FERNANDO PEREIRA MONTEIRO

COLONIZATION OF BEAN PLANTS BY *Bacillus amyloliquefaciens* AND ITS POTENTIAL AS A BIOLOGICAL CONTROL AGENT

LAVRAS - MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitopatologia, área de concentração em Fitopatologia, para a obtenção do título do doutor

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COLONIZAÇÃO DAS PLANTAS DE FEIJÃO POR *Bacillus amyloliquefaciens* E SEU POTENCIAL COMO AGENTE DE CONTROLE BIOLÓGICO

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitopatologia, área de concentração em Fitopatologia, para a obtenção do título do doutor

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Dedico

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RESUMO GERAL

Entre os agentes de controle biológico, *Bacillus amyloliquefaciens* é destacado por seu sucesso em controlar doenças de plantas. As moléculas responsáveis por isso são conhecidas como lipopeptídeos, substâncias-chaves que dão algumas vantagens para as bactérias. Objetivou-se, neste estudo, compreender o comportamento bacteriano enquanto coloniza plantas de feijão sob duas temperaturas (28º e 20ºC) e a produção de lipopeptídios nos meios de cultura (Otimalizado, MB1, PDB e Luria) e nos exsudatos de raízes e seiva dos caules que foram testados como substratos. O pH ótimo foi também avaliado dentro de uma gama variando de 5 a 9, usando MB1. Entre todos esses substratos testados o único capaz de produzir todos os lipopeptídios (surfactina, fengycina e iturina), foi o PDB. Usando a seiva do caule e exsudato de raiz como substrato foi observada uma fonte de alimento que pode suportar o crescimento bacteriano. Surfactina e iturina também foi produzido após 48 horas. O melhor pH para a produção de lipopeptides foi 6. No que diz respeito à colonização, a bactéria foi encontrada em todos os órgãos da planta (folhas, caules e raízes) a 28ºC, sendo discriminados de acordo com o comportamento endofítico e epífita, mas a 20°C não foi encontrada qualquer bactéria em folhas, em nenhum dos métodos de introdução da bactéria à planta empregados. Quando a bactéria foi aplicada sobre a superfície das folhas foi observado que a população epífitas foi extremamente reduzida após 24 horas. O controle biológico de *Botrytis cinerea* in vitro e em morangos também foi avaliada num segundo artigo. Uma experiência para atestar o antagonismo contra *B. cinerea*, foi realizado, utilizando três meios diferentes (PDA, otimizado e MB1) e também três diferentes temperaturas (15, 25 e 30ºC). A produção de voláteis também foi avaliada em cinco meios (PCA, otimizados, Luria, MB1 e NAA), realizando-se a identificação dos principais compostos. O efeito dos metabólitos produzidos por B. amyloliquefaciens foi também realizado, para estudar como seria nocivo sobre os esporos. *In vivo*, os morangos foram tratados com metabólitos bacterianos, sendo infectados pelo fungo, sucessivamente. No antagonismo direto foi observado o controle micelial apenas para o substrato PDA. Para os compostos voláteis, o melhor substrato que controlou o crescimento do fungo foi o meio otimizado. Os principais compostos produzidos foram 2,3-butanodiol eacetoína. A germinação de esporos foi inversamente proporcional à concentração de metabolitos. Os tratamentos com os metabólitos foram capazes de proteger os morangos contra *B. cinerea*. Com base nos resultados,*Bacillus amyloliquefaciens* pode desempenhar um papel importante contra doenças de plantas.

Palavras-chave: Surfactina. Fengicina. Iturina. Lipopeptídeos.Voláteis.

