC grade methanol (MeOH) to obtain a stock solution (2 ml) at 500 µM. Concentrations of 7.5, 15, 20, 30 and 50 µM were tested for which, 1 mL of solution (sterile PDB supplemented with CLPs at each concentration) was prepared. PDB medium and PDB supplemented with MeOH (same volumes added with treatments) were used as positive and relative controls respectively. PDB supplemented with each compound (MeOH, Srf, Fng, Itu) not inoculated with FOX-Map5 were used as blanks. The fungus was inoculated in each solution after adding the blank wells, adjusting the concentrations to $1x10^5$ and $1x10^7$ microconidia.mL^{-1} (for growth and germination, respectively) and 250 mL were dispensed in triplicate. The incubation conditions and the measurement of variables were carried out as described above.

3.2.6. Gnotobiotic system for measure antagonism against FOX

The antagonism of *B. amyloliquefaciens* Bs006 on *F. oxysporum* Map5 was validated using two germinated cape gooseberry seeds which were put on a gelified nutrient solution (45 mL) described by Nihorimbere *et al*. (2012) in sterile square Petri dishes (Greiner Bio-One, 120 x 120 x 17 mm). Germinated seeds were located 3 cm apart from the edge and separated from one another by 6 cm. Germinated seeds were incubated for 7 days at 30 °C and 12L:12D photoperiod in a vertical position to promote positive geotropism of the root. After this time, 4 µL of bacterial suspension in SDW ($1x10^8$ cfu.mL^{-1}) were inoculated at the crown of one seedling while in the other seedling SDW was inoculated as a control and left to incubate for four days. After this period 5 µL of the FOX-Map5 suspension of ($5x10^5$ microconidia.mL^{-1}) were placed in the space between the two seedlings. Once the drop of inoculum was dried the system was incubated for four days and the distances between the main root of each seedling to the edge of the *Fusarium* colony were measured. Inoculum suspension of Bs006 was prepared in SDW from a 24 h old culture in LBA, and the cells were washed three times with SDW. Meanwhile, inoculum suspension of FOX-Map5 was prepared from a 7 days old culture in PDB, and it was also washed three times with SDW. Data were used for calculating the efficacy in reducing the growth of the FOX-Map5 colony towards the root of the plant treated with Bs006 by following the equation: Inhibition (%)= (1- (NT/T))*100 where, NT: distance between the fungus and the root of the

control plant and T: distance between the fungus and the root of the plant treated with Bs006.

3.2.7. Quantification of CLPs in antifungal activity tests

Quantification of CLPs in inhibition zones. One agar plug was sampled from each inhibition zone between FOX-Map5 and Bs006 with a cork borer (5 mm diameter) (two plugs per plate) near to the edge of the fungal colony. Three Petri dishes from treatment and controls were sampled. Agar plugs from a plate were placed in 500 µL of HPLC grade ACN 50 % and formic acid 0.1% solution in HPLC grade water in Eppendorf tubes (2 mL). Samples were stirred two minutes and then incubated overnight in cold room (4 °C). Subsequently each sample was vortexed for two minutes, centrifuged (10000 rpm, 10 min) and the supernatant was filtered (Millipore®, 0.22 µm). The resulting filtrates were directly injected in UPLC analyzer, it means no previous solid phase extraction of CLPs was made. Moreover, the protocol of chromatographic analysis, extraction and mass detection was modified from that described above using retention time of 5 min.

Quantification of CLPs in the gnotobiotic system. Samples from root and agar near to the edge of the FOX-Map5 colony were taken to determine the presence of CLPs, and to identify and quantify the compounds produced during antagonist-plant-pathogen interactions. In the first case, 2 cm of the elongation zone of the main root were taken, whereas in the case of the agar samples, two cylinders of agar were taken with a cork borer (0.5 cm diameter) near the edge of the FOX-Map5 colony. Samples were submerged in an ACN (50%) and FA (0.1%) solution in HPLC grade water. It was homogenized vigorously using a Vortex for two minutes and kept overnight at 4 °C. After this time, the sample was stirred and centrifuged and the obtained supernatant was filtered (0.22 µm). The resulting solution was analyzed directly by UPLC-ESI-MS.

3.2.8. *In vivo experiments*

Experimental conditions and inoculation of microorganisms. Experiments under greenhouse were carried out with the aim to compare the efficacy of Bs006 cells, filtered supernatant and pure CLPs in the reduction of FW of cape gooseberry in pot conditions

(plastic trays of 750 cm^3 cones) usually used for rooting trees. Plants were grown in a substrate consisting of a mix of soil (Andisoil, pH 5.7, OM 12.90, P 11 $mg.Kg^{-1}$, K 0.69 $cmol(+).Kg^{-1}$) and rice husk (3:1 ratio). This substrate (hereafter referred to as soil) was moistened with tap water up to 60 % of the maximum moisture holding capacity before the pots were filled. It was then inoculated with a suspension of FOX-Map5 ($1x10^6$ $microconidia.mL^{-1}$) so that the final concentration of pathogen in the soil was $1x10^4$ $cfu.g^{-1}$ of soil. Cones were filled with 700 g of soil/cone and 60 days old seedlings of cape gooseberry were transplanted (one seedling per cone). Biological treatments and controls were applied by drench immediately after transplant. The plants were watered by hand once a day and fertilized once a week using a commercial nutrient solution (Tottal® 2 to 4 mL/L, 30 mL/plant). Nutritional regime started 2 weeks post-inoculations. Temperature and relative humidity (HR) in the greenhouse were not controlled. Mean air temperature in the greenhouse was 25 °C, minimum and maximum temperature were 12 °C and 42 °C respectively and the mean RH was 60 % with minimum and maximum of 45 % and 75 %. Rhizosphere temperature ranged from 15 to 33 °C under this environment conditions.

Effect of Bs006 and supernatant on FW development. Growth of Bs006 was carried out in liquid LB, Landy and PZN media (150 rpm, 30 °C, 48 h) and the biocontrol activity of Bs006 cells, supernatant free of bacteria and the mix of cells and supernatant against FW of cape gooseberry was evaluated. The bacteria were applied as a washed cell suspension ($1x10^8$ $cfu.mL^{-1}$); the supernatant free of bacteria was applied at 10 % in tap water; and for the mixture the concentration of Bs006 cells was adjusted in 10 % of supernatant solution. Treatments were applied drenching 30 mL/plant. As controls we used plants growing in soil inoculated with FOX-Map5 and treated with non-fermented sterile media (relative control), plants without treatment against FW (negative control), plants growing in soil free of pathogen, and no treatment application (absolute control).

Effect of pure CLPs on FW development. The effect of surfactin, iturin and fengycin compounds, and their mixture, were evaluated on disease development. The compounds were provided as dry powder by AIBI, asbl Inc. (Belgium). The concentrations of CLPs used corresponded to those found in the filtered supernatant from the PZN medium in which Bs006 produced the highest concentration of CLPs (0,3 mM (srf), 2,5 mM (itu) and 8 mM (fng)). Each compound was diluted initially in 2mL MeOH and subsequently in tap water for a total volume of 1500 mL. 30 mL of each solution were applied by drench to each plant

immediately after transplant. Plants with no treatments against FW (negative control) and plants growing in soil free of pathogen, and no treatment application (absolute control) were included.

Pathogenicity test of microconidia of FOX-Map5 exposed to CLPs. From the fermented FOX-Map5 broth the required volume of microconidia suspension to prepare the inoculum that would be added to the soil to get $1x10^4$ cfu.g^{-1} was taken. This suspension was centrifuged (10000 rpm, 15 min) and the biomass obtained was washed two times with SDW. The obtained pellet was resuspended during 12 h in filtered supernatant solution from PZN broth fermented by Bs006 (150 rpm, 30 °C, 48 h). After that time the suspension was centrifuged and obtained biomass was washed two times with SDW. The resulting pellet was suspended in the necessary volume of tap water to inoculate the soil as mentioned above. Trays were filled with the inoculated soil and 40-day old cape gooseberry seedlings were transplanted. The effect of exposure of FOX-Map5 microconidia to several concentrations of filtered supernatant (2 %, 5 %, 10 % and 20 %) on pathogenic ability in cape gooseberry was tested. The controls of the experiment were microconidia exposed to sterile non-fermented PZN broth (relative control), washed microconidia, not exposed to supernatant nor the culture medium (negative control) and soil free of pathogenic inoculum (absolute control).

3.2.9. Experimental design and data analysis

Experimental design of *in vitro* tests. The experiments *in vitro* had a completely randomized experimental design. The experiments to measure the effect of the supernatant and pure CLPs on germination and growth of *Fusarium* had a factorial structure of treatments. In the first case, the factors were the medium used for fermentation of Bs006 (LB, Landy and PZN), the type of supplement in PDB (supernatant and sterile medium) and the concentrations of the supplement (0.5, 1.0, 2.0, 5.0 and 10 %). In the second case, the factors were the type of supplement in the broth (Solution of CLPs and MeOH) and the concentrations of the supplement (7.5, 15, 20, 30 and 50 µM). The experimental unit in both cases was a well with 250 µL of medium inoculated with the pathogen and there were three replicates per treatment. In the gnotobiotic system the experimental unit consisted of a Petri

dish and there were 10 replicates. Six Petri dishes were sampled for CLPs analysis in this system.

Experimental design in the greenhouse tests. The three experiments had a randomized complete block experimental design with four replicates per treatment and 10 plants per experimental unit. However, the experiment in which the biocontrol activity of Bs006 cells and supernatant were compared had a factorial structure (2^3 + 2). The factors were the culture media with three levels (LB, Landy and PZN) and the biological treatment with three levels also (cells, supernatant and cells + supernatant). The experiment in which the pathogenicity of microconidia exposed to supernatant was evaluated, also had a factorial structure (2^4 + 2). Here the factors were the exposure medium with two levels (supernatant and sterile medium) and the concentrations (2 %, 5 %, 10 % and 20 %).

The Incidence and severity of FW of cape gooseberry was recorded once a week during 6 to 7 weeks after inoculations. The incidence expresses the proportion of cape gooseberry plants with typical symptoms of FW, whereas the severity of the disease expresses the intensity of symptoms in affected plants. The severity was graded based on a scale 0 - 5 degrees, modified from Sánchez and Forero (2009) where: 0 = no evident symptoms of the disease, expanded leaves, turgid, without foliar bending, green leaves; 1 = bending of young leaves, slight epinasty, mild chlorosis of mature leaves ; 2 = foliar bending, epinasty in 30-50% of the leaves, moderate chlorosis in the oldest leaves and slight chlorosis in leaves from the middle of the plant, clear stunting of the plants; 3 = 60-80% of leaves show epinasty, clear loss of turgidity, moderate chlorosis in young leaves, abscission of oldest-chlorotic leaves; 4 = all the leaves present epinasty, severe chlorosis, moderate defoliation, the plant is clearly in wilting state; 5 = twisted leaves, severe wilting, severe defoliation, bended stem, dead plant. Incidence and severity were calculated by follow the formulas described by Zhang *et al*. (1996). Incidence= $[(n_1 + n_2 + n_3 + n_4 + n_5)/n] \times 100$ and Severity= $[(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5)/5n] \times 100$. Where 0 – 5: Degrees of disease; $n_0 – n_5$: Number of plants in each degree of the disease; n: Total number of plants in experimental unit. The area under the progress curve of incidence and severity were calculated using the formula described by Campbell and Madden (1990) and the efficacy of treatments in reduction of development of incidence and severity of the disease was estimated with the formula Efficacy= [(A-B)/A]*100. Where A: Value area under the disease curve of the negative control; B: Value area under the disease curve of a given treatment.

Data analysis. Data were submitted to Shapiro Wilk normality test (*P*> 0.05) and Bartlett variance homogeneity test (*P*> 0.05). Significant effects of the treatments were determined by ANOVA using the GLM procedure of SAS. Duncan's multiple range and Tukey tests (α= 0.05) were used for means comparisons between treatments *in vivo* and *in vitro* experiments respectively. All analyses was conducted using the Proc GLM of Statistical Analysis Software System (9.4 version; SAS institute, Cary, NC).

3.3. Results

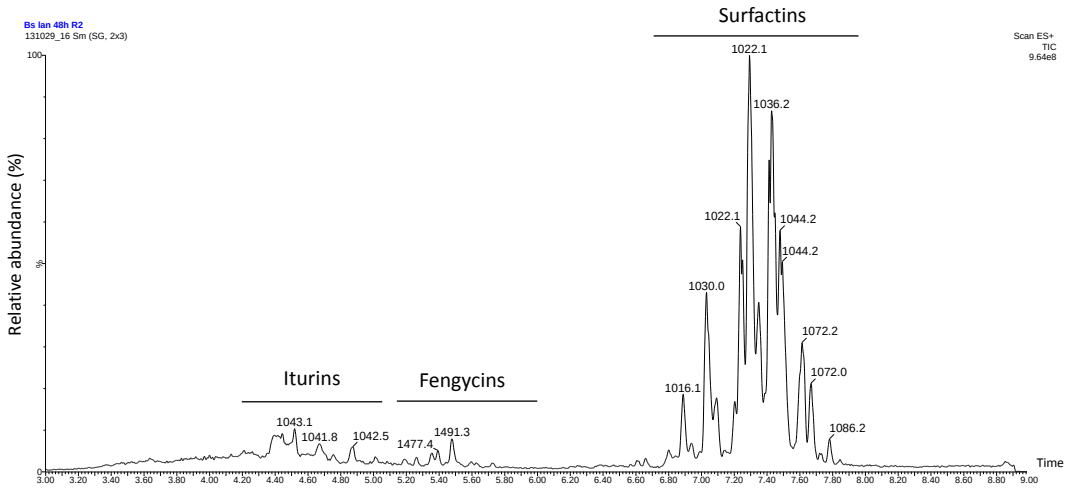
3.3.1. CLPs produced by *B. amyloliquefaciens* Bs006 in liquid media

The UPLC-ESI-MS analysis of the supernatant free of bacterial cells in the Landy medium revealed that Bs006 has the ability to synthesize several homologous compounds to all three CLPs families. Mass spectrometry (MS) analysis of the obtained peaks showed compounds of the families of CLPs, iturins (retention time from 4,375 to 4,880 min), fengycins (5,242 to 5,760 min) and surfactins (6,826 to 7,842 min) (Figure 3-1). Five ions were detected in the first group, with mass to charge (m/z) value of 1042, 1057, 1065, 1080 and 1093 Da. Based on literature information and pure compounds analysis the 1042 $[M+H]^+$ and 1057 $[M+H]^+$ m/z peak value corresponds to a homologous compound of iturin A, with a lipidic tail of 14 and 15 carbons (C14 and C15 respectively). Ions of m/z 1065 and 1080 are their sodium adducts (Na^+) respectively (Argüelles-Arias *et al*., 2009). Meanwhile the compound with m/z 1093 represents the adduct $[M+Na]^+$ of the analogue compound iturin A C16 with mass of 1071 $[M+H]^+$ which was also detected by the MS analysis (Figure 3-2A).

In the fengycins group, several molecules were detected, which differed from each other mainly by residues of CH_2, reflecting the difference in lipidic tail length. Compounds with masses (m/z) of 1449.2, 1463.3 and 1477.4 Da were assigned to homologous compounds of fengycin A (C15, C16 and C17 respectively); the molecule with mass of 1491 corresponds to C16-fengycin B ; the molecule with mass 1499.7 is $[M+Na]^+$ adduct of C17-fengycin A; the compound with mass 1505.5 corresponds to C17-fengycin B; the molecules

with m/z value 1513.2 and 1529 Da are adducts $[M+Na]^+$ and $[M+K]^+$ respectively of C16-fengycin B (Figure 3-2B).

Figure 3-1: Chromatographic profile of the supernatant free of *B. amyloliquefaciens* Bs006



Note[9]

In the third group of compounds detected in the mass spectrometry analysis, C12, C13, C14 and C15 homologues of surfactins with m/z $[M+H]^+$ 994.1, 1008.1, 1022.1 and 1036.2 Da, respectively, were found (Leclère *et al*., 2005; Monaci *et al*., 2016). The peaks with m/z value of 1016.1, 1030.1, 1044.1 and 1058.1 are sodium adducts $[M+Na]^+$ of these homologous compounds respectively. Peaks with m/z masses of 1072 to 1074 are potassium adducts $[M+K]^+$ of C15-surfactin (Figure 3-2C).

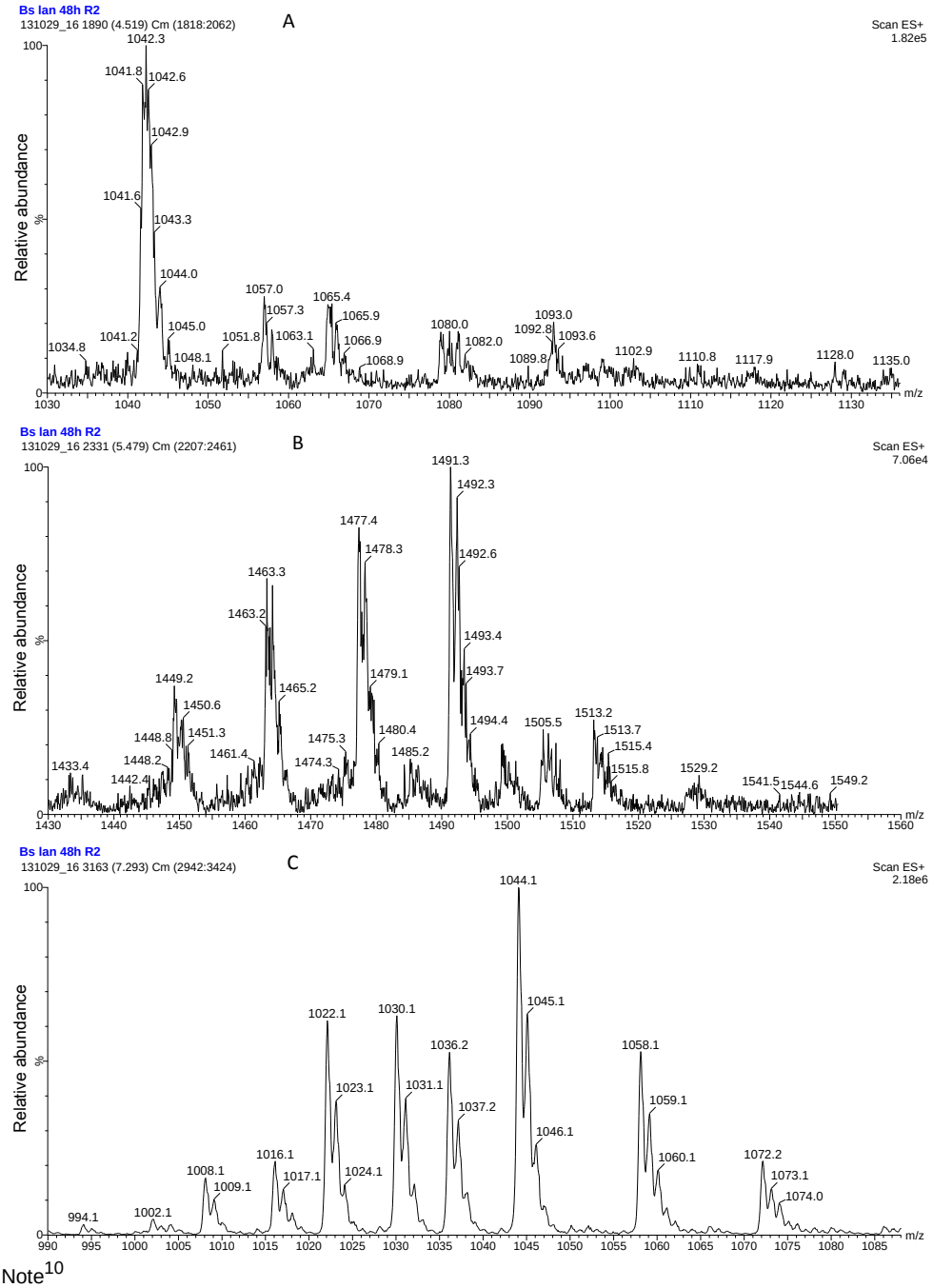
UPLC-ESI-MS analysis of the supernatant from PZN broth fermented by Bs006 showed differences in the variety and abundance of synthesized compounds, particularly in the iturins group compared to that found in Landy medium. In this regard, the two main peaks of m/z 1031 and 1045 $[M+H]^+$ were detected in the iturins group produced in PZN medium

[9] Sample was taken from a 48 h old culture of Bs006 in Landy medium incubated under 30 °C and 150 rpm conditions. Pre-purification of CLPs through solid extraction in C_{18}-carttridge was made to filtered supernatant before UPLC-ESI-MS analysis. Numerical values of peaks correspond to mass to charge (m/z) of detected ions.

which correspond to analogous compounds of bacillomycin D with lipidic tails of C14 and C15. Other ions found in lower abundance showed m/z value of 1057 $[M+H]^+$, 1079 $[M+Na]^+$ and 1097 $[M+K]^+$ which correspond to analogous of C15-mycosubtilin (Appendix 1-A). Unlike in Landy medium in which iturin A was the dominant compound, in PZN medium no clear peaks of iturin A were found and bacillomycin D was the dominant compound. The profile of detected ions of fengycins and surfactins groups in PZN were the same as those found in Landy (Appendix 1-B and 1-C respectively). However, differences in the relative abundance of the compounds were found. In addition to CLPs, polyketides (PKs) such as macrolactin and dificidin compounds were detected in the supernatant from Bs006 culture. However we focused on CLPs analysis in this work.

The concentration of each CLPs family present in the supernatant of the liquid media fermented by Bs006 was calculated based on the calibration curves of iturins, fengycins and surfactins available in the laboratory. In this way, it was found that the proportion among CLPs families varied among the culture media. In PZN medium, the highest concentration of iturins (51 µL/mL) was found, followed by surfactins (35 µL/mL) and fengycins (29 µL/mL). Bs006 produced lower concentration of CLPs in Landy medium compared with PZN, where the concentration of surfactins was the highest (25 µL/mL), followed by iturins (11 µL/mL) and fengycins (8 µL/mL).