GENERAL ABSTRACT

Among the biological control agents, Bacillus amyloliquefaciens is highlighted by its success in controlling plant disease. The molecules responsible for it are the lipopeptides, key substances that give some advantages to the bacteria. Surfactins are bioemulsifing molecules that act lowering surface tension and favor bacterial spread. Fengycins and iturins are the active substances against the pathogens. This study aimed to understand bacterial behavior while colonizing bean plants under two temperatures (28ºC and 20ºC) and its lipopeptides production in culture medium (Optimized, MB1, PDB and Luria) and exudates from roots and stems fluid as substrates. The optimum pH was also evaluated within a range from 5 to 9 using MB1 as substrate. The temperature and substrate type influenced the amount of lipopeptide produced, affecting it both qualitatively and quantitatively. Among all the substrates tested, the only one capable of producing all the lipopeptide (surfactin, fengycin and iturin) was PDB. By using the stem fluid and root exudate as a food source, it was found that it can support the growth. Surfactin and iturin were also produced after 48 hours. The best pH for lipopeptide production was 6. Concerning colonization, the bacteria were found in all plant organs (leaves, stems and roots) at 28ºC, being discriminated according to endophytic and epiphytic behavior, but at 20°C no bacteria were found on leaves. When the bacterium was applied on the surface of the leaves, it was observed that the epiphytic population was extremely reduced after 24 hours. The biocontrol of Botrytis cinerea in vitro and over strawberries was also evaluated. An experience to attest antagonism against B. cinerea was performed using three different media (PDA, Optimized and MB1) and also three different temperatures (15, 25 and 30ºC). The volatile production was also evaluated in five different media (PCA, Optimized, Luria, MB1 and NAA), with the identification of the major compounds. The effect of the metabolites produced by B. amyloliquefaciens was also performed to see how harmful it would be on the spore. In vivo the strawberries were treated with bacterial metabolites, being infected by the fungus, successively. Regarding direct antagonism, only the PDA substrate showed control. For the volatile compounds, the best substrate to control fungus growth was optimized. The major compounds produced were 2,3-butanediol and acetoin. The spore germination was proportionally inverse to the metabolite concentration. Treatments with the metabolites were capable of protecting the strawberries against the B. cinerea. Based on the results Bacillus amyloliquefaciens can play an important role against plant diseases.

Keywords: Surfactin. Fengycin.Iturin.Lipopeptides.Volatile.

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FIRST PART

1 INTRODUCTION

Bacillus sp. is a widespread bacteria present mainly in cultivated soils. As a microorganism it can act in different environments, with influence on its dynamics. Industrial use is hugely employed for specific strains. It has been exploited for agricultural practices. Within this group, *Bacillus subtilis* is one of the most known prokaryotes concerning molecular and cell biology, making this microorganism a model to study prokaryotes. The success of this group can be seen in the successful commercial formulation applied in agricultures nowadays, protecting different kinds of plants in a huge variety of pathosystems.

B. amyloliquefaciens belongs to *B. subtilis* group and stands out for its application in crop protection. It can play different roles such as direct antagonism, plant growth promoter and ISR induction. Among the substances produced, cyclic lipopeptides are the main ones responsible for control. They are a class of secondary bacterial metabolites based on a polypeptide chain, generally composed of seven amino acids and a 3-hydroxy or 3-amino fatty acid, connected in two places, forming a macro-cyclic structure (Romano et al., 2013). The most common families are surfactins, fengycins and iturins. Surfactins act like a bioemulsificant that lowers the surface tension and favors bacteria spreading. Fengycins and iturins are direct linked to phytopathogens control.

It can also produce a wide range of volatile compounds. These substances are also produced by Bacillus spp., preventing fungal growth. As they are secondary metabolites, the type of volatile compounds depend on the substrate promoting or not a harmful effect against fungus. The boiling point determines how volatile the compound will be in environmental conditions. This

is important in practical uses, because a closed environment is required to give time for the volatile compound to be effective against the pathogen.

The effect of environment plays an important role on the secondary metabolites produced. The main variables that change the bacterial metabolism dynamics are temperature, light and ph. Regarding temperature, it can have influence not only on the population, but also on the amount of molecules produced. Light seems to have qualitative influence, because a huge difference in the substances produced is observed when treatment in contact with light is compared to those that remain in the dark. PH also has influence on both population and substances produced. For instance, pH 6 is the best for production of lipopeptides.

Once seen that it can act against pathogens, many efforts are required to apply the bacteria in vivo conditions. Efficient bacteria would be able to colonize all organs (leaves, stems and roots). Larger populations may create a protection against harmful pathogens avoiding the infection. Generally, it has demonstrated a common ability to colonize roots, but few papers have shown its behavior in colonizing other plant organs.

Based on this, the objective was to understand the bacterial behavior while colonizing bean plants under two temperatures (28ºC and 20ºC) and its lipopeptides production, with culture medium (Optimized, MB1, PDB and Luria) and exudates from roots and stems fluid as substrates. The optimum pH was also evaluated within a range from 5 to 9 using MB1 as substrate. The temperature and substrate type influenced the amount of the lipopeptide produced, affecting it both qualitatively and quantitatively.

The biocontrol of *Botrytis cinerea* in vitro and on strawberries was also evaluated. An experience to attest antagonism against *B. cinerea* was performed using three different media (PDA, Optimized and MB1) and also three different temperatures (15, 25 and 30ºC). The volatile production was also evaluated in

five different media (PCA, Optimized, Luria, MB1 and NAA), identifying major compounds. The effect of the metabolites produced by B. amyloliquefaciens was also tested to see how harmful it would be over the spore. In vivo, the strawberries were treated with bacterial metabolites being infected by the fungus, successively.

2 THEORETICAL FRAMEWORK

2.1 *Bacillus subtilis* group metabolites

Bacillus spp. are bacteria with rod-shaped, Gram-positive, variable length measuring 0,5 x 1,2 µm until 2,5 x 10 µm, member of the Firmicutes phylum. Generally, it moves due to peritrichous cilia. Species in the genus can be obligate aerobes or facultative anaerobes. It is able to produce enzymes and toxins. Ubiquitous in nature, it normally plays a role of biological control agent in the environment. Under stressful environmental conditions, it can produce endospores (one vegetative cell may generate a single endospore), oval structures that remain in a dormant state for long periods until conditions become favorable again. The ability to produce endospore, growth under a variety of temperatures and substrates, and produce a diverse range of bioactive molecules renders *Bacillus subtilis* a promising microorganism to be used in the biological control of various plant diseases. A particular interest in studying these organisms is the ability it has to produce many antibiotics, an average 4 to 5% of its genome is dedicated to antibiotic synthesis (Stein, 2005). Not surprisingly, many authors have obtained successful disease control by using Bacillus-based products. Nihorimbere et al. (2010) showed in vitro antagonistic activity of Bacillus spp. against *Fusarium* sp. Among the compounds produced, the most versatile were lipopeptides, characterized by presence of peptides linked to fatty acids, and the amino acids are frequently disposed in a cyclic structure. A property common to all of them is the ability to change the physical and chemical traits, mainly in liquid surfaces.

There are two ways *Bacillus subtilis* synthases lipoptides:, either by non-ribosomal peptide synthetases (NRPSs) or by hybrid polyketide synthases and non-ribosomal peptide synthetases, PKSs/NRPSs (Ongena & Jacques 2007).

Although many metabolites are produced, the most studied ones are surfactin, iturin and fengycin (Peypoux et al. 1978, 1999; Vanittanakom et al. 1986).

Surfactin is a kind of molecule included in a large lipopeptide family excreted by some strains of Bacillus sp.. There is little diversity in the substances produced by different strains due to the ramification of the fatty acid chain, including differences in the peptide sequence (Dufoura et al. 2005), but all members are heptapeptides with a β –hydroxyl fatty acid to form a cyclic lactone ring structure (Peypoux, et al.,1999). The fungi-toxicity properties are based on membrane disruption or solubilization, but surfactin has this toxicity when acting synergistically with iturin (Maget-Dana et al. 1992). Studies indicate that this lipopeptide has an unique effect on the formation of a stable biofilm in the roots in some plants, which is not observed for iturin and fengycin, maybe due to it being more amphiphilic than others (Kinsinger et al.2003; Hofemeister et al.2004). According Bais et al. (2004) the surfactin acts in the reduction of the infection caused by *Pseudomonas syringae* on Arabidopsis and is accept as anti-viral compound to cause disintegration of the envelope.

According Peypoux et al. (1999) the srf operon encodes genes related in the surfactin biosynthesis. Nihorimbere et al. (2009) published that lipopeptide secretion still remains active in nutrition solution given to tomato plantlets, showing that exudate components are conducive for srfA operon expression, even in oxygen-starved culture conditions. They also reported the first demonstration of efficient lipopeptide gene expression in the rhizosphere during Bacillus – plant interaction. Ongena et al. (2007) report that surfactin can be perceived by plant cells to trigger a signal to initiate defense mechanisms. They also report the surfactins and fengycins involvement in Induced Systemic Resistance (ISR) acting as elicitors. Jourdan et al. (2009), working with tobacco cells, showed that surfactin treatment lead to major changes in the phenolic

pattern. These authors also showed that the relative H_2O_2 accumulation increased with surfactin concentration. It is also known that surfactin synthetase expression is pH-dependent and its production is influenced by the composition of the culture medium.

Another lipopeptide is iturin, which has a large number of variants that are characterized as heptapetides, linked to a β-amino fatty acid chain with 14 to 17 carbons. Its activity differs from surfactins, presenting a strong antifungal action against some yeasts and fungi, but there is no effect on bacteria and virus. The fungi-toxicity probably acts by providing an osmotic perturbation allowing the formation of ion-conducting pores. These are considered derivative forms of the iturin family, mainly the molecules: iturin A and C, bacillomycin D, F, L and LC and mycosubtilin. According to Bonmatin et al. (2003) the presence of ergosterol in fungi and yeasts plasma membranes helps the activity of iturin.