Figure 3-2: Mass spectral of iturins (A), fengycins (B) and surfactins (C) produced by *B. amyloliquefaciens* Bs006 in Landy broth



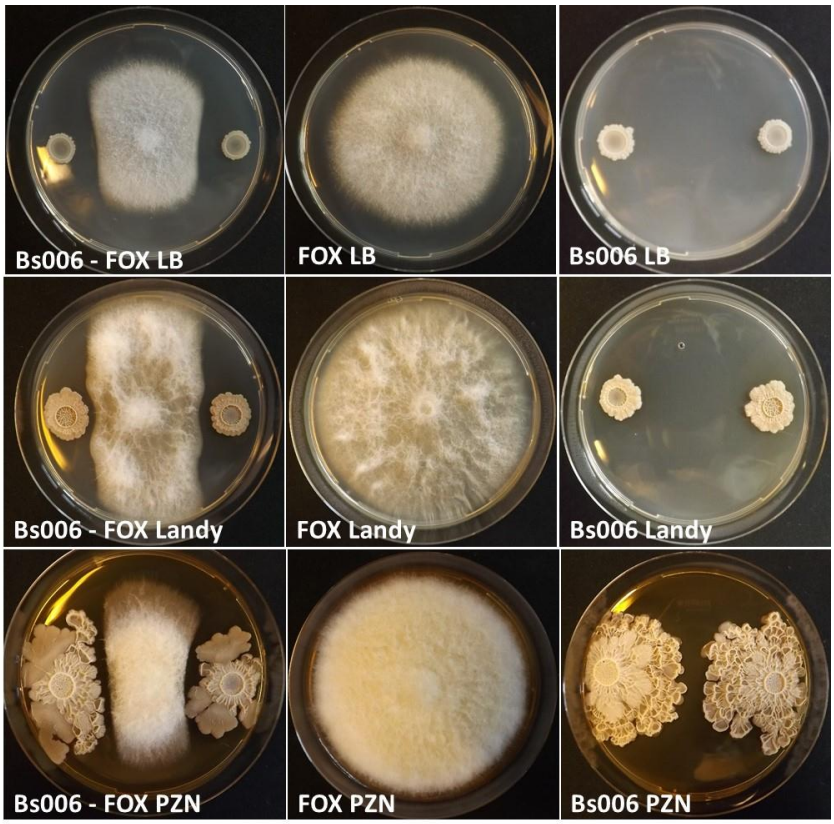
Note[10]

[10] Data are from a sample of supernatant submitted to pre-purification by solid extraction in C18-cartridge then analyzed by UPLC-ESI-MS.

3.3.2. *In vitro* antifungal activity of Bs006 against FOX-Map5

Dual confrontation test. The *in vitro* antagonism test showed that Bs006 significantly reduced the growth of FOX-Map5, which was evident in the reduction of the diameter of the fungal colony and the inhibition zone between the bacterium and the fungus (Figure 3-3). However, the medium significantly ($P < 0.05$) affected the efficiency of Bs006 to reduce the growth of FOX-Map5 (PZN (68%), Landy (55%) and LB (47%)) (Figure 3-4). Bs006 showed a slight reduction in its own development in PZN medium in presence of FOX-Map5 as compared to Bs006 growing alone (Figure 3-3).

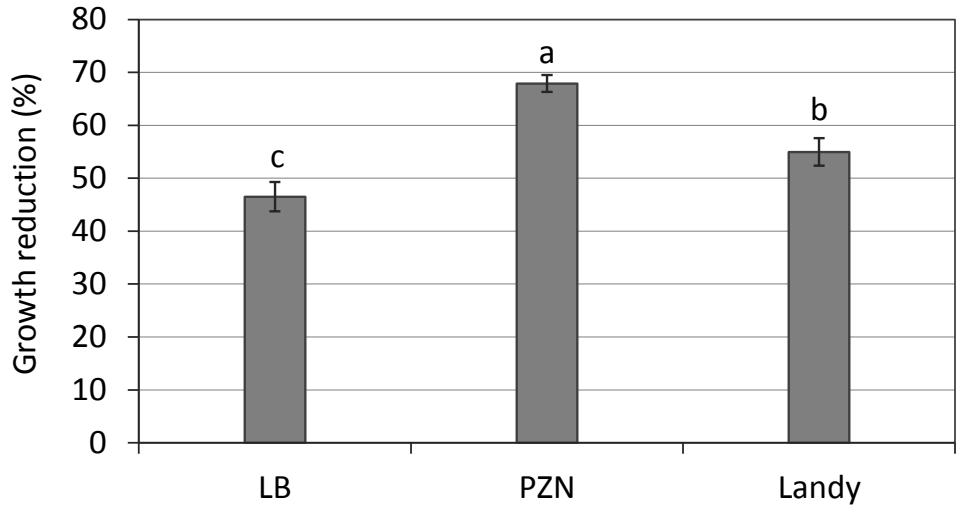
Figure 3-3: Antagonistic activity of *B. amyloliquefaciens* Bs006 against *F. oxysporum* MAP5 (FOX) in dual confrontation test on LB, PZN and Landy media



Note[11]

[11] Pictures were taken at 7 day post-inoculations. The experiment was incubated at 30 °C and darkness.

Figure 3-4: Efficacy of *B. amyloliquefaciens* Bs006 to inhibit the growth of FOX-Map5 in dual confrontation test



Note[12]

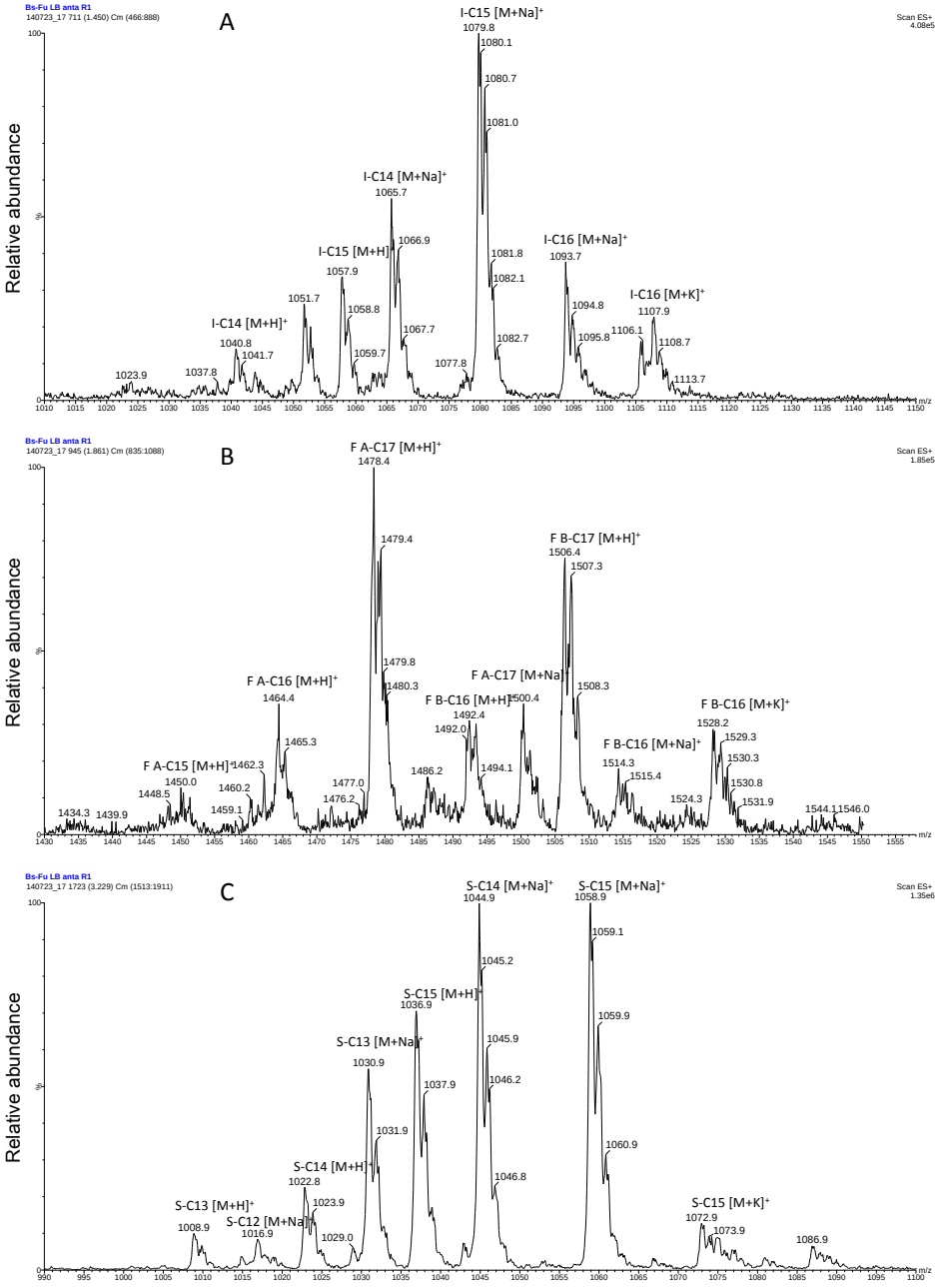
CLPs profile in inhibition zones. Bs006 synthesized various homologous compounds of each CLP family in inhibition zones during interactions with FOX-Map5 in the dual confrontation test, showing similar profile in the three media used (Appendix 2). Detected ions in inhibition zones from LB medium suggest that iturins produced by Bs006 in these conditions correspond to iturin A group (C14-itu A 1043 $[M+H]^+$ and 1066 $[M+Na]^+$, C15-itu A 1057 $[M+H]^+$ and 1080 $[M+Na]^+$; C16-itu A 1093 $[M+Na]^+$ and 1108 $[M+K]^+$ (Figure 3-5A). Homologous compounds of fengycin A (C15-fng A 1450 $[M+H]^+$, C16-fng A 1464 $[M+H]^+$, C17-fng A 1478 $[M+H]^+$ and 1500 $[M+Na]^+$) and fengycin B (C16-fng B 1492 $[M+H]^+$, 1514 $[M+Na]^+$ y 1528 $[M+K]^+$, C17-fng B 1506 $[M+H]^+$) were found (Figure 3-5B). In relation to surfactins, homologous compounds with lipidic tail from twelve to fifteen carbons were found (C12-srf 1016 $[M+H]^+$, C13-srf 1008 $[M+H]^+$, 1030 $[M+Na]^+$, C14-srf 1022 $[M+H]^+$, 1044 $[M+Na]^+$, C15-srf 1036 $[M+H]^+$, 1058 $[M+Na]^+$, 1072 $[M+K]^+$) (Figure 3-5C). The profiles of CLPs molecules found in Landy (Appendix 3 D, E, F) and PZN (Appendix 3 G, H, I) were similar than profile found in LB medium but there were differences in relative

[12] Dual confrontation test was performed in LB, PZN and Landy media. Columns with the same letter are not significantly different according to LSD test (α= 0.05). Bars on the columns represent standard deviation of data (n= 5).

abundance of the compounds which explain differences in CLPs concentration between the media.

The production of iturins was higher compared to the production of fengycins and surfactins in inhibition zones in the three media (Figure 3-6). In relation with total amount of CLPs produced in inhibition zones, the proportion of iturins was 71% on LB and 76% on PZN, and 45% in Landy. The proportion of fengycins was 17% in LB, 15% in PZN and 43% in Landy. Lastly the proportion of surfactins was the lowest in the three media, 12% in LB, 9% in PZN and 13% in Landy. The concentration of CLPs detected in the inhibition zones was similar when Bs006 grew in Petri dishes inoculated with FOX-Map5 compared to dishes free of the fungus, except on PZN medium in which iturins production was significantly lower in presence of *Fusarium* (Figure 3-6).

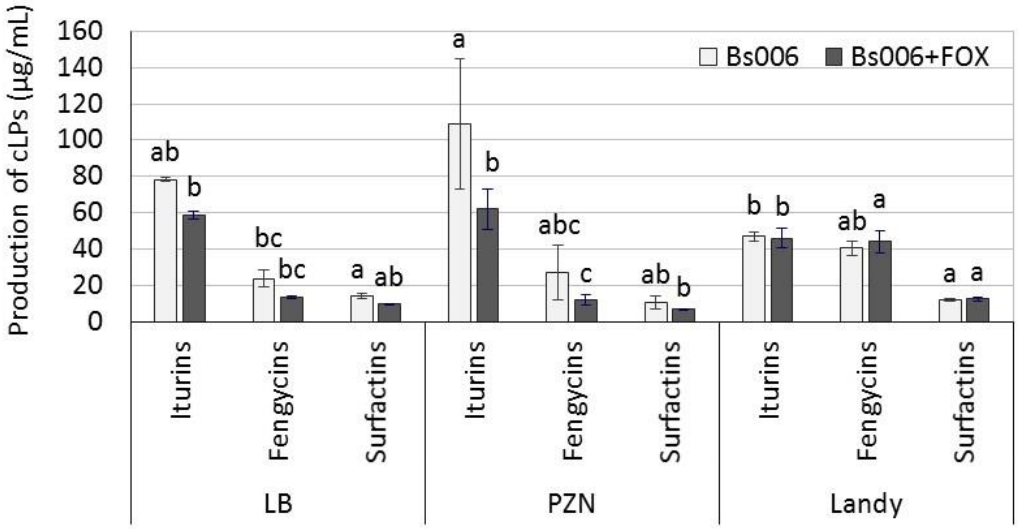
Figure 3-5: Mass spectral of CLPs produced by Bs006 in inhibition zones



Note[13]

[13] Iturins (A), fengycins (B) and surfactins (C) detected in inhibition zones of growth against FOX-MAP5 during confrontation dual test with Bs006 on gelified LB. Analysis was performed by UPLC-ESI-MS. Homologue compounds of each CLP family are indicated according to lipid tail length from 12 to 17 carbons as I C14-17 (iturins); F C15-17 (fengycins); S C12-16 (surfactins).

Figure 3-6: Concentration of CLPs produced by Bs006 in the inhibition zones



Note[14]

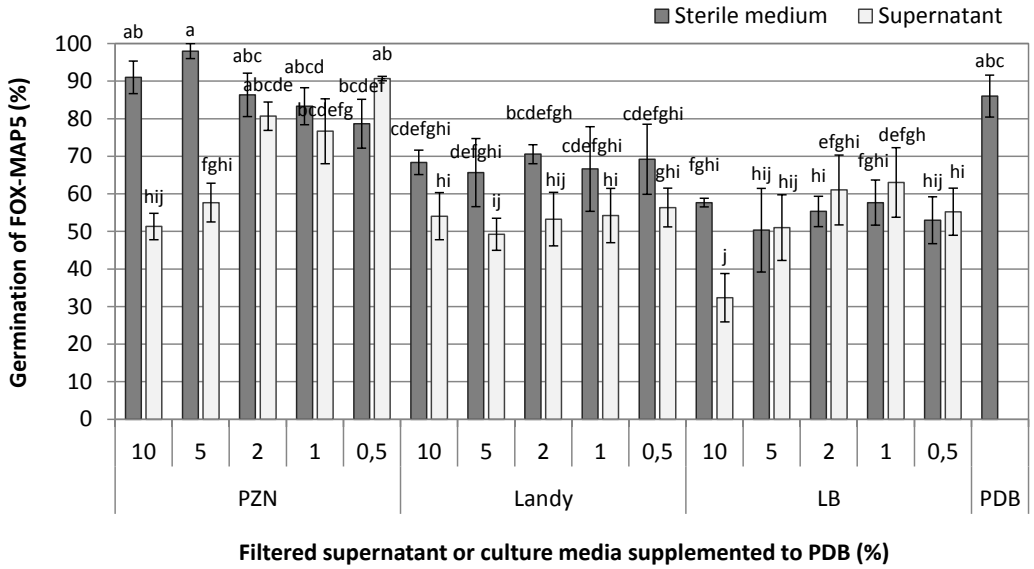
3.3.3. Germination and growth of FOX-Map5 under the effect of CLPs

Effect of supernatant from Bs006 liquid cultures. Germination of FOX-Map5 microconidia was reduced by filtered supernatant of the Bs006 liquid cultures in LB and PZN media in a concentration-dependent manner (Figure 3-7). Supplementing the PDB broth with 10 % of the supernatant from cultures grown in LB significantly reduced the germination of FOX-Map5 microconidia ($P < 0.05$) compared to the germination in its respective control (LB broth at 10 %). The same effect was observed with the supplement of 10 and 5 % of the supernatant from PZN to the PDB medium. Supplementing PDB medium with the supernatant of PZN and LB media at lower concentrations, or the addition of the supernatant from Landy medium at any of the concentrations tested did not show

[14] Pattern of total CLPs production by *B. amyloliquefaciens* Bs006 on inhibition zones of growth against FOX-Map5 during confrontation dual test with Bs006 on gelified LB, PZN and Landy media. Samples were taken from plates inoculated with bacteria (Bs006) or with bacteria and fungi (Bs006+FOX). Samples were taken at 7 days after incubation under 30 °C and darkness. Columns in each CLP family with the same letter are not significantly different according to Tukey test (α= 0.05). Bars on the columns represent the standard deviation of data (n= 3).

significant reductions on the germination of FOX-Map5, compared to its respective control (Figure 3-7). The dilution of the PDB with sterile Landy and LB broths also significantly reduced the germination of FOX-Map5 ($P < 0.05$), compared to the non-supplemented control.

Figure 3-7: Effect of filtered supernatant from liquid culture of Bs006 on germination of FOX-Map5 microconidia



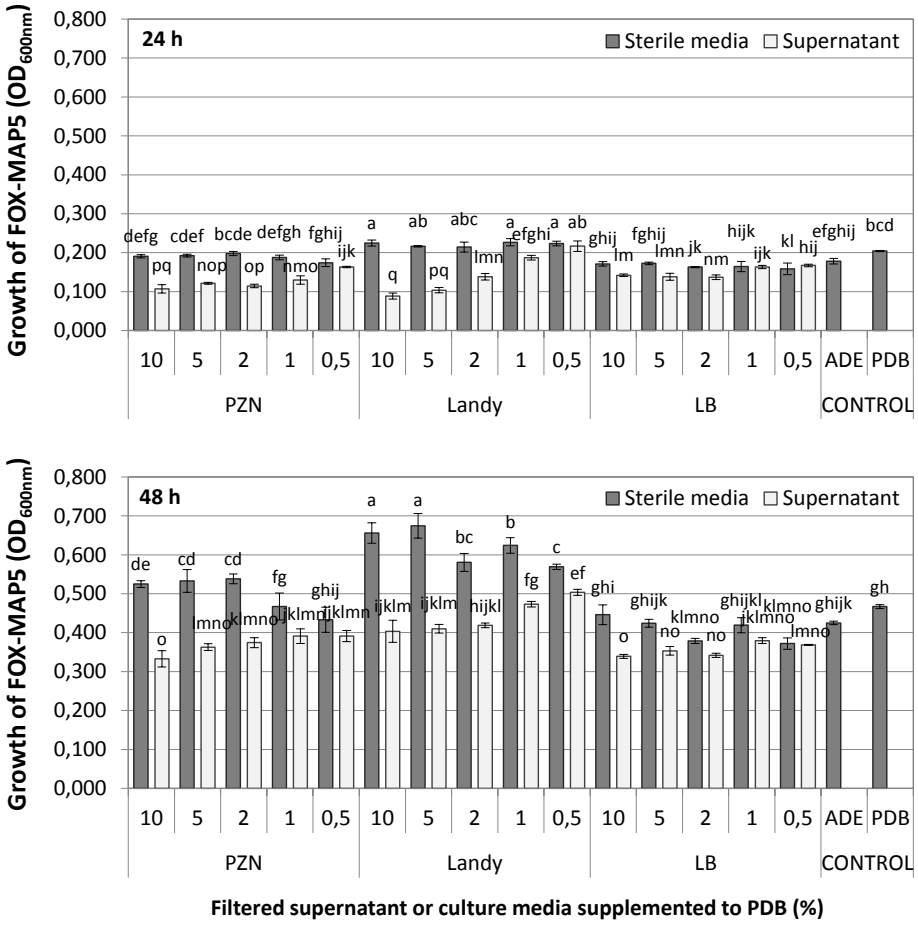
Note[15]

Growth of FOX-Map5 measured by optical density at 24 and 48 h after contact with the supernatant of media fermented by Bs006 was also reduced in a concentration-dependent manner (Figure 3-8). In general, the effects of the supplement to the PDB medium (sterile medium or supernatant), the evaluated concentration and the interaction of these two factors (supernatant and concentration) were highly significant ($P < 0.0001$) in both measurement times. At 24 h the supernatant from PZN medium significantly reduced the growth of the fungus from 1 % concentration while the supernatant from Landy and LB media did from 2 % in comparison to its respective controls (PDB supplemented with each

[15] Supernatants from cultures in PZN, Landy and LB media were used as supplement to PDB medium at 0.5, 1, 2, 5 and 10 %. Germination was measured 24 h after incubation. Columns sharing the same letter are not significantly different according to Tukey test (α= 0.05). Bars on the columns represent the standard deviation of data (n= 3).

sterile media, PDB supplemented with SDW 10 % and PDB without supplement) (Figure 3-8).

Figure 3-8: Effect of filtered supernatant from liquid culture of Bs006 on growth of FOX-Map5



Note[16]

[16] Supernatants from Bs006 cultures media were used as supplement to PDB medium at 0.5, 1, 2, 5 y 10 %. Sterile-distilled water (10%) and PDB medium without supplement were used as controls. Growth of FOX was measured as optical density in microplate reader at 24 and 48 h after incubation. Columns sharing the same letter are not significantly different according to Tukey test (α= 0.05). Bars on the columns represent the standard deviation of data (n= 3).