The third most studied lipopeptide is fengycin, which is known as plipastatins also. There are two variations in this family: A and B distinct from different amino acids on its chain. It is lipodecapeptide with an internal lactone ring in the peptidic moiety and with a β – hydroxyl fatty acid chain with C14 to C18(carbons) which can be saturated or unsaturated. The molecules have strong fungi-toxicity, mainly towards filamentous fungi. It acts probably by interacting with lipid bilayers or altering cell membrane structure and permeability.

Fengycin and surfactin are known to interact with plants, leading to an immune response, a phenomenon called induced systemic resistance. According with Ongena et al. (2007) each family has a specific skill to stimulate different plant cells. These authors showed that surfactins can interact with bean, tomato and tobacco, but not with potato, whereas fengycins produced effects in all them.

These lipopeptides may act synergistically for interactions between surfactin and iturin (Maget-Dana et al.1992), surfactin and fengycin (Ongena et

al. 2007), as well as iturin and fengycin (Koumoutsi et al.2004; Romero et al.2007).

Biological control of plant diseases is especially attractive because of the diversity of metabolites it produces. We have shown that a *Bacillus amyloliquefaciens* strain (ALB 629) was able to control the disease and also inhibit pathogen growth in vitro, inducing an increase in the activity of phenylalanine-ammonia lyase, the content of total phenols and lignin in the shoot (Martins, 2012). However in the presence of the pathogen, a decrease in the induced responses was observed instead of an expected priming of defense response. When bean plants were grown inoculated with the pathogen and treated with the biocontrol agent (both at the seed level) and subsequently maintained at 20 or 30ºC (in average), there was consistent control of the disease but the bacterium was restricted to the root at the lower temperature and colonized the whole plant at the higher one. Since the bacterium is confined to the roots at the lower temperature and it controls the disease, a possible induction of resistance may be enough to cope with the disease and at the higher temperature a possible combination of antibiotics with direct activity would complement the induction of resistance in disease management. The most commonly found antibiotics produced by *Bacillus* spp.are lipopetides and it has recently been demonstrated that the temperature may influence the diversity and abundance of lipopeptides produced (Ongena, 2012).

2.2 Plant colonization

Bacillus spp. are generally sensitive to amoxicillin-clavulanic acid, gentamicin, amikacin, kanamycin, fluoroquinolones, tetracycline, chloramphenicol, rifampicin and vancomycin. It has shown itself resistant to lincomycin, to colistin and fosfomycin. The production of beta-lactamase by

countless strains limits the use and interest in penicillin and cephalosporins. Normally, a pioneer method is to isolate strains resistant to a specific antibiotic. Once this has succeeded, it is possible to study the population that is colonizing the plant. An additional method is to proceed with sequencing using primers complementary to essential genes.

Lugtenberg & Kamilova (2009) published a review demonstrating important points concerning root colonization, such as nutrients for microbes in the rhizosphere and colonization genes and traits. During root colonization, bacteria can act directly as a plant growth promoter. Van Loon (2007) discussed plant responses to plant growth-promoting bacteria. In this capacity, some microorganisms can act as biofertilizers, rhizoremediators or phytostimulators.

B. subtilis can decreases plant susceptibility in some cases. Ongena et al. (2004) studying a interaction with *B. subtilis* and tomato seeds against the disease incited by *P. aphanidermatum* observed an increasing host resistance associated with differential gene expression. Sarosh et al. (2009) also observed different gene expression involved in microbial interactions while studying the pathosystem *Brassica napus* and *Botrytis cinerea* influenced by *B. amyloliquefaciens.*

2.3 Botrytis cinerea and grey mold: an widespread fungus and serious disease

Botrytis cinerea is aplant pathogen considered necrotrophic and able to incite disease in more than 200 crops worldwide. It has become an important model for molecular study of necrotrophic fungi as it is easy to deal with. Its taxonomy is as follows: kingdom: Fungi, phylum: Ascomycota, subphylum: Pezizomycotina, class: Leotiomycete, order: Helotiales, family: Sclerotiniaceae, genus: Botryotinia. The fungus is able to produce a wide range of cell-wall-

degrading enzymes, toxins and other low-molecular-weight (Williamson et al., 2007). The fungus is the etiologic agent that causes a disease known as grey mold, one of the most serious post-harvest diseases of fruit and vegetables; for instance, in strawberries it can cause serious problems, reducing its availability the market.