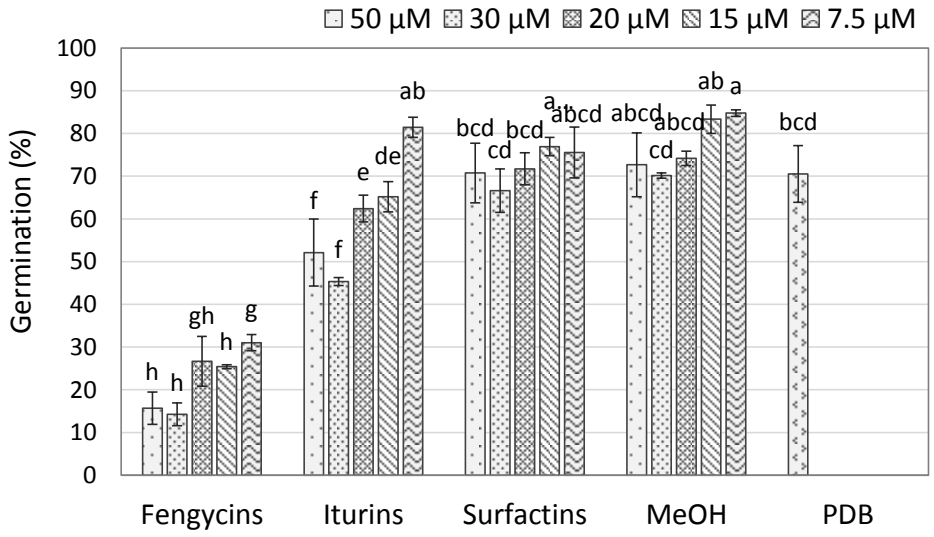
After 24 h the fungus continued growing in all treatments, since at 48 h the optical density value was higher than the value recorded at 24 h. At 48 h some of the concentrations that had shown a significant effect no longer did. For example, the supernatant from PZN and LB significantly reduced the growth of FOX-Map5 only at 5 and 10 % concentrations while supernatant from Landy medium did not reduce the growth of the fungus (Figure 3-8-48 h). It was observed that PDB supplemented with Landy medium stimulated the growth of FOX-Map5 after 48 h (Figure 3-8).

Effect of pure CLPs. The evaluation of the effect of lipopeptides on FOX-Map5 microconidia germination showed that only iturins and fengycins reduced germination and did so in a concentration-dependent manner. The presence of iturins in PDB medium significantly reduced the FOX-Map5 germination from 20 µM ($P < 0.05$), whereas in the case of fengycins, all the concentrations tested significantly ($P < 0.05$) reduced the germination of the fungus (Figure 3-9). Efficacy of fengycins at 50 µM for reduce FOX-Map5 germination was 78 % while 50 µM iturins showed an efficacy of 28 %.

After 24 h of incubation of FOX-Map5 in the presence of the homologous compounds from each cyclic lipopeptides family, iturins and fengycins reduced the growth of the fungus in a concentration-dependent manner, while surfactins did not affect the growth of FOX-Map5. The presence of iturins at concentrations of 30 and 50 µM and fengycins at 50 µM in PDB medium significantly reduced the growth of FOX-Map5 ($P < 0.05$) compared to the relative control (PDB supplemented with methanol) and absolute control (PDB without supplement) (Figure 3-10). Although surfactins at concentrations of 30 and 50 µM reduced fungal growth compared to the absolute control, this effect was not significantly different ($P > 0.05$) from the effect of methanol (Figure 3-10), so the effect cannot be attributed to the presence of surfactins in the PDB medium. However, surfactins at 50 µM caused a significant reduction of FOX-Map5 growth ($P < 0.05$) after 48 h of incubation, compared to the controls (Figure 3-10-48 h). Meanwhile, the negative effect of iturins was maintained only at the concentration of 50 µM, whereas the tendency in the case of fengycins was that all evaluated concentrations, caused a significant reduction of fungus growth (Figure 3-10). In terms of efficacy, the highest tested concentration of iturins, fengycins and surfactins reduced growth of FOX-Map5 in 63, 46 and 29 % respectively, after 48 h of treatment.

Correlation analysis between measured variables of FOX development (germination and growth) and CLPs concentration established consistent effects of fengycins and iturins on germination. However, a stronger correlation was found for all CLPs and growth of FOX measured by optical density. This analysis showed an inverse relationship between the concentration of CLPs and FOX development (Appendix 4).

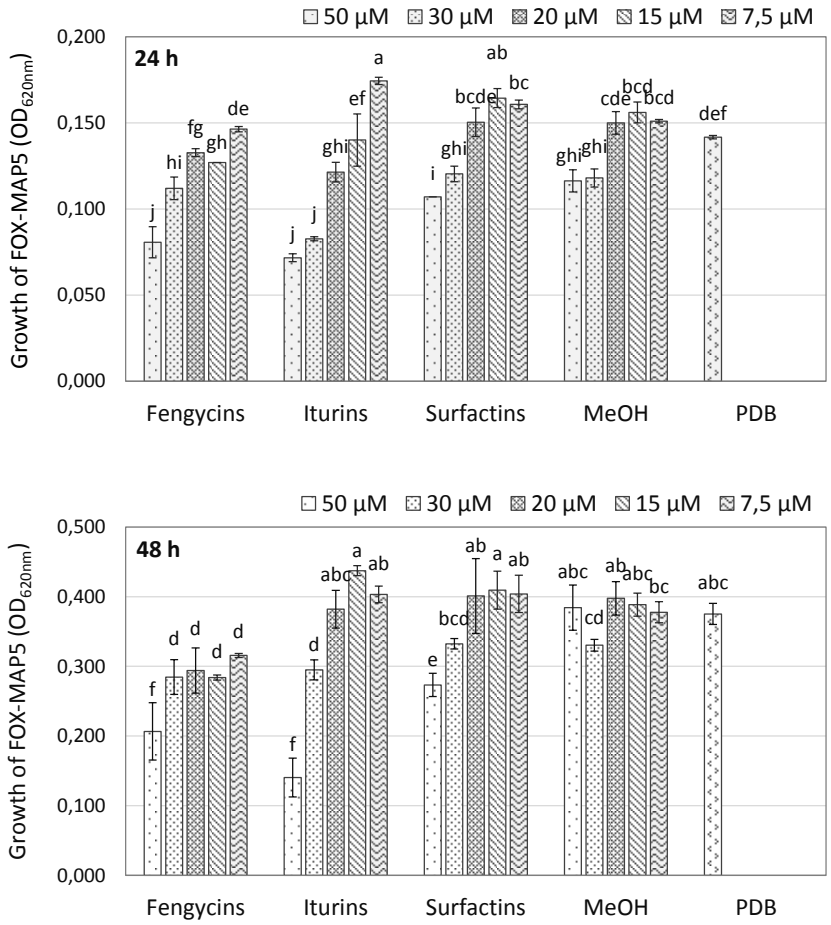
Figure 3-9: Effect of pure CLPs on FOX-Map5 microconidia germination



Note[17]

[17] Each CLP treatment consisted of homologue compounds mix as supplement to PDB medium at 50, 30, 20, 15 and 7.5 µM. Germination was measured 30 h after incubation. Pure methanol (MeOH) added to PDB at same volumes used in treatments and PDB medium without supplement were used as controls. Columns sharing the same letter are not significantly different according to Tukey test (α= 0.05). Bars on the columns represent the standard deviation of data (n= 3).

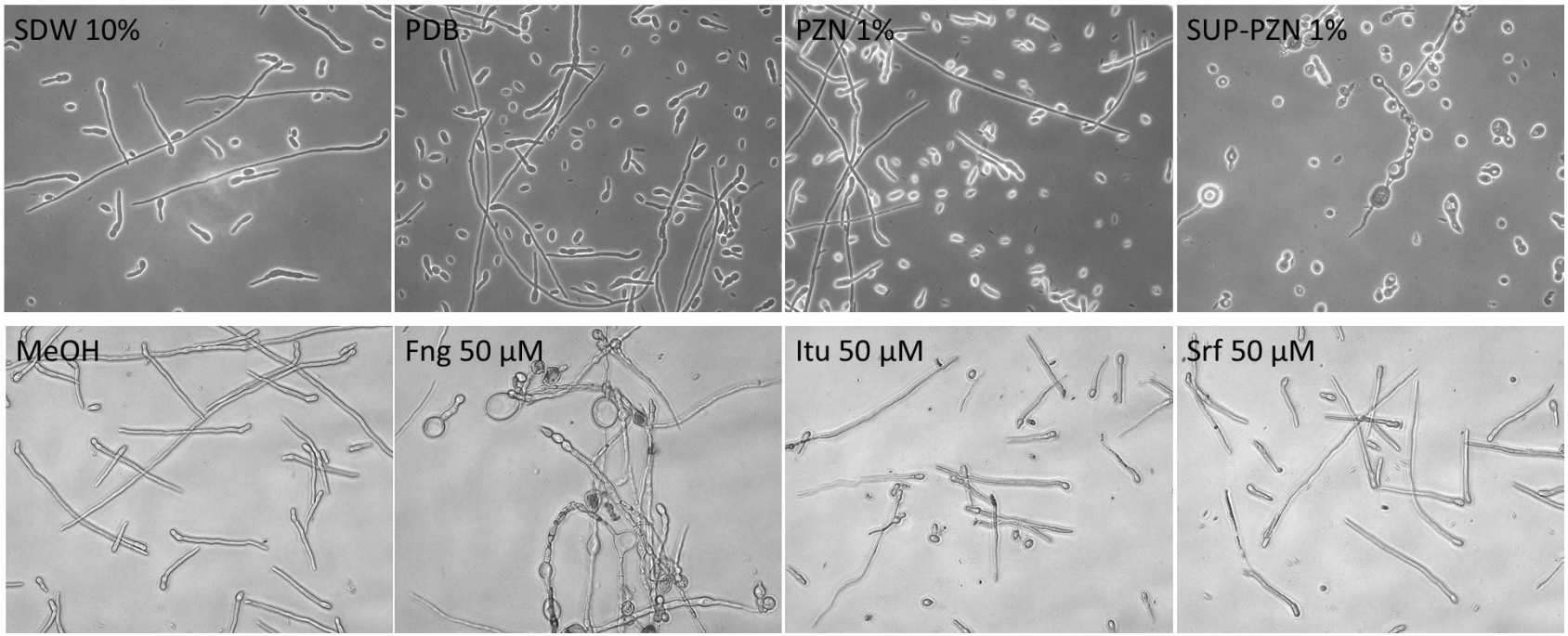
Figure 3-10: Effect of pure CLPs on FOX-Map5 growth



Note[18]

[18] Pure CLPs were added to PDB medium at 50, 30, 20, 15 y 7.5 µM. Pure methanol (MeOH) added to PDB at same volumes used in treatments and PDB medium without supplement were used as controls. Growth was measured as optical density in a microplate reader at 24 and 48 h after incubation. Columns sharing the same letter are not significantly different according to Tukey test (α= 0.05). Bars on the columns represent the standard deviation of data (n= 3).

Figure 3-11: Effect of pure CLPs on FOX-Map5



Note[19]

[19] Effects of filtered supernatant from liquid culture of *B. amyloliquefaciens* Bs006 in PZN added to PDB at 1% (SUP-PZN 1%) and the mix of homologous compounds of fengycins (Fng), iturins (Itu) and surfactins (Srf) at concentration of 50 µM on FOX-Map5 microconidia at 24 h after incubation under 30 °C, 125 rpm and darkness conditions. Sterile distilled water added at 10% (SDW 10%), sterile PZN medium added at 1 % (PZN 1 %), pure methanol (MeOH) as supplements to PDB medium and PDB with no supplement (PDB) were used as controls.

The concentration of fengycins produced by Bs006 after 48 h of fermentation at 30 °C was 377, 291 and 136 µg/mL in PZN, Landy and LB media respectively. The exposure of FOX-Map5 to the supernatant with the lowest concentration of fengycins (fermented-LB) showed significant effects used at 10 % (25 µL of supernatant in 250 µL of broth), which represents 3.4 µg of fengycins/well (~13.6 mg/L). In the case of the supernatant with the highest concentration of fengycins (fermented-PZN) the amount of this CLP used at 10 % is almost 3 times higher (9.4 µg/well ~37.6 mg/L) while used at 5 % represents an amount of 4.7 µg/well (~18.8 mg/L). On the other hand, exposure of FOX-Map5 to the pure fengycin solution showed significant effects from the 7.5 µM (~11.25 mg/L) concentration. Preliminary evaluations showed that concentrations lower than 7.5 µM did not significantly reduce neither germination nor growth of the fungus (data not shown). This suggests that the minimum concentration of fengycins required to significantly reduce FOX-Map5 development and cause physical damage to the conidia and germ tubes is 11.25 mg/L.

Description of the damage caused by the supernatant and pure CLPs on FOX-Map5. FOX-Map5 microconidia exposed to the filtered supernatant from media fermented by Bs006 showed damage to the microconidia and to the developed hypha of the fungus in a concentration-dependent manner. The observed damages consisted of pore formation, cellular swelling, lysis and inhibition of germination. Damage was stronger and affected a larger population of FOX-Map5 microconidia when higher concentrations of supernatant were used as a supplement to the PDB medium and also when supernatant from culture in PZN was used (Figure 3-11).

In the evaluation of the effect of pure lipopeptides the physical damages in the microconidia and in the hyphae exposed to fengycins, were similar to the damages caused by supernatants from Bs006 culture. Iturins and surfactins caused not physical damages to FOX-Map5 (Figure 3-11).

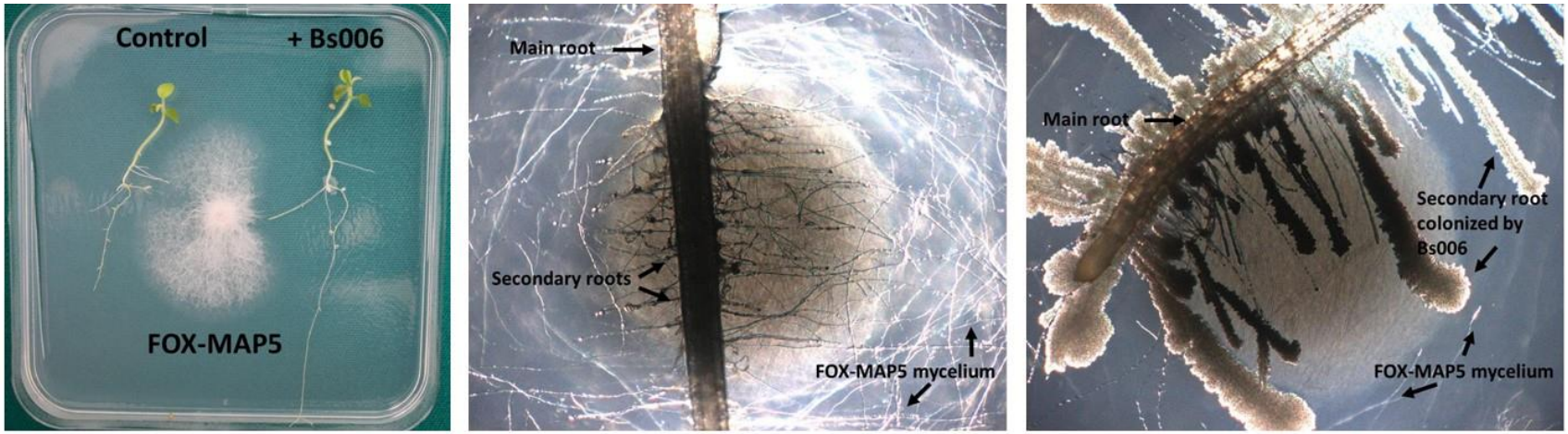
3.3.4. Antifungal activity of Bs006 in gnotobiotic system

The main root and secondary roots of the cape gooseberry plant inoculated with *B. amyloliquefaciens* Bs006 were colonized by this bacterium and a biofilm on the surface could be observed 4 days post-inoculation (Figure 3-12). The growth of the colony of FOX-Map5 occurred without restriction towards the root of the control plant (without Bs006) after

inoculation of the fungus in the center of the Petri dish. Whereas its growth was restricted towards the root colonized by Bs006 where an inhibition zone could be observed (Figure 3-12). The growth of FOX-Map5 was reduced by Bs006 root colonization by 62 %.

Pattern of CLPs in the gnotobiotic system. UPLC-ECI-MS analysis showed the presence of compounds of the three CLPs families on the surface of Bs006-colonized root and agar in growth inhibition zone. The concentration of iturins and fengycins was higher than the concentration of surfactins in both root and agar. However, iturins were found at a concentration significantly higher than the concentration of fengycins and surfactins in the agar (Figure 3-13). Homologous compounds of iturins from C13 to C16, fengycins from C15 to C18 and surfactins from C12 to C15 were found. Generally, the compounds found in the root were also found in the inhibition zone (Appendix 5). No CLPs were detected in the roots and close agar from control plants.

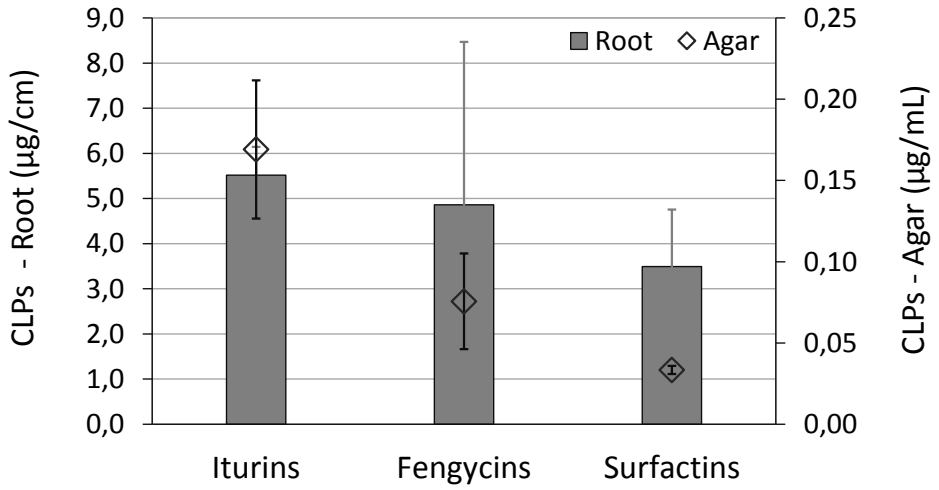
Figure 3-12: Gnotobiotic system used to study the interactions among Bs006, FOX-Map5 and cape gooseberry



Note[20]

[20] Control plant was inoculated with SDW (Negative control) and treated plant was inoculated with Bs006 (+Bs006). Notice the inhibition zone of growth of FOX-Map5 between fungus and inoculated root with Bs006. Picture of negative control show dense mycelium of FOX-Map5 around the root, while picture of root inoculated with Bs006 show few hypha approaching to the root. And biofilm of Bs006 on root surface. Pictures were taken five days after inoculation of FOX in the system.

Figure 3-13: Concentration of CLPs produced by Bs006 in the gnotobiotic system



Note[21]

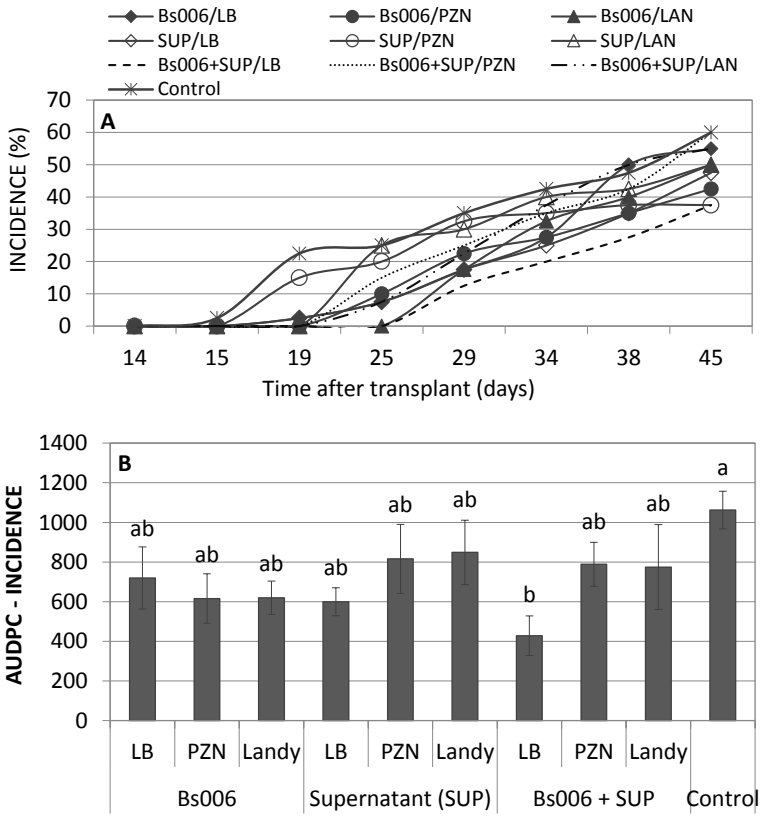
3.3.5. Biocontrol efficacy of Bs006 and supernatant in greenhouse

Symptoms of FW disease were initially presented in negative control plants (plants grown in FOX-inoculated soil without biocontrol treatment) 15 days after transplant and four days later in plants treated with the supernatant from Bs006 culture in PZN (SUP/PZN). In most of the treated experimental units, the symptoms of the disease appeared after 25 days, showing a delay in the incubation period of 10 days. The incidence of the disease showed a progressive increase in all treatments, which was similar in negative control, SUP/PZN and SUP/LAN treatments. In contrast, the application of the bacterium together with supernatant from LB medium (Bs006+SUP/LB) maintained the lowest level of disease incidence during the whole evaluation period (Figure 3-14A). However, there were no significant differences among treatments applied to control the disease with regards to the area under the progress curve of the disease incidence (Figure 3-14B). The application of

[21] Concentration of CLPs produced by *B. amyloliquefaciens* Bs006 on root Surface of cape gooseberry (Root) and inhibition zones between inoculated root and FOX-Map5 (Agar) in gnotobiotic system. Samples were taken 9 days after inoculation of Bs006 on Crown root of cape gooseberry seedlings (five days after FOX inoculation). Bars on the columns represent standard deviation of the data (n= 3).

Bs006 combined with supernatant from LB (Bs006+SUP/LB) was the only treatment that significantly reduced the progress of Fusarium wilt incidence during 45 dpi, compared to the negative control (Figure 3-14B). Figure 3-15 shows the symptoms of the disease in the experimental units and the differences in the level of the disease among treatments described above.