As for the bacteria, *Bacillus subtilis* groups are successful in controlling plant disease. It has been reported as a suppressor against a sort of pathogen. Specifically, B. amyloliquefaciens belongs to this major group, acting like a biological control agent when combined with *Rhizoctonia solani* (YU et al., 2002); *Botrytis cinerea* associated with fresh-market tomatoes (MARI et al., 1996); *Colletotrichum dematium* attacking mulberry (HIRADATE et al., 2002); *Thielaviopsis paradoxa*, *Colletotrichum musae* and *Fusarium verticillioides* (ALVINDIA & NATSUAKI, 2009); *Fusarium graminearum* (DUNLAP et al., 2013); *Sclerotinia sclerotiorum* (ABDULLAH, 2008); *Rhizopus stolonifer* and *Penicillium expansum* on peach fruit (ARREBOLA et al., 2010); *Penicillium digitatum* and *Geotrichum candidum* (HAO, et al., 2011). It can also be applied to bacteria as *Ralstonia solanacearum* (Hu et al. 2010) and *Erwinia amylovora* (Chen, et al. 2009).

2.4 Strawberries

The global production of strawberries in 2011 was approximately 4.2 million tons. The United States of America, Turkey, Spain, Egypt and Mexico are the leading producers. The five highest yields were obtained in the United States of America, Jordan, Morocco, Egypt and Cyprus. In Brazil, the harvest area was estimated in 376 ha, with three thousand tons and a yield of 80 thousand tons (Hg/ha) (FAOSTAT, 2011). Diseases are an important factor that limits yield and profit. Among them, gray mold is an important disease that

requires management to ensure good quality products on supermarket shelves.

3 OBJETIVES

3.1 General objectives

The objective was study the influence of environmental factors on lipopeptide accumulation, bean plant colonization and volatiles production by the bacterium *B. amyloliquefaciens*.

3.2 Specific objectives

a) Bacterial identification using Rec A and Gyr A primers;
b) Lipopeptides analysis in function of culture media and temperature;
c) Direct antagonism against *Aspergillus ochracius*, *A. niger* and *Fusarium oxysporum* in different temperature conditions;
d) Lipopeptides accumulation by *B. amyloliquefaciens* using stem sap;
e) Lipopeptides accumulation by *B. amyloliquefaciens* using root exudates
f) Lipopeptides accumulation influenced by different pHs levels;
g) Bean plant colonization using different inoculation methods;
h) Volatile compounds produced *B. amyloliquefaciens* varying culture media;
i) Effect of thermostable metabolites on conidial germination of *Botrytis cinerea*;

j) Biological control of grey mold using *B. amyloliquefaciens* as a biological control agent.

4 General Provisions

The study shows how temperatures and substrates are important to specific compound production. These results may be used for biological control applied in agricultural fields, as well as in the synthetic industry, which intends to produce it in commercial scale. Further, this shows how the temperatures can change the bacteria population dynamics.

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SECOND PART - ARTICLES

ARTICLE 1 **A Tropical strain of *Bacillus amyloliquefaciens*:** lipopeptides accumulation and different inoculation methods for bean plant colonization studies

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A tropical strain of *Bacillus amyloliquefaciens*: Lipopeptides accumulation and different inoculation methods for bean plant colonization studies

ABSTRACT

The lipopeptides surfactin, iturin and fengycin play a role in the colonization of plants providing a competitive advantage for the lipopeptide-producing bacterium. How culture media influences the accumulation of these compounds in *Bacillus* spp. as well as the relationship between lipopeptide accumulation and effective bacterial colonization in plants has not been previously investigated. Here, the influence of four growth media, including luria, potato-dextrose- broth (PDB), MB1 and optimized, three temperatures 30, 25 and 15ºC ,and varying pHs from 5 to 9 using only MB1 media were studied for lipopeptide diversity and abundance in *Bacillus amyloliquefaciens*. Lipopeptide accumulation was also measured using either a stem-sap or root-exudate. Finally, bacteria were identified as either endophytes or epiphytes when grown at 28 and 20º C, using a bean-plant colonization assay. Lipopeptide accumulation was influenced by temperature and media conditions in a quantitative and qualitative manner. Lipopeptide

accumulation was possible in MB1 at a pH of 6 and 7, while with other tested pH conditions, bacterial growth was observed without detecting lipopeptide. Endophytic and epiphytic *B. amyloliquefaciens* populations were influenced by the method of inoculation, showing differences in leaf, stem and root colonization. When a bacterial suspension was applied on the leaf surface, bacterial populations rapidly diminished within five days. These studies established the contribution of temperature, pH and culture media on lipopeptide accumulation and bacterial colonization in plants with potential applications to effective bacterial-plant interactions in a field setting.