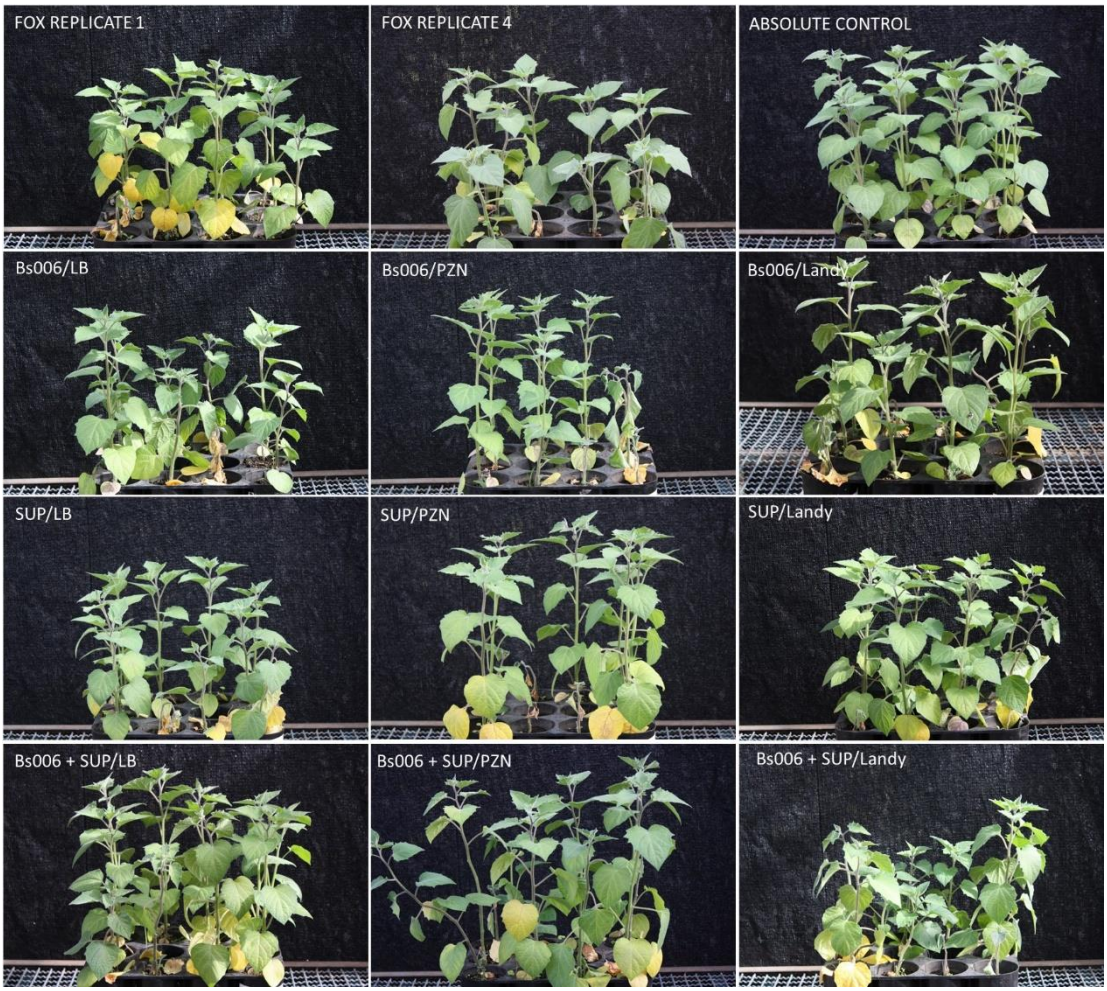
Figure 3-14: Effect of Bs006 and supernatant on Fusarium-wilt progress



Note[22]

[22] Effect of individual and mix application of Bs006 cells (Bs006) and filtered supernatant from liquid cultures (30 °C, 48 h, 150 rpm) of Bs006 (SUP) in LB, PZN and Landy media on the progress of Fusarium wilt incidence in cape gooseberry. A. Progress curves of disease incidence. B. Area under disease incidence progress curves (AUDPC) for 45 days post-transplant. Control: Grown plants in soil inoculated with pathogen, without biocontrol treatment. Bars on the columns represent standard error of the data (n= 4). Treatments sharing the same letter are not significantly different according to Tukey test (α= 0.05).

Figure 3-15: Symptoms of Fusarium-wilt in cape gooseberry plants treated with Bs006 and supernatant



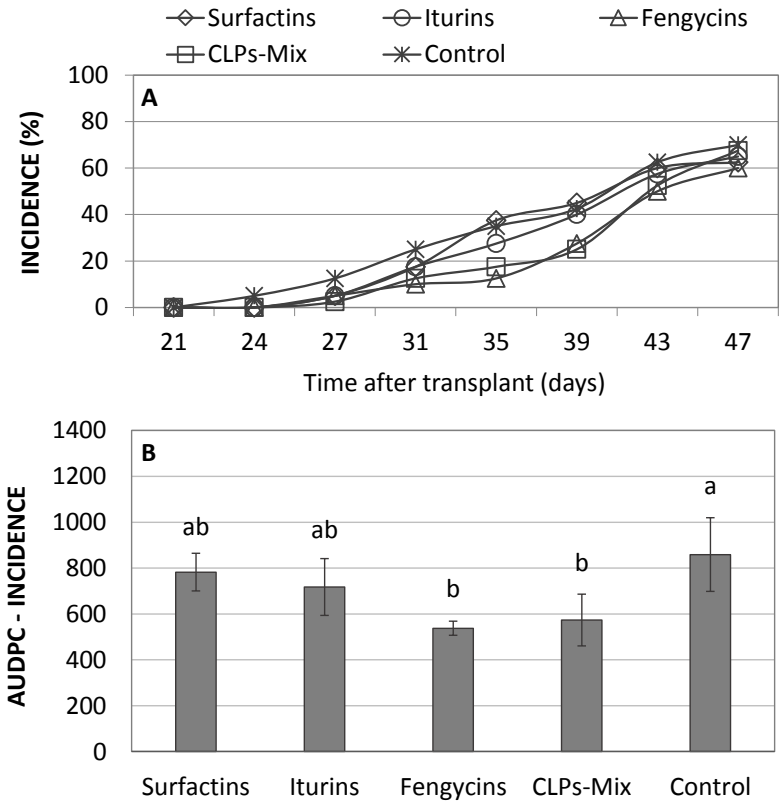
Note[23]

[23] Symptoms of Fusarium wilt in cape gooseberry plants inoculated with *F. oxysporum* Map5 [1x10^4 cfu/g of soil] (FOX) and treated with *B. amyloliquefaciens* Bs006 cells [1x10^8 cfu/mL], filtered supernatant from liquid culture of Bs006 diluted in water at 10 % (SUP) or with Bs006 cells and supernatant mix (Bs006 + SUP). Media used to growth Bs006 (30 °C, 48 h, 125 rpm) were LB, PZN and Landy. Treatments were applied at rate of 30 mL/plant immediately after transplant. Pictures correspond to 50 days after transplant.

3.3.6. Biocontrol activity of CLPs in greenhouse

The incubation period of the disease was three days longer in plants treated with cyclic lipopeptides than negative control (Control). However, only the application of fengycins and the lipopeptides mixture maintained the incidence of the disease at a level considerably lower than that recorded in plants treated with surfactins, iturins and those of the control during three weeks after inoculation of the pathogen (Figure. 3-16A). Therefore, the AUDPC of the incidence was significantly lower with the application of fengycins (Figure 3-16B) as compared to the control.

Figure 3-16: Effect of pure CLPs on Fusarium-wilt progress



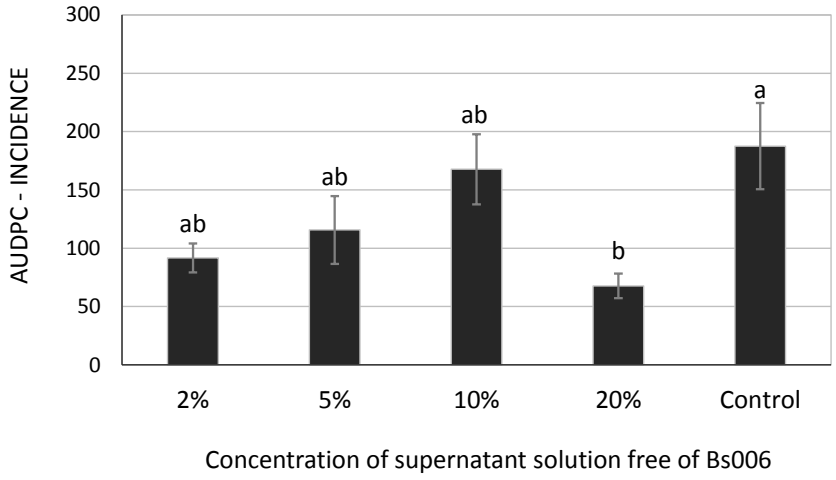
Note[24]

[24] Effect of the application of pure CLPs to cape gooseberry plants growing in soil inoculated with FOX-Map5 (FOX) on Fusarium wilt incidence progress during 47 days. A. Curves of disease incidence progress. B. Area under disease incidence progress curves (AUDPC). Control: Grown plants in soil inoculated with pathogen, without biocontrol treatment. Bars on the columns represent standard error of the mean (n= 4). Treatments sharing the same letter are not significantly different according to Tukey test (α= 0.05).

3.3.7. Pathogenicity of FOX-Map5 exposed to the supernatant

The exposure of FOX-Map5 to the supernatant from Bs006 culture in PZN reduced its pathogenic activity in cape gooseberry plants, since disease incidence was lower in experimental units inoculated with microconidia exposed to the supernatant, compared to the recorded incidence in the control (washed microconidia - not exposed to supernatant). However, only the exposure of microconidia to 20 % of concentration of supernatant showed a significant reduction of the incidence progress at 36 dpi, as compared to the control (Figure 3-18).

Figure 3-17: Pathogenicity of FOX-Map5 exposed to Bs006-supernatant free of bacteria



Note[25]

[25] Effect of exposition of *F. oxysporum* Map5 microconidia to filtered supernatant from liquid culture of *B. amyloliquefaciens* Bs006 on its pathogenic activity in cape gooseberry plants. Response variable is showed as area under disease incidence progress curve (AUDPC) during 36 days after transplant. FOX-Map5 inoculum was suspended in different concentrations of supernatant in water (2 %, 5 %, 10 % and 20 %). Washed pathogenic inoculum not exposed to supernatant was used as control. Bars on the columns represent standard error of the mean (n= 4). Treatments sharing the same letter are not significantly different according to Tukey test (α= 0.05).

3.4. Discussion

Members of the *B. amyloliquefaciens* species have the ability to synthesize compounds of the three non-ribosomal cyclic lipopeptides families (CLPs), iturins, fengycins and surfactins. Moreover, strains of *B. amyloliquefaciens* subsp. *plantarum* also have the ability to synthesize non-ribosomal polyketides (PKs) such as macrolactin and dificidin (Rückert *et al*., 2011). Since the secretion of these substances has been shown to be responsible for its protective effect against phytopathogens (Cawoy *et al*., 2014), this ability represents an important attribute in the context of the biological control of plant diseases. In the present study, we show that *B. amyloliquefaciens* Bs006 produces several homologous compounds to iturins, fengycins and surfactins and the polyketides bacillaene and macrolactin, suggesting that this strain has a high potential to be used as an alternative method to control plant diseases.

Compounds of the three types of CLPs were found in supernatant from Bs006 liquid culture, in the growth inhibition zones against FOX-Map5 in dual confrontation test and on the root surface of cape gooseberry in presence of FOX in the gnotobiotic system. Although the profile of homologous compounds produced by Bs006 was maintained in the three systems, the concentration was different in each model of study, where the highest concentrations of CLPs were found in liquid media, followed by solid media and the lowest levels were found on root surface. Moreover, the proportion among CLPs families found in the gelified media was different to the proportion found in liquid media. Thus, concentration of iturins was higher than the concentration of fengycins and surfactins in the agar diffusion zones (dual confrontation and gnotobiotic system) as well as in the root surface, whilst the proportion of fengycins was the highest in liquid media, indicating an influence of the state of the growth substrate on the efficiency in the biosynthesis of these compounds. It was also clear that the culture medium in which Bs006 grew influenced the production of CLPs, with the highest concentration found in PZN, followed by Landy and LB. These results contrast with that described by Cawoy *et al*. (2014) who found similar profiles of CLPs produced by different strains of *B. amyloliquefaciens*, *B. subtilis* and *B. pumilus* in both PDB and optimized medium and similar CLPs profile in liquid and gelified media.

Although CLPs profile of Bs006 in the gelified media was similar under both the presence and absence of FOX-Map5, iturins concentration was significant lower in the presence of

the fungus in PZN medium. This effect could be related to the difference in the size of the Bs006 colony among the treatments, since in the presence of FOX-Map5 in PZN the Bs006 colony showed lower development. This suggests that the fungus can also synthesize compounds that affect the growth of Bs006, and consequently, its ability to synthesize CLPs. Indeed, *F. oxysporum* strains have been reported to produce antimicrobial compounds such as enniatin, beauvericin, and fusaric acid, and the activity of these molecules are thought to enhance its survival and prevail when confronted with biological control agents (Bacon *et al*., 2004, 2006; Marzano *et al*., 2013). Similarly, the culture medium and the presence of FOX-Map5 modulated the amount of iturins synthesized by Bs006 in accordance with Cawoy *et al*. (2014) who showed that CLPs amount accumulated in the inhibition zones was modulated by the phytopathogen species interacting with *B. amyloliquefaciens*.

The proportions among CLPs produced by Bs006 in liquid media were different when the supernatant samples were injected directly into the UPLC compared to the injection of samples passed through C18 cartridges. With the implementation of the solid phase extraction protocol, the proportion of CLPs varied according to the culture medium used for the growth of the bacterium. However, direct injection of the supernatant free of bacteria showed a similar pattern in the proportions of CLPs for the three culture media tested, which has been consistent in subsequent analyzes of the new samples from the same media fermented by Bs006. Several studies have demonstrated that the solid phase extraction procedure is not necessary and that direct injection generates reliable results (Monaci *et al*., 2016; Yuan *et al*., 2012).

Knowing the mechanism behind the observed inhibition is essential for the appropriate development of commercial formulations and methods of application to maximize the potential use of biological control agents (Guleria *et al*., 2016). In this study, the *in vitro* antagonism showed a clear inhibition of the growth of FOX-Map5 in the presence of Bs006, whereas incubation of the fungus in the presence of supernatant free of Bs006 cells, reduced both FOX growth (germination and development) and caused alterations of the protoplasm and damages in the integrity of the cell wall. This suggests that Bs006 produces substances with fungistatic activity and substances with fungitoxic activity. This result is in agree with work reported by Kumar *et al.* (2012) who have shown that CLPs produced by

strains of *B. subtilis* / *amyloliquefaciens* species complex, are substances that can cause both types of effects on fungal cells in a concentration dependent manner.

The result of interactions among CLPs and phytopathogenic fungi depends on the nature of the target pathogen, as recently described Cawoy *et al*. (2014). They demonstrated that the growth inhibition of *F. oxysporum* and *Botrytis cinerea* was exerted by iturins, whereas in the case of *Cladosporium cucumerinum* it was due to fengycins. However, none of the CLPs had an inhibitory effect on *Pythium aphanidermatum* in dual confrontation tests. We observed no effects on the cellular integrity of FOX-Map5 when incubated with iturins or surfactins. However, effects such as swelling, lysis and total degradation of fungi cells have been reported for iturins (Peypoux *et al*., 1984; Kumar *et al*., 2012) and fengycins (Vanittanakom *et al*., 1986) as well as surfactins (Carrillo *et al*., 2003). These damages on fungi are due to the destabilizing action on the plasma membrane which has been demonstrated for iturins (Maget-Dana and Peypoux, 1994), fengycins (Patel et al., 2011), and surfactins (Carrillo *et al*., 2003). Furthermore, fengycins and iturins are consistently reported as causing these effects on a broad spectrum of filamentous fungi, including *Fusarium* spp. (Chitarra *et al*., 2003; Hu *et al*., 2007; Li *et al*., 2007; Vanittanakom *et al*., 1986; Yu *et al*., 2002)

Even though CLPs have the ability to interact with plasma membrane components of fungi, causing the formation of pores, ion channels and the loss of cellular integrity (Inès and Dhouha, 2015), the ability of *Bacillus* species to synthesize cell wall degrading enzymes, have also been demonstrated, and may be important in the process of antagonism (Baysal *et al*., 2013, El-Bendary *et al*., 2016, Chérif *et al*., 2002; Rocha *et al*., 2014). There may be a synergistic effect between chitinases and antibiotics during antagonism and that the alteration of host cell walls is a prerequisite for the diffusion of antibiotics (Di Pietro *et al*., 1993; Chérif *et al*., 2002). However, damage such as swelling of hyphae and microconidia, and to a lesser extent, loss of protoplasm through cell wall pores or complete disintegration of the cell wall during FOX-Map5 exposure to supernatant as well as pure fengycins were observed in the present study. Indicating that fengycins have the ability to cause lysis to FOX cells similar to the enzymatic action of chitinases. Although the presence of CLPs in agar inhibition halos have also been related to mycelial damage of the fungi (Torres *et al*., 2017), for the hydrolytic action of the enzymes, contact between *Bacillus* spp. and phytopathogen is necessary (Chérif *et al*., 2002). In the present work, we focused on CLPs

but a further work is required to determine if Bs006 produces cell wall degrading enzymes and their role in the control of phytopathogens.

We consistently detected high concentrations of iturins in the inhibition zones of FOX growth, rather than compounds of the other two CLPs families, suggesting that iturins play a main role in fungistatic activity against FOX-Map5. This result agrees with the work of Cawoy *et al*. (2014) who demonstrated that the antagonistic activity of *B. amyloliquefaciens* against *F. oxysporum* in dual confrontation test was mainly related to the presence of iturins. However, the results in the literature are variable, showing both fungistatic and fungicidal effects caused by both iturins and fengycins, depending on the target fungus and the CLPs concentration. Li *et al*. (2005) found that exposure of *Trichoderma harzianum* and *Gliocladium roseum* to low concentrations (0,01 to 0,025 mg/mL) of iturin A caused formation of chlamydospores in both fungi but, exposure to high concentrations (0,05 mg/mL) caused swelling and lysis of conidia. Kumar *et al*. (2012) described that bacillomycin D produced by *Bacillus* sp. A5F caused damage to the membrane of *Sclerotinia sclerotiorum*, in a concentration dependent manner, leading to cell death. Yuan *et al*. (2012) reported that iturins and fengycins produced by *B. amyloliquefaciens* NJN-6 had a fungistatic effect but non-fungicidal effect on *F. oxysporum* f.sp. *cubense.* Zhao *et al*. (2014) described that fengycin A of *B. amyloliquefaciens* Q-426 caused the inhibition of germination of conidia of *F. oxysporum* f.sp. *spinaciae* but did not cause cell damage. Chitarra *et al*. (2003) proved that the culture supernatant of *B. subtilis* YM 10-20 inhibited the growth of several fungi, including *Fusarium culmorum*, in agar-diffusion antibiosis tests. Specifically iturin A, the main compound produced by *B. subtilis*, caused reduction of germination, morphological changes and destruction of conidia of *Penicillium roqueforti* when it was incubated in liquid medium supplemented with the culture supernatant.

We determined in this study that the minimum inhibitory concentration of fengycins required to significantly reduce the germination and growth and to cause physical damage to FOX-Map5 is 11.5 mg/L in liquid media. However, there are variable data and effects in the literature showing the minimum inhibitory concentration of fengycins. For instance, in antagonism tests against *Fusarium* species on gelified medium, a fungistatic effect caused by fengycins has been reported but with high variability in the minimum inhibitory concentration. Here some examples are presented: 10 µg/mL on *Fusarium* sp. (Vanittanakom *et al*., 1986), 0.78 µg/mL on *Fusarium moniliforme* (Hu *et al*., 2007), 31

µg/mL on *F. oxysporum* f. sp. s*pinaciae* (Zhao *et al*., 2014). In these previous studies, the effect of fengycins on *Fusarium* spp. was described as inhibition of conidia germination and reduction of mycelial growth, but no cell membrane damage or loss of cytoplasmic content was reported.

When the effect of Bs006 supernatant on FOX-Map5 microconidia was evaluated, the addition of Landy and LB media to PDB significantly reduced the germination, compared to the non-supplemented control. However, dilution of PDB with sterile PZN medium did not cause reduction of fungus germination (Figure 3-7). This demonstrates the importance of including unfermented culture media as a control, on the contrary, it could be attributed false effects to the treatments. Under the fermentation conditions used (150 rpm, 30 °C, 48 h) the proportion in the CLP content is different among the three culture media (Appendix 6). In PZN, there is a higher proportion of fengycins than iturins and surfactins while on LB medium the content of surfactins is higher than iturins and fengycins, although in the two culture media compounds of the three types of CLPs are found. Although the three types of CLPs were also found in Landy medium, surprisingly its incorporation into the PDB medium did not reduce the germination of the fungus in a significantly different proportion to the incorporation of the unfermented medium, indicating that some component of the medium make it fungistatic against FOX. Considering that damages caused by supernatants and pure lipopeptides were present in a certain proportion of the population of microconidia, the results suggest that the microconidia that were not affected presented some mechanism of defense against fengycins and iturins. The tolerant fungal propagules continued its growth, which was evident by a higher value of the optical density at 48 h after incubation.

There is a large number of publications that show the ability of production of CLPs *in vitro* by members of the genus *Bacillus*, but the number of publications demonstrating the production of these compounds on the root surface or the rhizosphere is still very limited. It is also common to find publications that suggest the participation of CLPs in the phenomenon of antagonism based on *in vitro* studies, but there are few studies that correlate the biocontrol activity with CLPs. We developed a gnotobiotic system in order to evaluate the ability of Bs006 to colonize and produce CLPs on the root surface of cape gooseberry and its interaction with FOX-Map5, spatially separated from the root. Our results showed that Bs006 has the ability to colonize the root surface of cape gooseberry,

suggesting that cape gooseberry-root exudates served as a nutritional source for the bacteria to grow colonizing the root-surface and forming a biofilm (Figure 3-13). On the root surface the bacteria synthesized compounds from the three cyclic lipopeptides families (detected on both the root and the diffusion zone of the agar). Iturins and fengycins were the most concentrated and are attributed with the growth-prevention of FOX-Map5 in the direction of the colonized root by Bs006. Although at a lower proportion, under these conditions Bs006 also synthesized surfactins, which are attributed an important role in the process of colonization and biofilm formation of *Bacillus* spp. (Bais *et al*., 2004, Chen *et al*., 2007). This fact shows a huge potential for the use of Bs006 and its CLPs to protect cape gooseberry root against FOX infections.

In contrast with previous studies of the secretome of *B. amyloliquefaciens,* strains S499 and GA1 on tomato roots, tobacco and arabidopsis (Debois *et al*., 2014) and FZB42 on roots of *Lemna minor* (Fan *et al*., 2011) where the main CLPs found on the roots were surfactins, in our study a higher production of iturins was found. The concentration of CLPs produced by Bs006 on the root surface and diffused in the medium were sufficient to inhibit the progress of FOX-Map5 towards the plant. However, under natural rhizosphere conditions the situation is probably different, considering that the native microflora and environmental conditions may influence the colonization patterns of the bacteria, CLPs synthesis and the stability of these compounds (Debois *et al*., 2014; Pertot *et al*., 2013).

Since CLPs are likely produced in amounts below the detection threshold in the rhizosphere, the study of their dynamics at the target site has been difficult and its direct effect on phytopathogens under natural conditions has not been determined. *B. amyloliquefaciens* probably does not produce sufficient amounts of CLPs in the rhizosphere to exert a direct effective antagonism activity under natural conditions and perhaps the induction of systemic resistance (ISR) by CLPs plays an important role in disease control (Wu *et al*., 2015), although knowledge about the persistence of the elicitor effect of CLPs, and their impact on plant physiology is very limited. Taking this into account, a viable option for the control of vascular wilts may be to incorporate CLPs into a formulation for soil application. Our results from the *in vivo* evaluations suggest that the study of the interactions among the CLPs with the plant and the native microflora need to be further studied in order to find an optimal combination of Bs006 cells/spores and CLPs without promoting development of the disease. Taking into account that conidia germination and

growth of *F. oxysporum* and disease incidence is more successful under sterile soil conditions (Abawi and Lorbeer, 1972) and considering that CLPs and PKs can cause damage to fungi and bacteria respectively (Chen *et al*., 2009b), we suggest that the application of high concentrations of supernatant to the soil could have had a fungicidal and bactericidal effect, creating a biological vacuum, which could favor the rapid colonization of FOX-propagules that tolerated the effect of CLPs, causing high levels of disease (Figure 15). Although it has been demonstrated that the application of *B. amyloliquefaciens* FZB42 does not have a significant impact on the bacterial rhizosphere community (Chowdhury *et al*., 2013), the effect of the *in situ* application of CLPs on the microflora of the rhizosphere and its relationship with the development of the pathogenic inoculum has not been studied.

Although our results showed that the effect of CLPs on FOX depends on the dose, direct exposure of the fungus to high doses of CLPs is not sufficient to completely eliminate the initial inoculum of FOX, since some propagules may tolerate these effects, maintaining their pathogenic ability. FOX has a great versatility to tolerate the antibiotic and enzymatic attack of biological control agents (Bacon *et al*., 2004, Chérif *et al*., 2002, Duffy and Défago 1997, Marzano *et al*., 2013), adding to the difficulty in controlling it. In our research group we have determined that during the co-cultivation of FOX-Map5 with Bs006 the bacterium affects the viability of the fungus but, under favorable conditions for fungal growth, it can also reduce the viability of Bs006 and the production of CLPs (Moreno *et al*., unpublished) so it is possible to think that under soil conditions, the fungus could have come advantages over Bs006.

3.5. Conclusions

Overall the results of the present study show that *B. amyloliquefaciens* Bs006 has the ability to colonize the surface of cape gooseberry roots and to protect it against FOX infection through the synthesis of CLPs. Among these compounds, iturins had fungistatic activity while fengycins had fungicidal activity against FOX-Map5 in a concentration-dependent manner. The CLPs produced by Bs006 could be incorporated as part of the treatment to control the vascular wilt of cape gooseberry. However, additional studies are needed to measure the impact of its application to the soil on the interaction between the native microflora and the development of vascular wilt.

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Chapter 4. Influence of temperature and culture media on growth, lipopeptide production and *in vitro* antagonistic activity of *Bacillus amyloliquefaciens* Bs006

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Abstract

Bacillus amyloliquefaciens subsp. *plantarum* is one of the best known rhizobacteria due to its antagonistic activity against phytopathogens, through the production of non-ribosomal cyclic lipopeptides (CLPs), which exert direct (iturins and fengycins) or indirect (surfactins and fengycins) effects to reduce plant diseases. Previous experience has suggested that rhizosphere abiotic factors can affect the expression of the biocontrol traits of *B. amyloliquefaciens* Bs006 in the *Fusarium oxysporum* / Cape gooseberry pathosystem but not its plant growth promoting activity. These aspects have not been widely studied and require more attention in order to design strategies to increase the effectiveness of biological control agents (BCA). The aim of this study was to determine the effect of temperature on growth of *B. amyloliquefaciens* strain Bs006, its antagonistic activity against *F. oxysporum* strain Map5 (FOX-Map5) and the pattern of iturins (itu), surfactins (srf) and fengycins (fng) produced in LB, PZN, and Landy media. Growth of Bs006 in liquid media, its antagonistic activity in dual culture and CLPs profile varied with temperature-media

interaction. In liquid culture for instance Bs006 did not grow under 15 °C/Landy combination. Interestingly growth of Bs006 was slower at 15 °C in PZN which has higher nutritional level than LB. The highest population level of Bs006 was reached at high temperatures and rich media (25 and 30 °C / PZN). Fng were produced by Bs006 in the highest concentration followed by itu in PZN media at 72 h of fermentation. The highest concentration of srf was found in LB at 25 °C. Amounts of CLPs produced in solid media was different from those in liquid media, being higher iturins > fengycins > surfactins in the three tested temperatures, with the highest concentration at high temperature. Presence of *Fusarium* in solid media did not change the CLPs profile produced. Reduction of *in vitro Fusarium* growth by Bs006 was higher in PZN at all temperatures (49 – 55 %), followed by LB-30 °C (46 %) and Landy (31 – 33 %). Overall the results suggest that expression of biocontrol traits such as CLPs, by *B. amyloliquefaciens* are influenced by interaction between culture media composition and temperature of the growing environment. This response by *B. amyloliquefaciens* could partly explain the variability of its biocontrol efficacy in the *Fusarium* / Cape gooseberry pathosystem.

Key words: Biological control, Golden Berry, Fusarium-wilt, PGPR, Antibiosis.

4.1. Introduction

Biological control of plant pathogens is considered as an alternative or complementary method to chemical fungicides (Gerhardson, 2002). Plant growth-promoting rhizobacteria (PGPR) are one of the main groups of benefic microorganisms used against diseases caused by soil-borne phytopathogens (Weller, 1988; Pérez-García *et al*., 2011). Currently 75% of commercial products based on microorganisms are formulated based on bacteria (Lazarovits *et al*., 2014), of which several species of the genus *Bacillus* are used due to their ability to form endospores, structures that give them advantages to resist conditions such as exposure to chemicals, radiation, desiccation and nutritional deficit (Emmert and Handelsman, 1999; Ongena and Jacques, 2008; Pérez-García *et al*., 2011). *Bacillus amyloliquefaciens* is a rhizobacteria recognized for its antagonistic activity against phytopathogens, through the production of antibiotics, such as non-ribosomal cyclic lipopeptides (CLPs) and polyketides (PKs) (Argüelles-Arias, *et al.*, 2009; Raaijmakers *et al*., 2010), the induction of resistance in the host plant (Kloepper *et al*., 2004; Henry *et al*.,

2011; Desoignies *et al*., 2013; Pertot *et al*., 2013; Cawoy *et al*., 2014) and nutrient competition through siderophores production (Dunlap *et al*., 2013; Magno-Pérez *et al.,* 2015). CLPs from the iturin and fengycin families produced by *B. amyloliquefaciens* subsp. *plantarum* are known for their direct effects against phytopathogens (Cawoy *et al*., 2015; Malfanova *et al*., 2012). Moreover even though surfactins are known as elicitors of induced systemic resistance in plants (Pertot *et al*., 2013; Cawoy *et al*., 2014) fengycins have also demonstrated to stimulate plant defense responses (Ongena *et al*., 2005; Ongena *et al*., 2007).

The main limitations of biological control with microorganisms are that their efficacy is strongly influenced by environmental factors making them rarely as effective as chemical pesticides. Additionally the control activity is not as fast as that of agrochemicals (Lazarovits *et al.*, 2014). Temperature, pH and nutritional conditions affect the physiology and growth of antagonistic bacteria applied to the soil, which can limit root colonization and the expression of biocontrol traits (Compant *et al*., 2005). Specifically, the synthesis of antibiotics is related to the general metabolic state of the cell (Thomashow, 1996), which in turn depends on the availability of macronutrients and micronutrients, type of sources and supply of Carbon and Nitrogen and the pH, temperature, and other environmental factors (Compant *et al*., 2005). These aspects have not been widely studied and need more research to design strategies to increase the efficacy of biological control agents.

It has been shown that the variation among produced lipopeptides by *B. amyloliquefaciens* is highly dependent on the strain, its growing conditions and the composition of the culture media (Bonmatin *et al*., 2003; Das *et al*., 2009). In turn, variation in homologous molecules and the isoforms affect not only the physico-chemical properties of the lipopeptides but also their biological activity (Das *et al*., 2009; Shing *et al*., 2014). Carbon sources used for *B. amyloliquefaciens* growth may affect the type of lipopeptide, the yield and therefore the antifungal activity (Shing *et al*., 2014). Peypoux and Michel (1992) observed that presence of amino acids as nitrogen sources in Landy culture media (Landy, 1948) influenced the production of surfactin by *B. subtilis* S499 [now: *B. amyloliquefaciens* (Nihorimbere *et al*., 2012)].

B. amyloliquefaciens strain, Bs006 (Gámez *et al*., 2015), the focus of this study, was isolated from the rhizosphere of healthy plants of cape gooseberry (*Physalis peruviana*)

within a crop highly affected by *Fusarium oxysporum* (FOX), which is responsible for vascular wilt disease (Fusarium wilt ~ FW) of this crop. This rhizobacteria was selected for showing high plant growth-promoting activity and high antagonistic activity *in vitro* against *F. oxysporum* strain Map5 (FOX-Map5) (Caviedes, 2010), one of the most virulent isolate found in cape gooseberry (Rodríguez, 2010). Previous experiments carried out under greenhouse measured the plant growth-promoting activity and biocontrol activity of *B. amyloliquefaciens* Bs006 against FW of cape gooseberry and have shown a consistent behavior of its plant growth-promoting activity, but inconsistent biocontrol activity (Caviedes, 2010; Guacaneme, 2010; Torres, 2013). These results suggested that environmental factors typical of the rhizosphere may affect the expression of traits related to biocontrol activity, but not those related to plant growth promoting activity. However, plant growth promotion indicates the establishment of an active population of bacteria in the rhizosphere. As such, abiotic factors as pH, temperature, soil moisture or soil nutritional content among others, or biotic factors such as native microflora and root exudates, do not appear to have a negatively effect on the rhizosphere competence of *B. amyloliquefaciens* Bs006.

In Colombia, cape gooseberry is grown mainly in the Andean region, on soils, whose pH is acidic (6,0 – 6,5) to very acidic (4,5 - 5,5) (Roveda *et al*., 2012), at altitude from 1800 to 2800 m.a.s.l, and average ambient temperature from 14 to 20 °C (Fischer and Angulo, 1999). In order to enhance biological control efficacy microorganisms adapted to the target site conditions should be selected (Lucy *et al*., 2004), and as such the influence of biotic and abiotic factors of the agroecosystem on the biocontrol agent need to be studied.

Although lipopeptides with antifungal activity produced by *B. amyloliquefaciens* could be used as an active ingredient of an eco-friendly biopesticide, it would be ideal to have a population of bacterium established in the rhizosphere to prevent infection by phytopathogens and promote growth. As such, it is ideal to have a culture media that promotes growth of bacteria and lipopeptides production. Additionally rhizobacteria population requires the appropriate environmental conditions to express its mechanisms of action. Few works have measured the influence of the interaction between abiotic factors on growth and biocontrol activity of *B. amyloliquefaciens* (Pertot *et al*., 2013). In this context, we studied the effect of temperature and its interaction with culture media on the growth of

B. amyloliquefaciens Bs006, lipopeptides production, and its antagonistic activity against *F. oxysporum in vitro.*

4.2. Materials and methods

4.2.1. Microorganisms

FOX-Map5 was isolated from cape gooseberry plants with symptoms of vascular wilt and was purified through monosporic culture. The recovered isolate was highly virulent on cape gooseberry plants in pathogenicity tests (Rodríguez, 2010), and was selected to carry out all dual antagonism tests with the rhizobacteria in this investigation. Fungal isolate was preserved at -70 °C and from here it was reactivated in PDA media (Merk®) and incubated at 25 °C for 7 days. A subculture was grown in PDA and from that subculture, agar discs with young mycelium (one week old) were taken to inoculate sterile PDB (Difco®) (50 mL in an Erlenmeyer-250 mL). The inoculated broth was incubated for 7 days in continuous agitation (125 rpm), at 30°C and photoperiod of 12L:12D. Fermented broth was filtered using three layers of sterile muslin clothe. The suspension of FOX microconidia was collected and stored at -20 °C in sterile Eppendorf tubes to create a pre-inoculum stock which was used to inoculate sterile PDB at an initial concentration of $1x10^6$ microconidia/mL, to produce the inoculum that was used in the experiments, under the incubation conditions described above. After filtering the fermented broth, the obtained microconidia suspension was centrifuged at 15000 rpm for 15 min, and the biomass was washed twice with sterile distilled water (SDW). Microconidia were re-suspended in SDW and suspension was adjusted at $1x10^5$ microconidia.mL^{-1} by counting in a Neubauer chamber.

The rhizobacteria *B. amyloliquefaciens* Bs006 was preserved at - 70 °C in a sterile glycerol solution (10%), and reactivated in Luria-Bertani Agar (LBA). A cell suspension in SDW was prepared from the second sub-culture grown at 30 °C for 24 h, centrifuged, washed twice with SDW and the centrifuged biomass was re-suspended in SDW. To adjust the desired concentration, cell density was measured as optical density at 600 nm using a Optizen spectrophotometer (Mecasys Co, Banseok-dong, South Corea) at 600 nm wavelength (OD_{600}= 0,5 ~ $1x10^8$ cfu.mL^{-1}), according to a previously standardized calibration curve..

4.2.2. Effect of temperature on Bs006 growth, CLPs production and antagonistic activity against Fox-Map5

The effect of three different temperatures, 15, 25 and 30 °C on *B. amyloliquefaciens* Bs006 growth was measured in 30 mL of three different culture media: LB (Tryptone 10 g/L; yeast extract 5 g/L; NaCl 10 g/L; pH 7); PZN (soy peptone 40 g/L; Maltodextrin 40 g/L; KH_2PO_4 1,8 g/L; K_2HPO_4 4,5 g/L; $MgSO_4$ $7H_2O$ 0,3 g/L; Kelly solution 0,2 mL/L; pH 7. Kelly solution contains: EDTA 50 mg/L; $ZnSO_4$ $7H_2O$ 1 g/L; $MnCl_2$ $4H_2O$ 2,5 g/L; CaCl $2H_2O$ 7,34 g/L; CoCl $6H_2O$ 0,5 g/L; $(NH_4)_2MoO_4$ $4H_2O$ 0,5 g/L; $FeSO_4$ $7H_2O$ 5,0 g/L; $CuSO_4$ $5H_2O$ 0,2 g/L; pH was adjusted to 6 with NaOH solution); and Landy (glucose 20 g/L; glutamic acid 5 g/L; $MgSO_4$ 0,5 g/L; KCl 0,5 g/L; K_2HPO_4 1 g/L; yeast extract 1 g/L; $Fe_2(SO_4)_3$ 1,2 mg/L; $MnSO_4$ 0,4 mg/L; $CuSO_4$ 1,6 mg/L; pH 7) in 125 mL Erlenmeyers. Cultures were agitated on an orbital shaker at 125 rpm. Initial concentration of Bs006 in the culture media was adjusted to $5x10^6$ cfu.mL^{-1}. Bacterial growth was monitored by measuring the optical density (OD_{600nm}) after 24, 48 and 72 h of incubation. Lipopeptides extraction and quantification was carried out through UPLC-ESI-MS as described by Nihorimbere *et al*. (2012).

The antagonistic effect of *B. amyloliquefaciens* Bs006 against FOX- Map5 was measured through dual confrontation test in solid media LB, PZN and Landy in 90 mm diameter Petri dishes incubated in the dark at temperatures of 15, 25 and 30 °C. 10 µl of FOX-Map5 suspension [$5x10^5$ microconidia.mL^{-1}] were inoculated in the center of the Petri dish. Bs006 was inoculated 24 h later by placing 10 µl of cell suspension [$1x10^8$ cfu.mL^{-1}] at two opposite points located at 1 cm from the edge of Petri dish. Controls consisted in solid media inoculated with FOX-Map5 or Bs006. After seven days of incubation the diameter of FOX colony in the direction of bacteria inoculation points was registered. Growth inhibition of FOX was calculated using the formula GI = [(C – T)/C] * 100 where C represents the diameter of the fungus grown alone and T represents the diameter of the fungus grown in the presence of *B. amyloliquefaciens* Bs006. At this same time, agar samples from the inhibition zone were taken to determine the presence of cyclic lipopeptides by taking two agar cylinders (plugs of 5 mm diameter x 5 mm high) from each experimental unit (one from each inhibition zone) and suspending them in Eppendorf tubes with 500 µl of acetonitrile

(50%) and formic acid (0.1%) solution overnight. Samples were then, stirred vigorously in a vortex for 2 min and then centrifuged at 12000 rpm for 10 min. Supernatant was passed through a 0.22 µm filter, stored in glass vials and directly analyzed through UPLC-ESI-MS.

4.2.3. Experimental design and data analysis

The experimental unit for measuring Bs006 growth in liquid medium consisted of an Erlenmeyer (125 mL), while the experimental unit in dual confrontation tests consisted in a Petri dish. All experiments had a completely randomized design with factorial structure and with three replicates (bioassay in Erlenmeyers) and five replicates (bioassay in Petri dishes). Evaluated factors were medium and temperature, three levels each. Statistical analysis was performed with SAS software, version 9.4 (SAS Institute Inc., 2011). Data were submitted to normality (Shapiro-Wilk $P > 0.05$) and homogeneity of variance (Bartlett $P > 0.05$) tests followed by two way ANOVA and *post hoc* Tukey's test ($P= 0.05$).

4.3. Results

4.3.1. Growth analysis of *B. amyloliquefaciens* Bs006 at 15, 25 and 30 °C

Fermentations of Bs006 were carried out to measure the impact of incubation temperature on its growth and on cyclic lipopeptides produced during 72 h of incubation on LB medium, commonly used in laboratory for *Bacillus* spp. growth; Landy medium used by Landy *et al*. (1948) for production of bacillomycin and by Leclère *et al*. (2005 and 2006) for mycosubtilin production; and PZN medium used by Scholz *et al*. (2011 and 2014) for plantazolicin and amylocyclicin production, respectively.

The effect of the temperature x medium interaction on population growth of Bs006 was significant ($P < 0.05$). Bs006 did not grow on Landy medium at 15 °C, while the bacteria reached population levels of 9×10^8 cfu.mL^{-1} and 1.8×10^9 cfu.mL^{-1}on LB and PZN media respectively at the same temperature. Bs006 growth was stimulated by temperature increase, with the lowest population levels at 15 °C and the highest at 25 and 30 °C (2.8 x

10^9 cfu.mL^{-1} and 2.4 x 10^9 cfu.mL^{-1} respectively) regardless of the media and the incubation time.

The growth tendency of Bs006 also showed differences among culture media at temperatures of 15 and 25 °C but at 30 °C the growth rate was similar on the three culture media (Figure 4-1). At 15 °C Bs006 growth on LB medium reached its stationary phase before 48 h and after this time begun to decline. Meantime, on PZN medium growth was slower. Here, the cell density continuously increased even at 72 h of incubation.

Incubation of Bs006 at 25 °C caused earlier exponential growth (Figure 4-1), which was significantly higher ($P < 0.05$) on LB and PZN media (8.2 x 10^8 and 1.08 x 10^9 cfu.mL^{-1} respectively) compared to growth on Landy medium (2.2 x 10^8 cfu.mL^{-1}). At 25 °C, the time in the stationary phase was shorter on LB medium compared to that in the other two culture media and before 48 h it was already in decline phase. At 30 °C the growth of Bs006 had reached the stationary phase at 72 h of incubation on the three-culture media (Figure 4-1).

4.3.2. Impact of temperature on CLPs production in liquid media

The concentration of CLPs in filtered supernatant varied with incubation temperature, with culture medium and with fermentation time, being significant the interaction between these factors ($P < 0.001$) for iturins, fengycins and surfactins compounds. Higher temperatures and longer fermentation times generally increased production of the three lipopeptides families on PZN and Landy media. However, the concentration of iturins and fengycins on LB medium generally was similar from 24 to 72 h of fermentation in all tested temperatures while behavior of surfactins depended largely on the temperature and time evaluation (Figure 4-2). In overall CLPs production at 15 °C was strongly reduced in all three culture media but significantly there were no CLPs in Landy medium incubated at 15 °C, which is related to the absence of growth of Bs006. Interestingly incubation at 15 °C on Landy medium caused a bacteriostatic effect to Bs006, since 5th day after incubation, the experimental units were incubated at 25 °C resulting in evident Bs006 growth after 24 h of incubation.