RESUMO

Os lipopeptídeos surfactina, iturin e fengicina desempenham um papel relevante na colonização de plantas pois fornecem uma vantagem competitiva para a bactéria que os produz. Como os meios de cultura influencia a acumulação desses compostos por *Bacillus* spp., bem como a relação entre a acumulação de lipopeptídeos e a eficiência na colonização bacteriana em plantas, não foram anteriormente investigados. Aqui foram

estudadas a influência de quatro meios de crescimento incluindo Luria, batata-dextrose-caldo (PDB) , MB1 e otimizado, três temperaturas de 30, 25 e 15º C e diferentes pHs variando de 5 a 9 , utilizando o meio MB1, para avaliar a diversidade e abundância de lipopeptídio em *Bacillus amyloliquefaciens*. A acumulação de lipopeptídio também foi medida usando uma haste - seiva ou raiz - exsudato . Por fim , as bactérias foram identificadas como endófitos e epífitas , quando cultivadas a 28 e 20 º C ,utilizando-se um ensaio de colonização de feijão - planta . Acumulação lipopeptídio foi influenciado por condições de temperatura e de mídia, de forma quantitativa e qualitativa. Acumulação lipopéptido era possível em MB1 a um pH de 6 e 7 , enquanto que com outras condições de pH testados , o crescimento bacteriano foi observado sem lipopéptido detectado . Endofíticos e epífitas B. amyloliquefaciens populações foi influenciado pelo método de inoculação , mostrando as diferenças de licença , caule e raiz colonização . Quando uma suspensão bacteriana foi aplicada na superfície da folha , as populações bacterianas diminuíram rapidamente no prazo de cinco dias. Esses estudos determinaram a contribuição da temperatura , pH e cultura da mídia sobre a acumulação lipopeptídio e colonização bacteriana em plantas ,com potenciais

aplicações para interações efetivas bacterianas de plantas, em ambiente de campo.

INTRODUCTION

While protein synthesis is limited to the standard 20 amino acids via ribosomal catalysis, lipopeptides are biosynthesized from amino acid-lipid modules via non-ribosomal peptide synthetases (NRPS) that are then incorporated into cyclic structures. The presence of non-standard amino acids in lipopeptide distinguishes this biosynthesis from ribosomal metabolism (Finking & Marahiel, 2004). Among the many molecules produced by lipopeptide biosynthesis, surfactin, fengycin and iturin are the most studied with regard to the biological control of fungi and bacteria. The surfactin family is represented by heptapeptide variants of the esperin, lichenysin, pumilacidin and surfactin groups. The fengycin family is divided in fengycin A and B, usually referred to as plipastatins.

The main iturins produced are iturin A and C, bacillomycin D, F and L, with mycosubtilin being the more frequent variant. In fact, for all lipopeptides (surfactins, fengycins and iturins) different homologs are usually co-produced (Akpa et al., 2001). According to Duitman et al. (2007), two-component systems and quorum sensing function in

lipopeptide-biosynthesis regulation. Generally, surfactin gene expression is associated with cell-density increase and occurs in the transition between exponential to stationary growth, whereas fengycins and iturins biosynthesis occurs later in the stationary phase.

Surfactins are biosynthesized from three NRPS's called SrfA, SrfB and SrfC (Peypoux et al., 1999) in combination with the thioesterase/acyltransferase enzyme SrfD that catalyzes lipopeptide initiation (Steller et al., 2004). Surfactins are amphiphilic cyclic peptides composed of seven α-amino acids linked to a single β-hydroxyl fatty acid varying in length from 13 to 16 carbons. This amphiphilic property defines surfactin as emulsifying agent. Three functions have been proposed for these bioemulsifiers: (i) promotes an increase of surface area for hydrophobic water-insoluble growth substrates; (ii) promotes an increase in bioavailability of hydrophobic substrates by increasing solubility and (iii) promotes the attachment and detachment of bacterial to and from surfaces (Rosenberg & Ron, 1999). Carrillo et al. (2003) report that 20 µM of surfactin solution lowers the surface tension of water at 72 to 27 mN.m-1, demonstrating the strong detergent-like action. Surfactins also serve in bacterial motility facilitating cell spreading through a

substrate (Kinsinger et al., 2003) via reducing surface tension (Leclère et al., 2006).

Fengycin has considerable variation in some structural properties as cyclization and branching, and also unusual constituents in its chain. This amphiphilic cyclic peptides are composed of ten α-amino acids linked to β-hydroxy fatty acid, which vary from 14 to 18 carbon. It has strong antifungal activity. Usually it is specific to filamentous fungi (Stelleret al., 1999; Vanittanakom et al., 1986).

Iturins are amphiphilic cyclic-peptides with seven α-amino acids linked to one β-amino fatty acid, which has 14 to 17 carbons. This lipopeptide family is mainly related to antibiotic activity; many Bacillus spp. can act against a broad range fungus species. However the antibacterial activity is limited to a few species.

Temperature may interfere with lipopeptide accumulation, changing the amount produced. Vater et al. (2002) working with Bacillus subtilis showed that the best temperature for growth and surfactant accumulation was 30º C at minimum medium compared to 25º C and 45º C. The components of the culture medium may also change the amount of accumulated lipopeptides. Mizumoto & Shoda (2007) showed this

variation with iturin, with the accumulated amount channging with carbon and nitrogen source.

Plants can generally interact with the microorganisms surrounding their tissues. The part of the plant that has the strongest influence on that interaction is the root system. According to Uren (2007), plant roots release sugars, amino acids, organic acids/anions, inorganic ions, enzymes, molecules capable to change the rhizosphere, pH, antibiotics, quorum-sensing inhibitors and others substances. All those substances may interfere on the bacterial growth and lipopeptide signature according to the genome limits. Nihorimbere et al. (2012), working with B. amyloliquefaciens strain S499 showed that tomato roots exudate efficiently support surfactin, fengycin and iturin accumulation.

Surprisingly, there is no evidence of lipopeptide production within stems and leaves. The colonization of plants by bacteria is important to exert long-term benefits. Among the benefits most commonly reported are growth promotion (Richardson et al., 2009), increase in plant resistance to chemical and structural factors (Punja, 2010), and even direct antagonism against a harmful plant pathogens (Yoshida et al., 2001).

Based on these features, this work studied the ability of one particular tropical *B. amyloliquefaciens* ALB629strain on lipopeptide accumulation under different temperatures, pH and substrates, and also its ability to colonize common bean plants, considering different forms of bacteria introduction.

MATERIAL AND METHODS

Bacteria identification

Bacillus amyloliquefaciens (ALB629) was isolated as an endophyte from cocoa in the State of Bahia, in Brazil. Species level identification was made by sequence analysis using RecA (forward - TGA GTG ATC GTC AGG CAG CCT TAG and reverse - TTC TTC ATA AGA ATA CCA CGA ACC GC) and Gyr-A (forward - CAG TCA GGA AAT GCG TAC GTC CTT and reverse - CAA GGT AAT GCT CCA GGC ATT GCT). Total DNA was extracted and purified via a wizard genomic DNA purification kit (Promega), following the manufacturer's instructions. PCR samples were amplified using the program of 95ºC (5 minutes) followed by 26 cycles of 95ºC (30s), 55ºC

(30s) and 72ºC (2 min); subsequently a single cycle of 72ºC (10 min) and holding at 16ºC. Sequence analysis was performed at the Interdisciplinary Group For Applied Genoproteomics (GIGA). ALB629 identification was based on sequence comparisons using NCBI site and BLAST software.

Bacterial growth and bacterial suspension

ALB629 was grown on four solid media (MB1, Luria, PDA and optimized media) at 15º, 25º, 30ºC for 48h. Bacterial suspension was prepared according to temperature in the following experiment. Proceeding this way, the bacteria were already adapted to the specific temperature.

Lipopeptide analysis in relation to culture media and temperature

Influence of lipopeptide signatures considering four different culture media and three temperatures was observed by adding 25 mL of the optimized medium (casein peptone 30g/L, sucrose 20 g/L, yeast extract 7 g/L, KH2PO4 1.9 g/L, MgSO4 0.45 g/L, citric acid 10 mg/L, CuSO4 0.001 mg, FeCl3.6H2O 0.005 mg, NaMoO4 0.004 mg, KCl 0.002 mg, MnSo4.H2O 3.6 mg, ZnSO4.7H2O 0.014 mg e H3BO3 0.01 mg; the

pH was adjusted to 7 with KOH), MB1(sucrose 10 g/L, casein peptone 8 g/L, yeast extract 4 g/L, K2HPO4 2 g/L, MgSO4.7H2O 0.3 g/L), Luria (casein peptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L and glucose 1 g/L) and PDB (24 g of the powder substrate in one liter of sterile water). Concentrated suspension adjusted to 0.5 of absorbance was prepared with

Bacillus amyloliquefaciens previous culture, adding 50µL per treatment. It was grown for 72 hours at 30º and 25ºC. When at 15ºC it was grown for 120 hours. The Erlenmeyer (125 mL) was inserted in a shaker at 100 rpm (rotation per minute).