Surfactins were produced in lower concentration compared to iturins and fengycins in the three-tested media. LB was the media in which surfactin production was the highest. It was observed that concentration of this CLP was uniform (13.1 – 14.7 $\mu g.mL^{-1}$) from 24 to 72 h under incubation of 25 °C while at 15 °C its production tended to significantly increase (5.2 – 13.2 $\mu g.mL^{-1}$), as incubation time increased. On the contrary surfactins concentration tended to decline from 11.6 to 0.84 $\mu g.mL^{-1}$, as incubation time increased (Figure 4-2).

On the other hand, fengycins were the CLPs produced by Bs006 in the highest concentration in the three media. PZN was the culture medium in which this CLP was produced in the highest concentration (300 $\mu g.mL^{-1}$) follow by Landy (123.5 $\mu g.mL^{-1}$) and LB (75.5 $\mu g.mL^{-1}$). Production of fengycins significantly increased in PZN medium as incubation time and temperature increased, while in overall this was similar in Landy at 25 and 30 °C. Production of fengycins in LB medium was not significantly different between treatments but the highest production was observed under 25 °C (Figure 4-2). Regardless of culture media and incubation temperature, *B. amyloliquefaciens* Bs006 produces up to 20 times more fengycins than surfactins and up to five times more iturins.

Production of iturins by Bs006 had a similar behavior to fengycins under tested factors of temperature, culture medium and incubation time. Briefly, iturins concentration was higher than surfactins and lower than fengycins. The highest concentration of iturins was found in PZN medium at 72 h under 25 and 30 °C (49.5 and 60.8 $\mu g.mL^{-1}$ respectively). In Landy medium, iturins was produced in a similar concentration at 25 and 30 °C up to 35.9 $\mu g.mL^{-1}$ after 72 h. Finally, production of iturins in LB was similar in all temperature and incubation time (Figure 4-2).

Figure 4-1: Effect of incubation temperature and culture media on growth of *B. amyloliquefaciens* Bs006

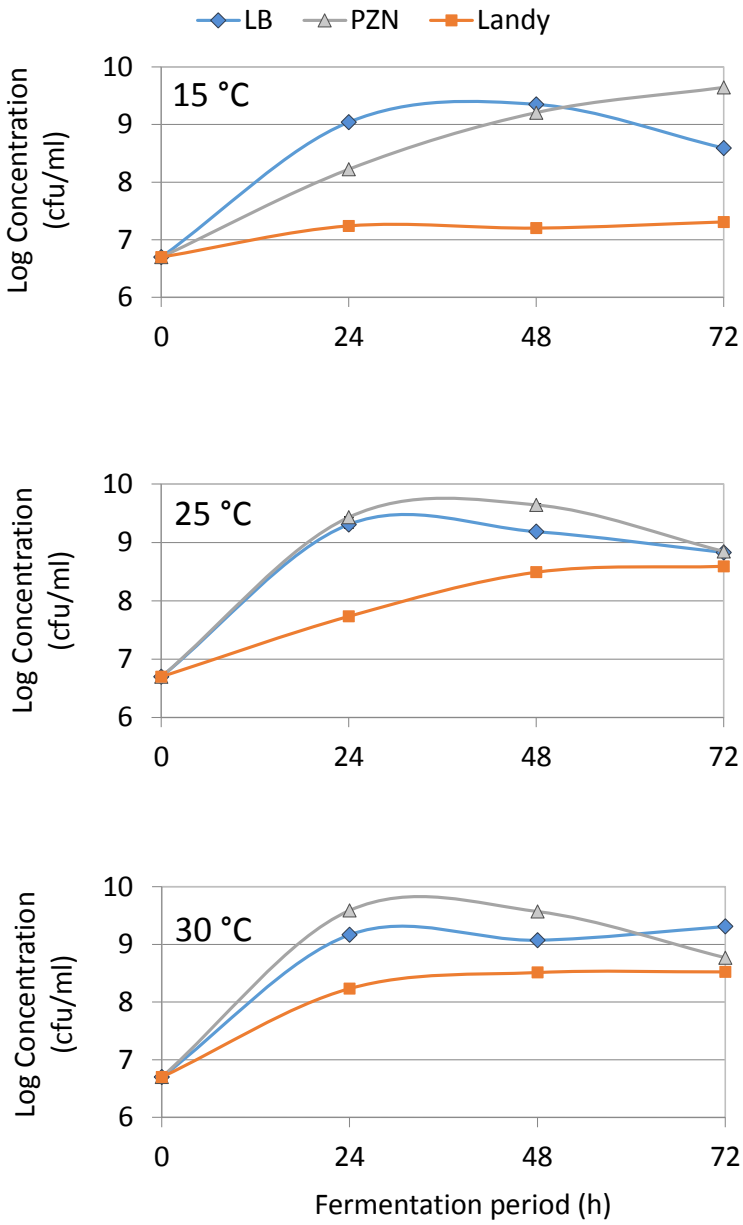
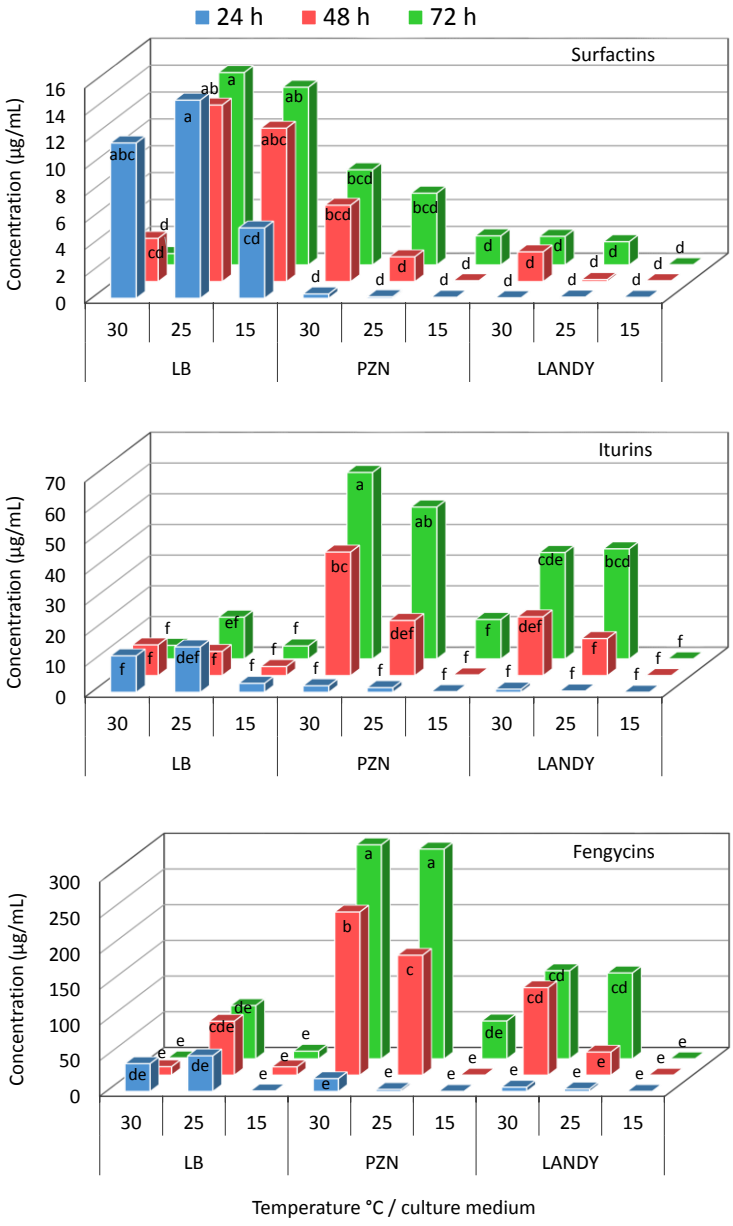


Figure 4-2: Effect of incubation temperature and culture media on production of CLPs by Bs006



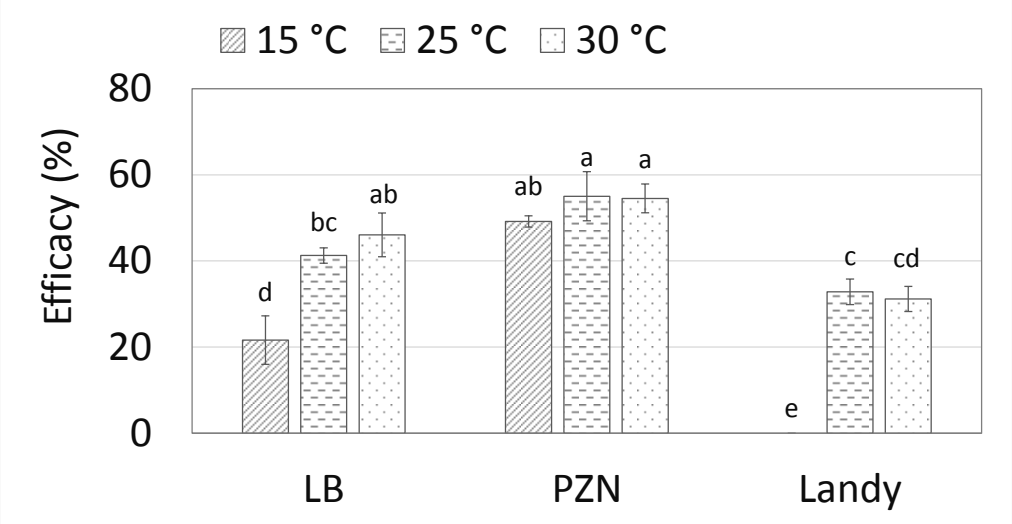
Note[26]

[26] Production of CLPs by *B. amyloliquefaciens* Bs006 in LB, PZN and Landy broth at 24, 48 and 72 h after incubation at 15, 25 and 30 °C. Columns within each CLP family with the same letter are not significantly different according to Tukey test (α= 0.05). Data are the mean from three biological replicates.

4.3.3. *In vitro* antagonistic activity of Bs006 against FOX-Map5

Both the culture medium and the incubation temperature affected the antagonistic activity of Bs006 against FOX-Map5 in the dual confrontation test (Figure 4-3). There was a significant interaction between temperature x medium ($P < 0.05$). There was no reduction of the growth of *Fusarium* in Landy - agar at 15 °C since Bs006 hardly grows on the medium (Figure 4-4, Appendices 7 and 8). On LBA at 15 °C the efficacy of Bs006 in reducing FOX-Map5 growth was significantly lower (22 %) than in the rest of the treatments where growth inhibition halos were presented. The efficacy of bacteria on reducing fungal growth was not significantly different between temperatures of 25 and 30 °C, regardless of culture medium. PZN showed significantly higher activity (55 %) against FOX-Map5 ($P < 0.05$) independent of growth temperature (Figure 4-3).

Figure 4-3: Effect of temperature and culture media on antagonistic activity of Bs006



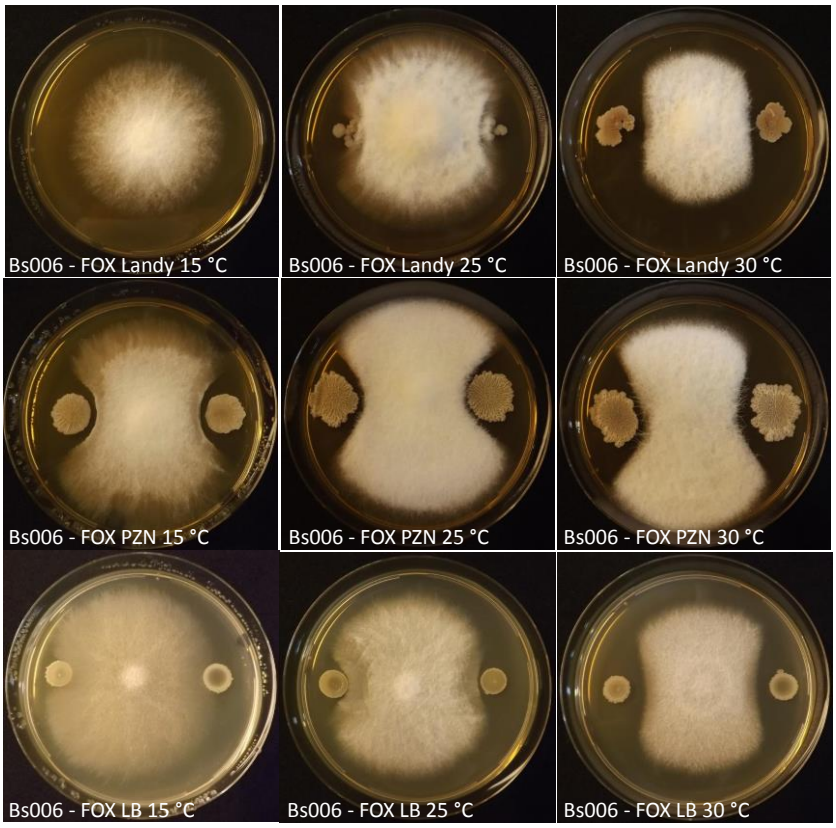
Note[27]

[27] Effect of incubation temperature and culture media on FOX-Map5 growth reduction efficacy by *B. amyloliquefaciens* Bs006. Columns with the same letter are not significantly different according to Tukey test (α=0.05). Bars on the columns represent standard deviation of data (n= 3).

4.3.4. Analysis of lipopeptides in inhibition zones

The concentration of the CLPs produced by Bs006 on solid media was similar when it was inoculated in absence and presence of FOX-Map5 (Figure 4-5). However, significant effects of the culture medium and temperature on the amount of lipopeptides produced by Bs006 were observed ($P < 0.001$ for iturins and fengycins and $P < 0.05$ for surfactins). Consistent with the results on liquid media, PZN produced the highest amounts of iturins and fengycins, while concentration of CLPs was similar in LB and Landy media. Regarding the nature of the lipopeptides, in contrast with liquid media, in all solid media iturins were produced in the highest amounts, followed by fengycins and lastly, surfactins (Figure 4-5).

Figure 4-4: Growth inhibition of FOX-Map5 by Bs006 under different temperatures



Note[28]

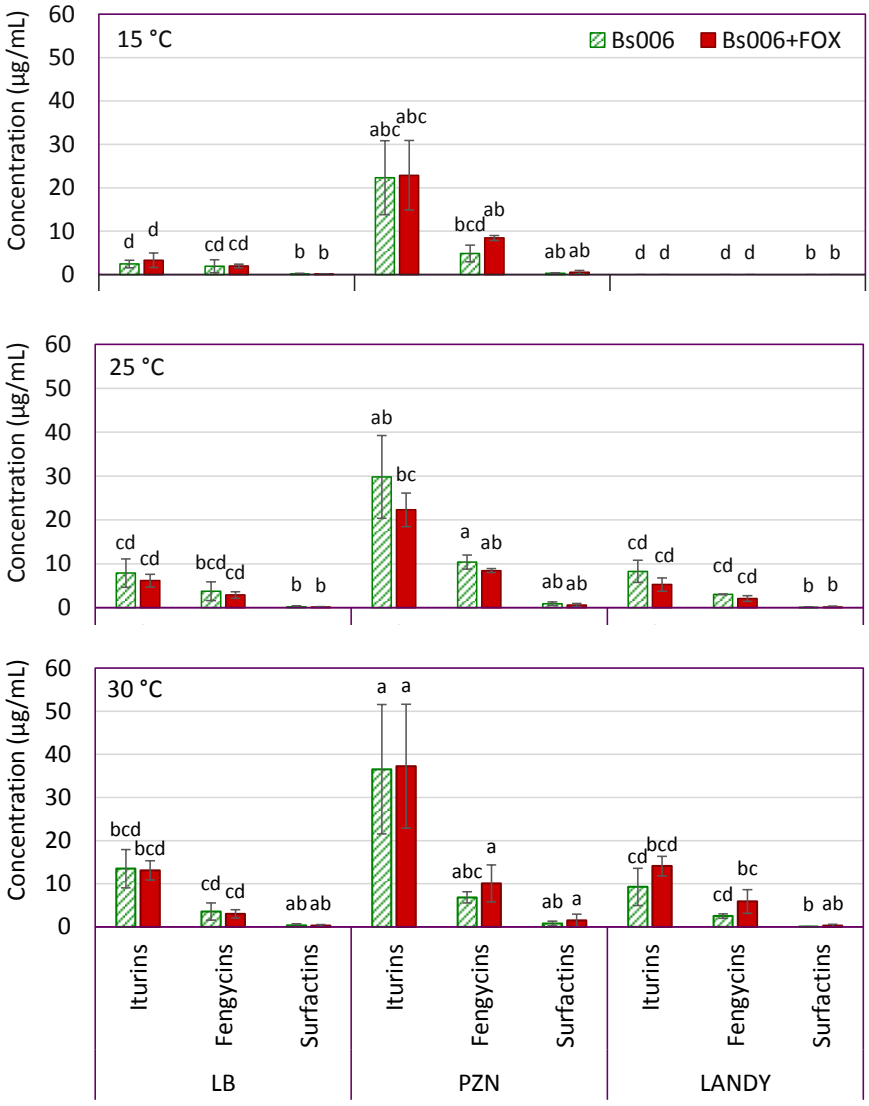
[28] Dual confrontation test between *B. amyloliquefaciens* Bs006 and *F. oxysporum* Map5 on LB, PZN and Landy media at 15, 25 and 30 °C. Pictures were taken on 7th day after inoculation of microorganisms.

Although no significantly different in all treatments, it was observed that concentration of CLPs increased as temperature increased. Thus concentration of iturins was significantly higher in PZN at 30 °C (37.2 µg.mL^{-1}) than 25 °C (29.8 µg.mL^{-1}) and 15 °C (22.9 µg.mL^{-1}). Concentration of fengycins (8.44 to 10.1 µg.mL^{-1}) were similar in all temperatures in this medium. Concentration of iturins in LB and Landy media ranged from 2.42 to 13.49 µg.mL^{-1} regardless the temperature, while fengycins ranged from 1.92 to 3.74 µg.mL^{-1} (Figure 4-5). However, no CLPs were produced in Landy incubated at 15 °C and no Bs006 colony was developed under this condition (Figure 4-4). No significant effect of temperature x media interaction was observed in this experiment.

4.4. Discussion

In a previous study Caviedes (2010) isolated rhizobacteria (fluorescent *Pseudomonas*, sporulated *Bacillus* and Actinomycetes) from the rhizosphere of healthy cape gooseberry plants, within a crop highly affected by vascular wilt caused by *F. oxysporum*, under the hypothesis that microorganisms present in these plants fulfilled a role of disease suppression. Isolate Bs006 from sporulating *Bacillus* group identified as *B. amyloliquefaciens* (Gámez *et al.,* 2015) was chosen due to its antagonistic potential against *F. oxysporum* based on the formation of inhibition halos in *in vitro* dual confrontation test on potato dextrose agar medium (PDA) (Caviedes, 2010), suggesting that the production of bioactive compounds with antifungal activity by Bs006. Recently Moreno-Velandia *et al.* (unpublished) confirmed the ability of Bs006 to produce the most known compounds of the three families of cyclic lipopeptides, which is common among the isolates of this specie (Cawoy *et al*., 2015).

Figure 4-5: Effect of the temperature and culture media on CLPs production by Bs006 in inhibition zones



Note[29]

[29] Concentration if CLPs produced by *B. amyloliquefaciens* Bs006 on inhibition zones from dual confrontation test against *F. oxysporum* Map5 at 15, 25 and 30 °C. Columns within each CLP family with the same letter are not significantly different according to Tukey test (α= 0.05). Bars on the columns represent the standard deviation of data (n= 3).

Application of the rhizobacteria to the soil in *Fusarium* / cape gooseberry pathosystem has shown high variability on the reduction of the disease under greenhouse conditions, whose temperature ranges between 10 to 42 °C (Moreno-Velandia *et al.*, 2015). In these environmental conditions, the temperature in the rhizosphere is highly variable during the day, ranging between 15 and 30 °C. Under these conditions it has been observed that the plant growth promoting activity by Bs006 is consistent among experiments, while its biocontrol activity on Fusarium wilt is variable. Therefore, the present study determined the influence of temperature on growth of Bs006, production of cyclic lipopeptides and antagonistic activity against FOX-Map5 *in vitro*. Three different culture media were included with the aim to know the response of those variables under different nutritional levels.

Considering that, the production of antifungal compounds and siderophores are the main mechanisms of diseases suppression by *Bacillus* spp. and *Pseudomonas* spp. (Wulff *et al.,* 2002) it is convenient to elucidate the biotic and abiotic factors that modulate the synthesis of these metabolites (Raaijmakers *et al*., 2002). Temperature is a factor that affects the expression of biocontrol mechanisms significantly (Burpee, 1990), which has been previously shown (Abushady *et al*., 2005; Fickers *et al*., 2008; Landa *et al.*, 2004; Pertot *et al*., 2013) and was demonstrated in this work. However, to the best of our knowledge, this is the first study that measured the influence of temperature and it's interaction with the culture medium on the growth, lipopeptides production and antagonistic activity of *B. amyloliquefaciens* against *F. oxysporum*.

According to the components and quantities used in this study, the nutritional level of LB, Landy and PZN media could be classified as low, intermediate and high respectively. LB is widely used for the growth of *Bacillus* spp. in the laboratory and is popular in microbiology since it allows rapid growth and has a good yield for many species of microorganisms (Sezonov *et al*., 2007). Landy and PZN media have been used for lipopeptide production by *B. amyloliquefaciens* (Arguelles-Arias *et al.*, 2009; Chen *et al*., 2009; Scholz *et al*., 2011; Scholz *et al*., 2014) and were incorporated in this study in order to determine their influence on the antagonistic efficacy. In this regard, it seems that the conditions of low nutritional level (LB) in interaction with low (15 °C) and moderate (25 °C) temperatures allow to *B. amyloliquefaciens* Bs006 to produce surfactins and iturins (Fig 3). In contrast, the interaction between a high nutritional level (PZN) with low temperature (15 °C) affected

significantly the growth rate of Bs006 and the production of surfactins and iturins. At the same time, the production of fengycins by Bs006 is affected negatively in low temperature conditions, independently of the nutritional level of the growth medium. Our results suggest that under conditions of low nutritional level, temperature is a determining factor of the efficacy of *B. amyloliquefaciens* Bs006 on *F oxysporum* Map5 but in conditions of high nutritional level the bacterium shows high antagonistic activity at low as well as high temperatures.

Nihorimbere *et al*. (2009) and Pertot *et al*. (2013) showed that the growth of *B. subtilis* / *amyloliquefaciens* is slow at low temperatures and the production of surfactins is favored under this condition. In contrast, we showed a rapid growth at 15 °C in LB broth and low concentrations of surfactins (7 µg/mL). Even on PZN and Landy media in which the concentration of carbon sources is higher than LB medium, the production of surfactins was very low at 15 °C.

Landa *et al*. (2004) observed that at 15 °C *Bacillus megaterium* RGAF 51 and *Paenibacillus macerans* RGAF 101 did not grow on PDB, phenomenon that are partially agree with that found in the present work, where at 15 °C, *B. amyloliquefaciens* Bs006 did not grow in Landy broth. In PZN/15 ° C broth, Bs006 presented a prolonged lag phase and delayed its growth rate, while in LB broth presented fast growth. This result suggests that the interaction of the components of PZN and Landy media with low temperatures, delay or stop the metabolic activity of *B. amyloliquefaciens* Bs006.

Previous studies have shown that lipopeptides production is influenced by the nutritional sources and carbon concentration used for the growth of strains of *Bacillus* spp. Not only the source but the concentration of the carbon sources influences the lipopeptides production. For example, Willenbacher *et al*. (2015) determined an optimal concentration of glucose to obtain a maximum production of surfactins by *B. subtilis* DSM 10^T cultivated on Cooper medium at 30 °C. Shing *et al.* (2014) showed that *B. amyloliquefaciens* AR2 produces a mixture of surfactins, iturins and fengycins incubated at 30 °C in minimal salts medium supplemented with dextrose, sucrose or glycerol, while under maltose, lactose or sorbitol as supplement the bacterium produced iturins only. Nihorimbere *et al*. (2009) used carbon sources from root exudates and determined that the surfactins production by *B. subtilis* BGS3 was higher in the presence of organic acids (citric acid and succinic acid) and

amino acids (aspartic acid and glutamic acid) compared to the presence of sugars (glucose, fructose, and maltose) as only sources of carbon in a minimal medium. Nitrogen sources are composed primarily of a variety of oligopeptides and free amino acids (Tryptone in LB; Soy Peptone in PZN; Glutamic Acid in Landy) and carbon sources (Yeast Extract in LB; Maltodextrin in PZN; glucose and Yeast Extract in Landy) employed in the culture media of the present study, allowed the production of the three families of lipopeptides by *B. amyloliquefaciens* Bs006. However, it is clear that PZN medium provides more availability of nitrogen and carbon due to its high amounts of soy peptone and maltodextrin (40 g/L in both cases) which probably favored both the growth of the bacteria and the production of iturins and fengycins in comparison with the other two culture media, where availability of carbon was probably limiting for the synthesis of lipopeptides.

Consistent with our results, Singh *et al*. (2014) suggested that the lipopeptide production is directly correlated with biomass production and that a substrate that favors good growth, also favors the high production of biosurfactants. In the present study maximum yield of biomass and lipopeptides (iturins and fengycins) was observed in the media with greater availability of carbon and nitrogen. Sezonov *et al*. (2007) showed that the early depletion of limited carbon on LB medium stopped growth of *Escherichia coli* K-12. Abushady *et al*. (2005) showed a linear increase in surfactin production by *B. subtilis* with an increase of the initial concentration of glucose in the culture medium. A rapid depletion of the carbon sources in the LB medium would explain the stability of the population of Bs006 after 24 h of fermentation and the limited production of cyclic lipopeptides. Considering that Bs006 population levels were similar on LB, PZN, and Landy media, the concentration of available nutrients in the LB medium, were probably sufficient to support cell growth but limited the metabolic activity. Furthermore, lipopeptide production is affected by the presence of minerals salts in the growth medium (Abushady *et al*., 2005), which are also limited in the LB medium compared with PZN and Landy media.

The expression of genes involved in the biosynthesis of surfactins is associated with an increase in the density of cells, which occurs particularly in the transition from the exponential phase to the stationary phase (Cosby *et al*., 1998), while the biosynthesis of iturins and fengycins occurs in the stationary phase (Jacques *et al*., 1999; Koumoutsi *et al*., 2007). Even though our results agree with those described above we observed that the duration of the growth phases varied with the culture medium and its interaction with

incubation temperature. As such, genes expression for biosynthesis of lipopeptides should vary with the growing medium and the incubation temperature accordingly.

In addition, Torres *et al*. (2016) showed that *B. amyloliquefaciens* produced mainly surfactins and iturins on Mueller-Hinton broth and all three families on the same solid medium. In contrast, we observe that *B. amyloliquefaciens* Bs006 produced all three families of lipopeptides in both liquid and solid medium even though they were produced in different proportions and amounts. Analysis of lipopeptides on the agar close to *B. amyloliquefaciens* Bs006 colonies in Petri dishes without *F. oxysporum* Map5, indicate that the fungus neither stimulated nor suppressed lipopeptide production, suggesting that expression of genes for the biosynthesis of these cyclic lipopeptides is constitutive. Considering that the lipopeptide profile of Bs006 on solid media differed from that in liquid medium, in the rhizosphere, whose growth conditions are more similar to a solid ambient, lipopeptides production profile of Bs006 in the rhizosphere may be similar to that on agar. However, the determination of lipopeptides production by this bacterium in the cape gooseberry rhizosphere in presence of *F oxysporum* requires further investigation. Lipopeptides production may also vary according to the strain. Yu *et al*. (2002) observed that *B. amyloliquefaciens* B94 produced only iturins in LB broth, whereas Bs006 produced iturins, fengycins and surfactins in the same culture medium.

Considering that the rhizosphere is characterized by low nutrient content, *B. amyloliquefaciens* Bs006 must compete with the native microflora for space and nutrients, and as such, lipopeptide production would be expected to be less compared to what was produced *in vitro*. Low lipopeptide production in the rhizosphere might explain the low consistency of biocontrol activity of *B. amyloliquefaciens* Bs006 against *F oxysporum* in cape gooseberry. Additionally considering that the soil temperature areas where cape gooseberry is grown ranges from 10 to 20 °C, our results would suggest that the rhizosphere would have a low concentration of fengycins, compared with the production of iturins and surfactins. Low levels of fengycins, could enable *F oxysporum* to colonize the rhizosphere effectively and make it difficult to contain the infection. However, this study will help to design strategies that favor the success of the biological control agent. In this particular case, the application to the soil of Bs006 cells and nutrients that promote their establishment in the rhizosphere and lipopeptide production could generate better

competition to *F oxysporum*. Alternately, the complementary application of *in vitro* produced lipopeptides could reduce the pathogenic activity of the initial inoculum of the fungus.

During the screening process of antagonistic rhizobacteria the antibiosis mechanism is considered as indicator of the biocontrol potential. However, this potential could be masked due to the influence of nutritional conditions of the substrate, by temperature or other growth conditions on the expression of biocontrol traits. This experiment measured the influence of the temperature on the efficacy of *B. amyloliquefaciens* Bs006 to inhibit *in vitro* growth of *F. oxysporum* Map5 on LB, PZN, and Landy media, and on the profile of cyclic lipopeptides in the inhibition zones.

The variation of metabolic activity among isolates of *Bacillus* spp. and even among isolates of *B. amyloliquefaciens* reported in the literature, suggests that results in this area cannot be extrapolated and that each specific interaction needs to be investigated to generate successful recommendations for their use and to reduce the variability of biological control. As such, it is urgent to standardize methodologies to reliably detect and quantify the lipopeptides produced by *Bacillus* isolates in the rhizosphere and to study the persistence and the efficacy of these molecules in the soil in order to design successful strategies for their application.

4.5. Conclusions

It was demonstrated that interaction between temperature and growth substrate for *B. amyloliquefaciens* Bs006 significantly affected its growing phases, lipopeptides profile production and antagonistic activity against *F. oxysporum* Map5. The available nutrients on LB medium allowed to Bs006 to get a high cell population but the fast growth limited the production of cyclic lipopeptides, as compared to what happened on PZN and Landy media that allowed high cell and lipopeptides concentration. Globally, our data showed that cold environment reduced lipopeptides production and antagonistic activity by *B. amyloliquefaciens* Bs006 against *F. oxysporum* Map5. However, combination of low temperature with limited content of nutritional sources accentuates this effect. Since rhizosphere environment is characterized by low availability of nutrients and low to moderate temperatures, the results of this work in part might explain the variability of

biocontrol activity of *B. amyloliquefaciens* Bs006 on cape gooseberry wilt disease caused by *F. oxysporum* Map5. Further studies about plant-pathogen-Bs006 are necessary to explain clearly the *in vivo* behavior of this rhizobacteria.

Acknowledgements

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Chapter 5. Conclusions and recommendations

5.1. Conclusions

Certainly, the conditions in which *B. amyloliquefaciens* Bs006 has been experimentally evaluated against Fusarium-wilt are extreme and difficult for a biological control agent as follow. A host highly susceptible (cape gooseberry), a pathogenic strain highly virulent (FOX-Map5), a pathogenic inoculum density considerably high (10^4 cfu.g^{-1} of soil). Moreover, in the present study Bs006 was applied to the soil at the same time with the pathogen (the day of the transplant), thus putting them in the same competitive conditions. The reduction of the disease rise by Bs006 in those conditions indicates a big potential of this antagonist as biocontrol agent. However, the variable results found in previous experiments motivated to study the antagonist-pathogen-host-environment interactions, having in mind that its plant growth activity had been consistent in absence of FOX.

In the present study it was determined that biocontrol activity by Bs006 is affected by biological vacuum in the soil, what supposes an optimal environment for fast rhizosphere colonization by FOX but at the same time an insufficient antagonistic activity of Bs006 to avoid infections in the host.

On the other hand, under natural soil conditions (non-sterile soil) it was determined that biocontrol activity of Bs006 is influenced by its own dose and the concentration of the pathogen in the soil. Where under low densities of pathogenic inoculum such as 10^2 cfu.g^{-1} of soil, low doses of Bs006 (10^7 cfu.mL^{-1}) efficiently control the disease but under high pressure of pathogenic inoculum such as >10^4 cfu.g^{-1} of soil, Bs006 show low efficacy. Interestingly, it was found that under the density of FOX 10^4 cfu.g^{-1} of soil, the dose of Bs006 10^8 cfu.ml^{-1} showed consistent reduction of the Fusarium-wilt.

In the present study, it was found that Bs006 is an efficient rhizobacteria as producer of iturins, fengycins and surfactins lipopeptides, confirming its enormous potential as biocontrol agent. However, the biological treatment used in the efficacy tests here in which the dose of Bs006 was adjusted diluting the fermented broth allowed to observe that application of high concentrations of the supernatant can favor the disease development. In this regard two hypothesis have been proposed to explain this effect. The first hypothesis supposes that the application of supernatant has the potential to create a biological vacuum in the soil owing to antimicrobial compounds in the treatment, which favor the fast colonization of tolerant propagules of FOX or those not reached by the treatment. Second hypothesis supposes a depression in the defense system of the host by high concentration of elicitors (surfactins and fengycins), leading to inefficient response to pathogen attack. Answer these questions result an interesting challenge for further research.

Supernatant free of Bs006 caused reduction of germination and growth of FOX and physical damage of the conidia in a concentration dependent manner. Evaluation of pure CLPs allowed to relate such damage with the effect caused by fengycins. Thus the fungistatic effect observed on FOX-Map5 was attributed to iturins while fungicide effect to fengycins.

Relevantly it was determined the Bs006 has high affinity for cape gooseberry root exudates which provided enough nutrients to the rhizobacteria, allowing surface colonization and synthesis of CLPs in the rhizosphere. Thus, the gnotobiotic system developed in this study allowed prove that facts as colonization and production of CLPs inhibited the colonization of FOX.

Temperature significantly affected the ability of Bs006 to synthesize CLPs, reducing its potential at cold temperatures. Nevertheless, even under cold temperature, optimal availability of nutrients could allow to the bacteria produce these antibiotics.

5.2. Recommendations

Next recommendations are studies whose responses will contribute to generate accurate directions for use of Bs006 in FOX-cape gooseberry pathosystem:

Demonstrate the impact of Bs006-supernatant and Bs006-CLPs on rhizosphere-microbiome of cape gooseberry.

Study the persistence of Bs006 in the rhizosphere and determine the ability to synthesize CLPs *in situ*.

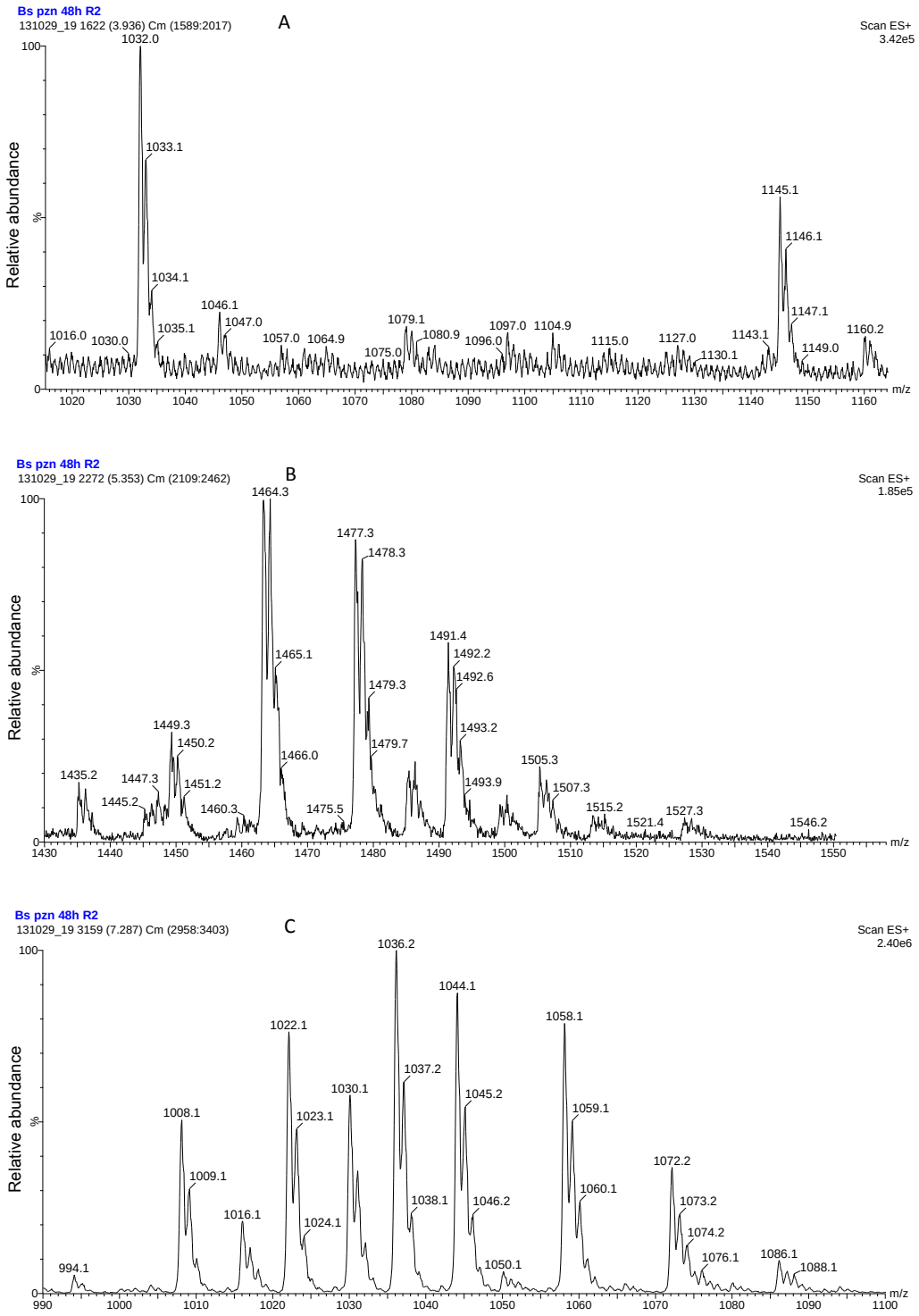
Generate Bs006-mutants impaired in CLPs biosynthesis to demonstrate clearly their influence on the antibiosis against FOX-Map5

Study the genic response from the host to Bs006 and Bs006-CLPs and to the Bs006 and FOX interaction.

Validate the results of this study using only spores of Bs006.

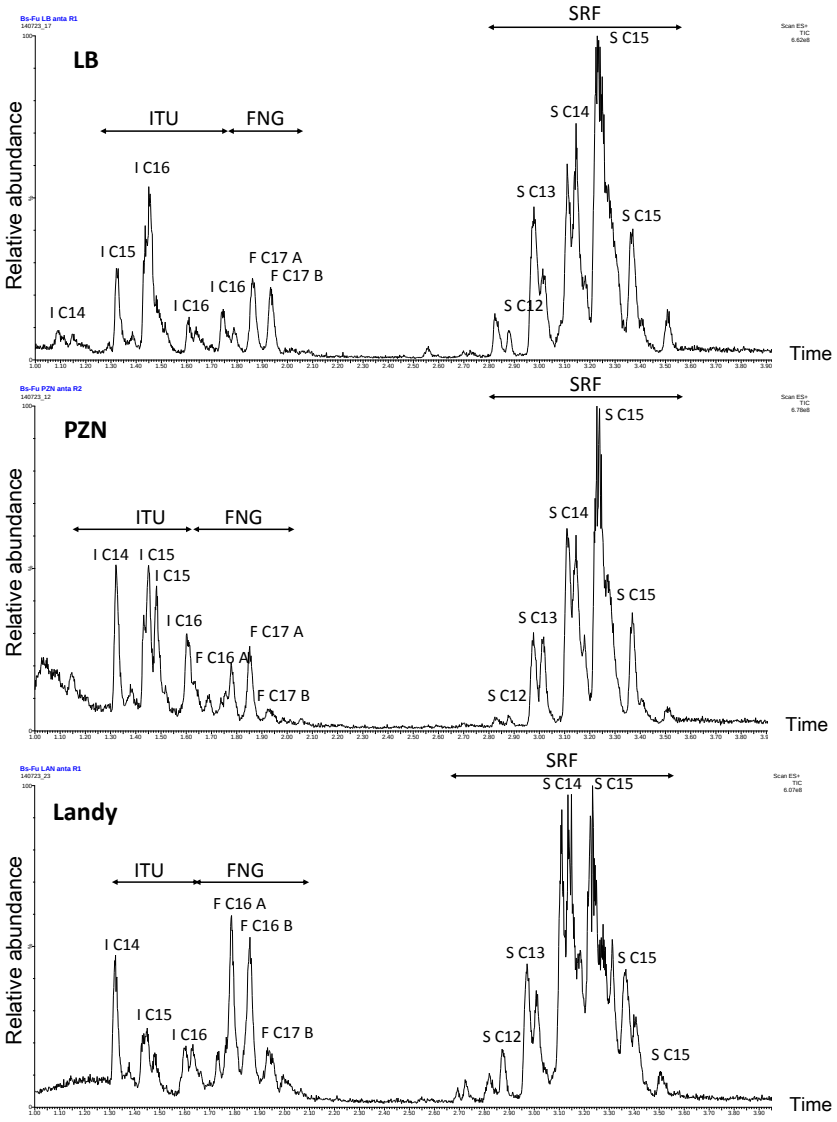
A. Appendix 1: Mass spectral of CLPs produced by Bs006 in PZN broth.

Iturins (A), fengycins (B) and surfactins (C) detected by UPLC-ESI-MS



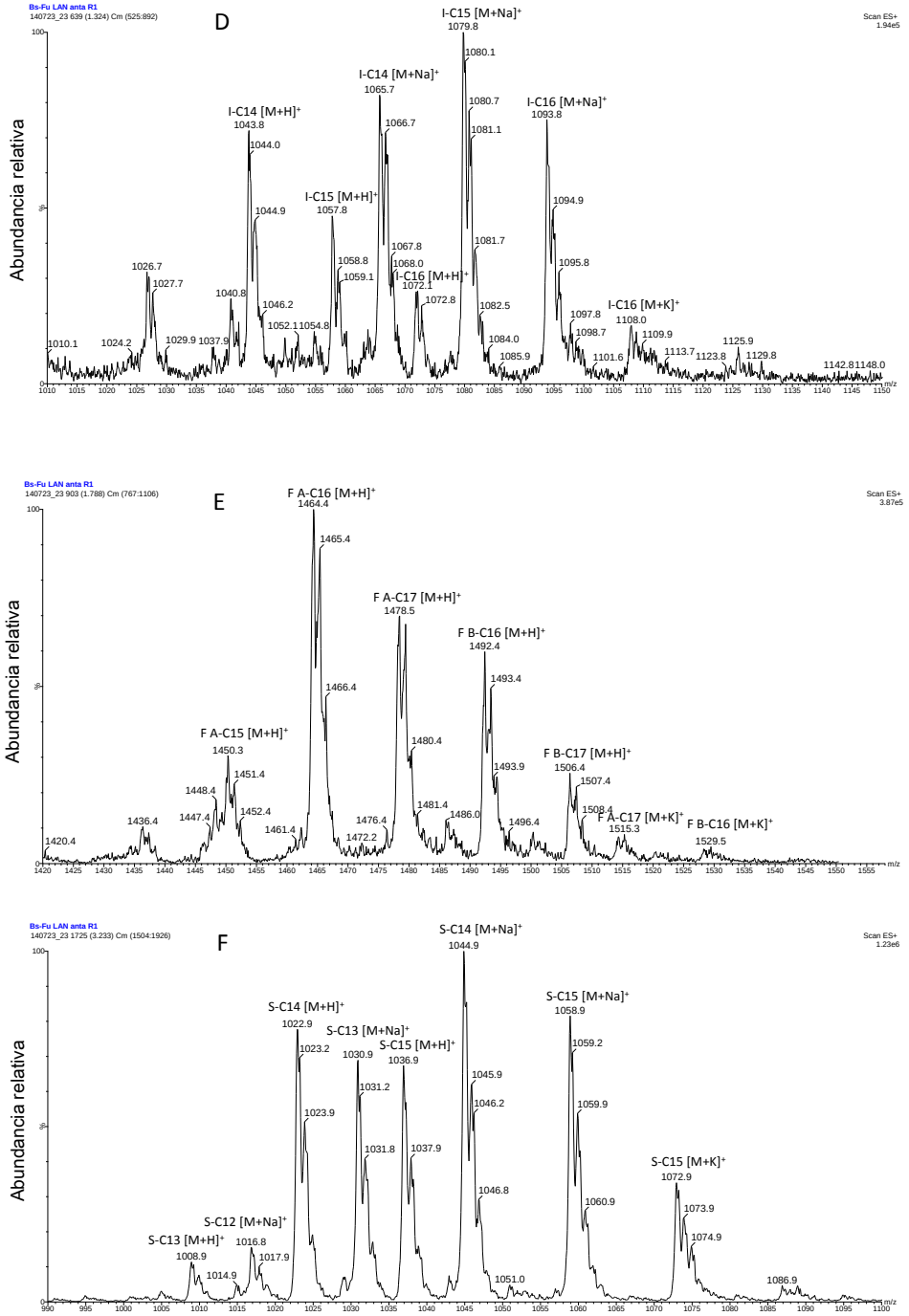
B. **Appendix 2:** Comparison of representative LC-MS profiles

Iturins (ITU), fengycins (FNG) and surfactins (SRF) produced by Bs006 in the inhibition zones of FOX-Map5 growth in LB, PZN and LANDY media. Homologue compounds of each CLP family are indicated according to lipid tail length from 12 to 17 carbons as I C14-17 (iturins); F C15-17 (fengycins); S C12-16 (surfactins).



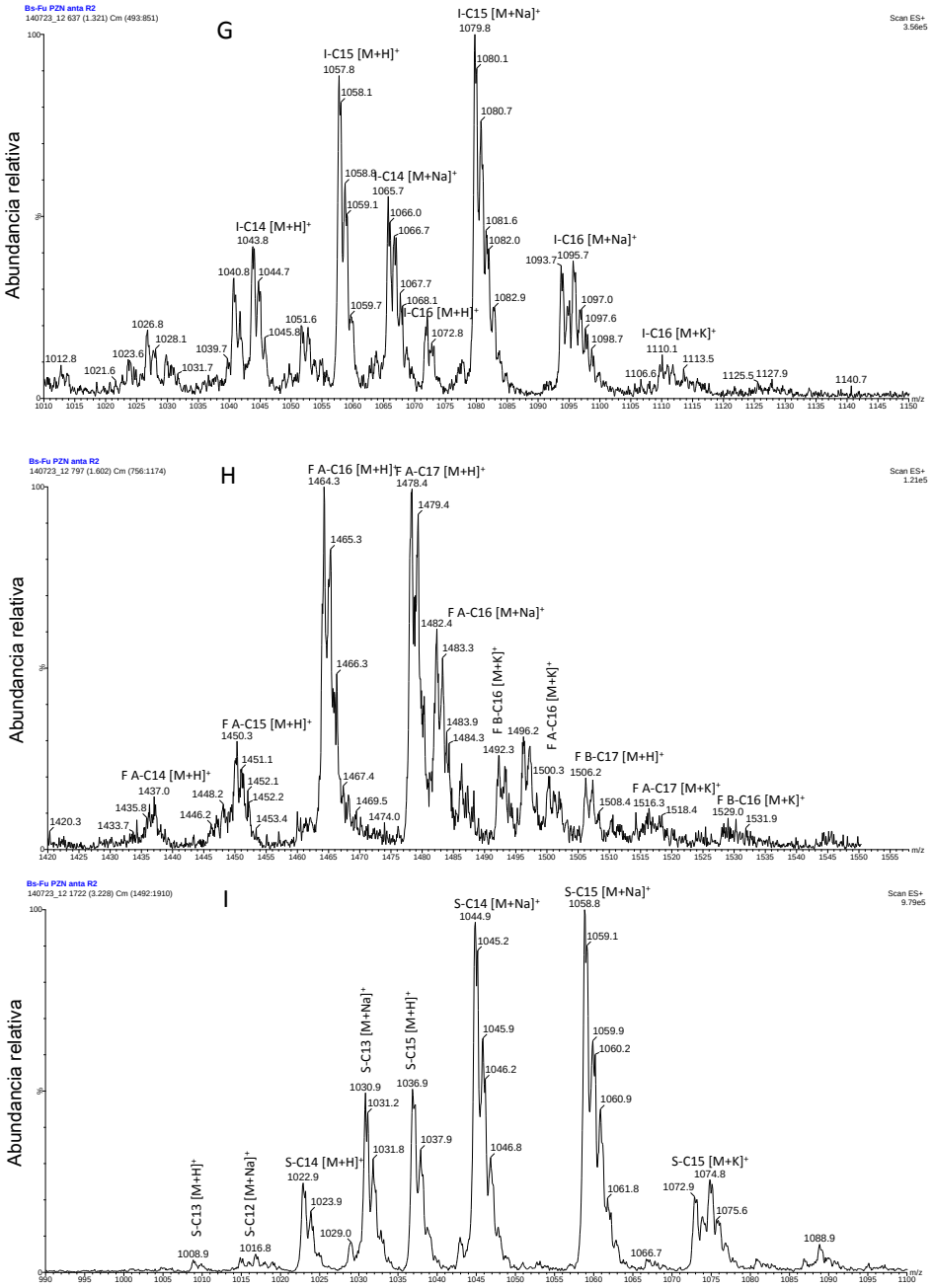
C. **Appendix 3:** Mass spectral of CLPs produced by Bs006 in inhibition zones (Landy)

Iturins (D), fengycins (E) and surfactins (F) produced by Bs006 in inhibition zones of FOX-Map5 growth in Landy-Agar obtained by ESI-MS analysis. Homologue compounds of each CLP family are indicated according to lipid tail length from 12 to 17 carbons as I C14-17 (iturins); F C15-17 (fengycins); S C12-16 (surfactins).



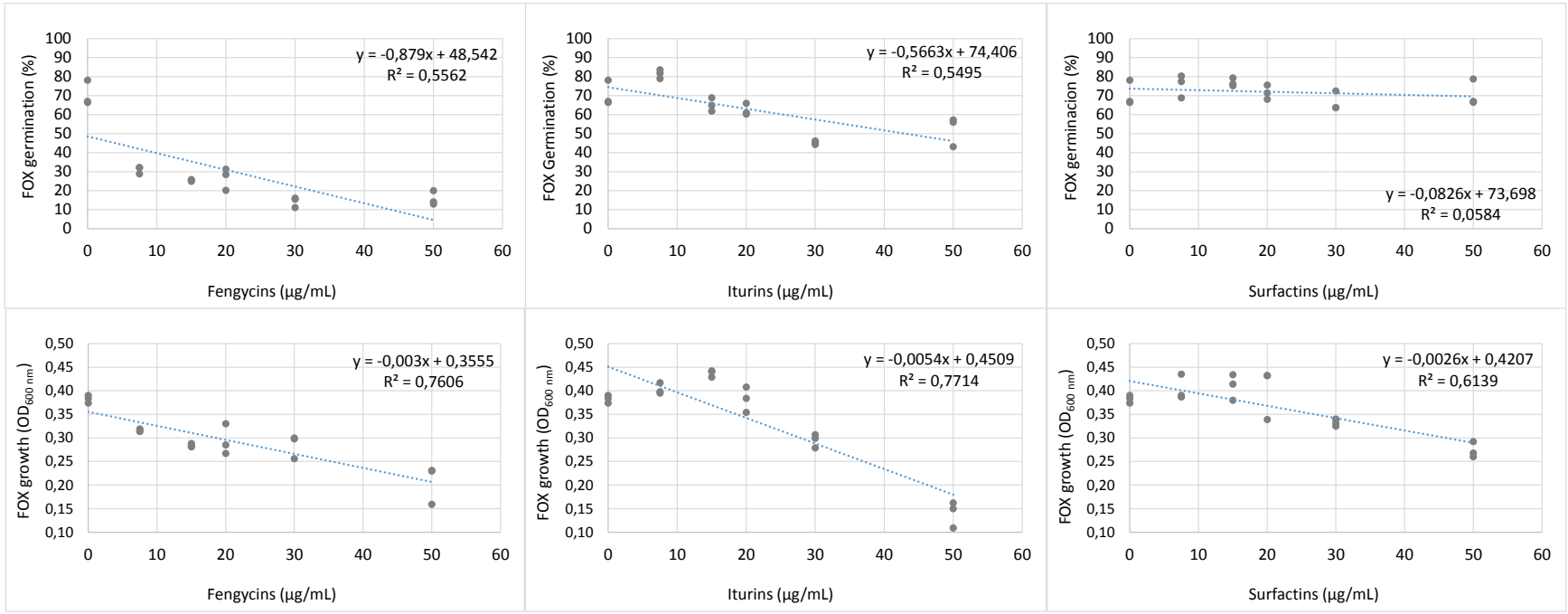
D. **Appendix 3:** Mass spectral of CLPs produced by Bs006 in inhibition zones (PZN)

Iturins (G), fengycins (H) and surfactins (I) produced by Bs006 in inhibition zones of FOX-Map5 growth in PZN-Agar obtained by ESI-MS analysis. Homologue compounds of each CLP family are indicated according to lipid tail length from 12 to 17 carbons as I C14-17 (iturins); F C15-17 (fengycins); S C12-16 (surfactins).

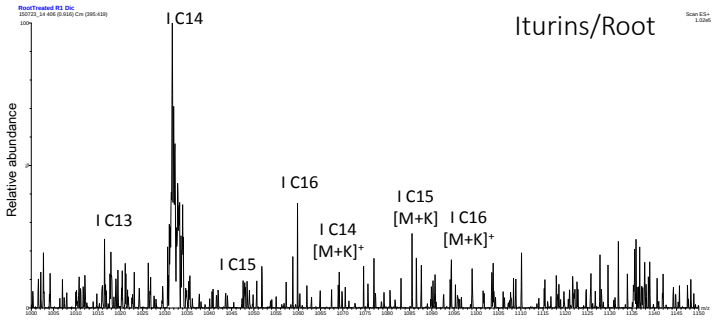


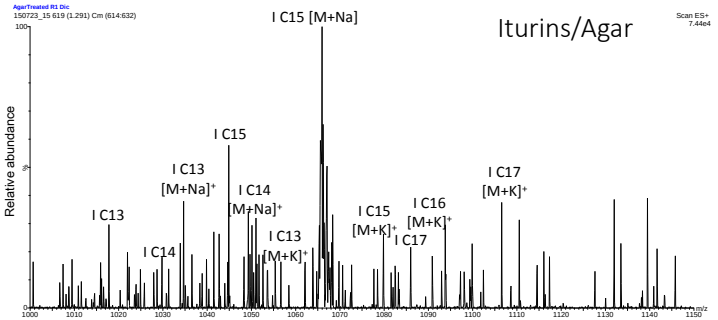
E. Appendix 4: Correlations between CLPs concentration and measured variables of FOX development.

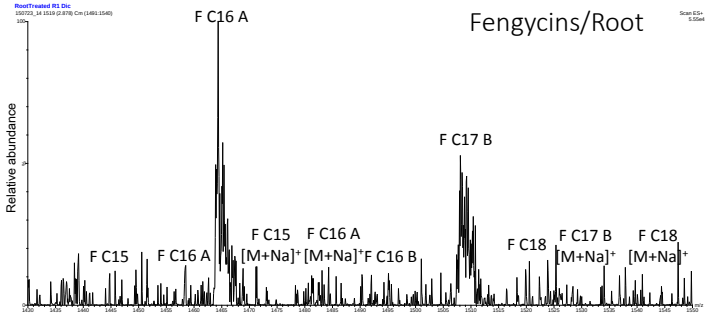
Presented data are from three biological repeats.

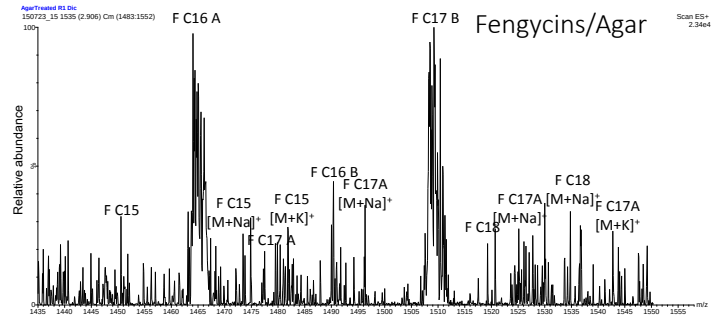


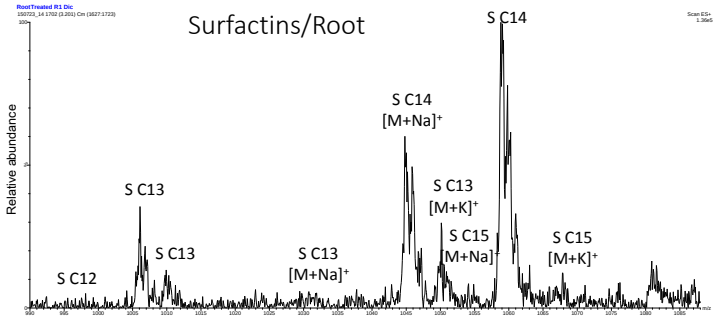
Appendix 5: CLPs produced by Bs006 on the cape gooseberry root surface and diffused on the agar in the gnotobiotic system

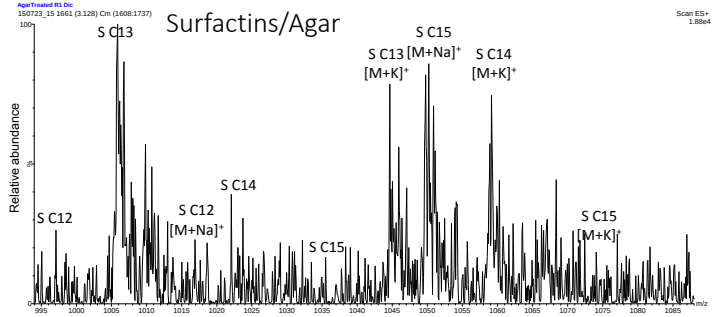




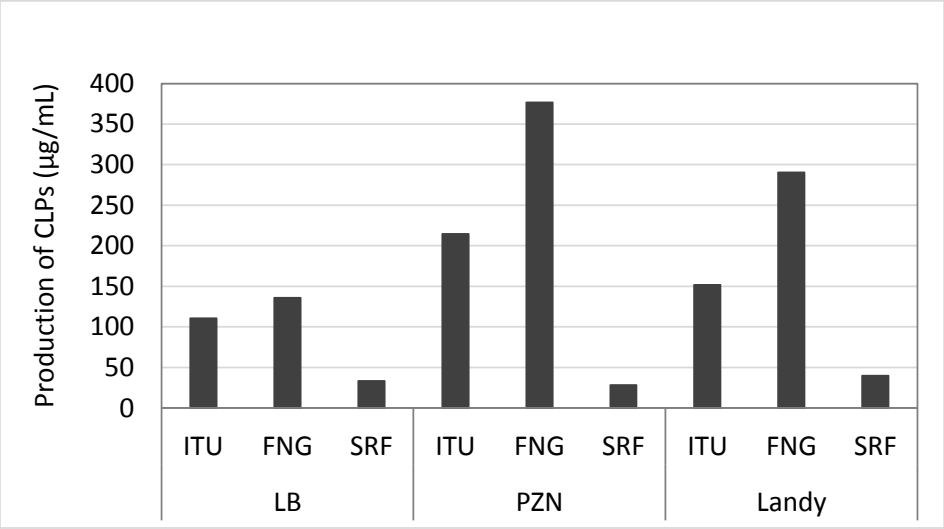






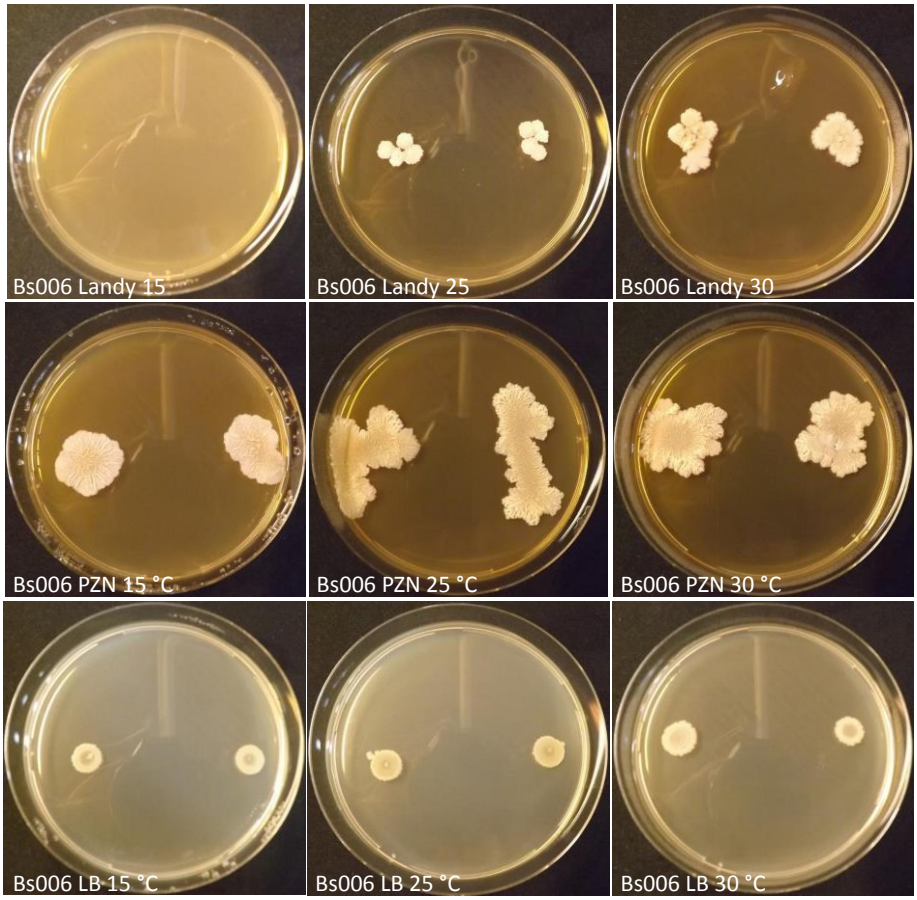


A. Appendix 6: Typical amount of CLPs produced by Bs006 in liquid culture



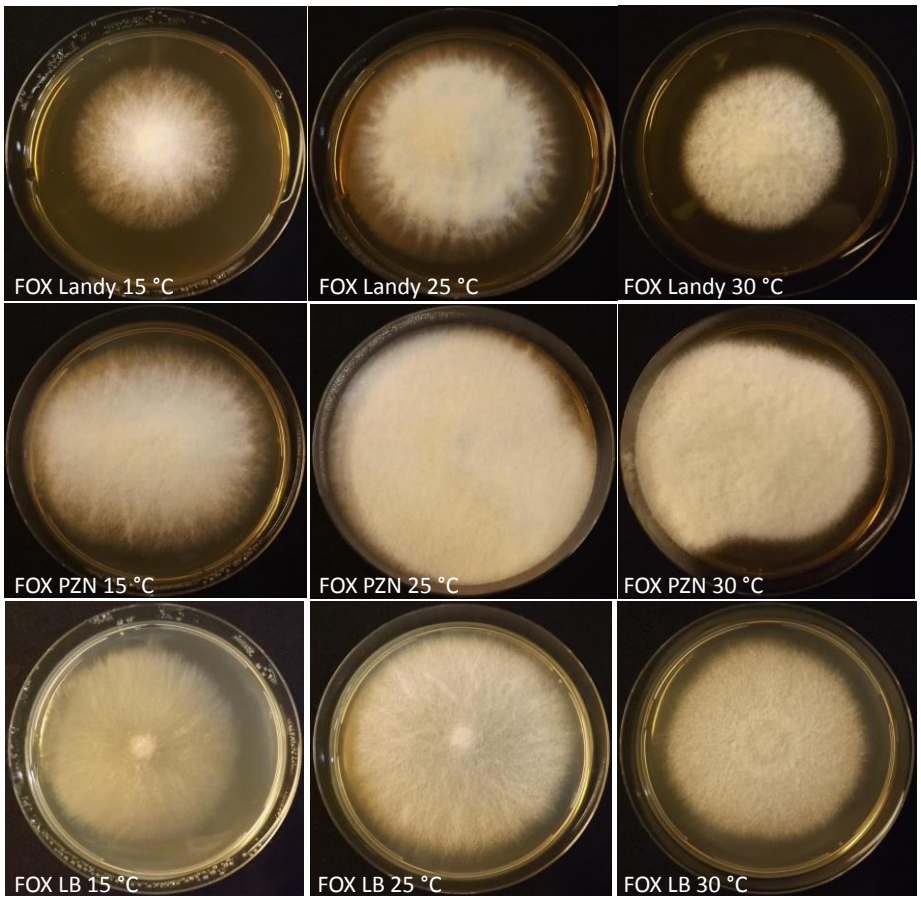
CLPs produced by Bs006 grown during 48 h in LB, PZN and Landy media under 30 °C and 150 rpm conditions. Samples of fermented broth were centrifuged (12000 rpm, 10 min) and filtered (0.22 µm). Harvested supernatant was directly injected to UPLC-ESI-MS equipment. These supernatants were used to evaluate their effect on the germination and growth of FOX-Map5.

B. Appendix 7: Control - *B. amyloliquefaciens* Bs006



Bs006 growing in absence of *F. oxysporum* Map5 on LB, PZN and Landy media at 15, 25 y 30 °C. Pictures were taken on 7th day after inoculation of microorganisms.

C. Appendix 8: Control - *F. oxysporum* Map5



FOX-Map5 growing in absence of *B. amyloliquefaciens* Bs006 on LB, PZN and Landy at 15, 25 y 30 °C. Pictures were taken on 7th day after inoculation of microorganisms.

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