Lipopeptides analyses were performed after the incubation period. 12 mL of each sample was collected from the supernatant, adding it into Falcon tubes and centrifuge for 10 minutes at 15,000 rpm. While this process was running, the cartridge (C18) apparatus was rinsed off by injecting 20 mL of methanol followed by 15 mL of Milleq water 10 mL were collected from those Falcon tubes, and it was passed through the cartridge apparatus to concentrate to 10x. During washing out of the sample, 5 mL of Milleq water was added. For lipopeptide concentration, the sample was passed through the cartridge. It was transferred to plastic tubes (Eppendorf) by passing 1 mL of methanol through the cartridge.

The sample was centrifuged once again for 10 minutes at 15,000 rpm. Finally, 300 µL was collected from 1 mL, which was added to a special tube for UPLC analyses. Lipopeptide analyses were performed by UPLC (Acquity Hclass; Waters s.a., Zellik, Belgium) coupled to a single mass spectrometer (Waters SQD mass analyzer) on an ACQUITY UPLC BEH C18 1.7 µm column. Elution was performed at 40°C with a constant flow rate of 0.6 mL/min, using a gradient of ACN in water acidified with 0.1% formic acid as follows: one min at 30 %, from 30% to 95% in 3.4 min, and maintained at 95% for 1 min.

Compounds were detected in electrospray positive ion mode by setting SQD parameters as follows: source temperature: 130 °C; desolvation temperature: 400 °C, and nitrogen flow: 1000 L.h–1. A cone voltage of 70 V was used. After the analyses, lipopeptides were identified in the chromatogram and spectrogram by mass comparison and retention time.

Direct antagonism

Further experiments were done to show the direct antagonism among fungi and bacteria. The experiment was performed using

Fusarium oxysporum in three different temperatures (30º; 25º and 15º C); *Aspergillus niger* and *A. ocracious* at 30º and 25ºC. The culture media employed was PDA, MB1 and optimized. Fungus was placed at the middle of each Petri dish containing different media. At the same time, ALB 629 was streaked at four spots at equal distance to the pathogen and crosswise. The experiment was finished, when the first inhibition halo appeared.

Lipopeptide accumulation using stem sap

This was performed with a common bean (*Phaseolus vulgaris* - Perola cultivar). Forty seeds were sown in a vermiculite-based substrate and incubated in chamber adjusted to 28ºC (+/- 2ºC) for 10 days. After this period, the stems were separated from leaves and roots. The stem tissue was disinfested with alcohol 70% for 30 seconds, sodium hypochlorite 2% for 1 min, and washed three times with distilled water. It was transferred to a Becker (1L) with 600 mL of sterile distillated water.

Aluminum paper was used to fill the empty spaces on the upper part, preventing the contamination. It was incubated in a chamber at 28ºC for 48h. It was sterilized by passing through a 0.22µm-diameter filter. An

aliquot of 20 mL of the sap was added to Erlenmeyer (125 mL) and incubated in an orbital shaker set for 100 rpm at 30ºC for 120 hours. At the end of the bacterial growth, the lipopeptides were analyzed as described above.

Lipopeptide accumulation using root exudates

Forty seeds were sown in the vermiculite substrate and incubated in a chamber adjusted to 28ºC for 10 days. After this period, the roots were sterilized as described above. The whole plant was immersed entirely in the Becker (1L) with 600 mL of sterile distilled water. It was incubated in a chamber at 28º for 48h. Cotton was used to fill the empty spaces on the upper part preventing the contamination. The solution containing the root exudate released in the Beaker was filtered by passing it through a 0,25µm-diameter filter. This exudate was used as a substrate for growth and lipopeptide accumulation. It was added 20 mL of this substrate into Erlenmeyer and incubated in a shaker adjusted for 100 rpm to 30ºC for 120 hours. The lipopeptide analyses was then performed, as described above.

Lipopeptides accumulation at MB1 culture media with different pHs

Lipopeptide signature based on bacterial growth at different pHs was performed by growing ALB629 in a range from five to nine. The MB1 culture media was prepared as before and adjusted to the different pHs to be tested. Accurately, 50 mL of the substrate was transferred to a Erlenmeyer (125 mL). Each pH was adjusted with NaOH and H3PO4.

From the bacterial suspension (2x105 cfu/mL), 100µL was added to each flask. Theses flasks were attached to an orbital shaker set for 100 rpm and 30ºC for 72 hours. After this period, the pH was measured again and lipopeptides extraction and analyzes were done as before.

Bean plant colonization cultivated in vermiculite using glass bottles

This experiment was performed in glass bottles opened on the top and lined with vermiculite. Hoagland's solution was the source of nutrient and water. Seeds were pre-germinated in the vermiculite for 5 days. The method employed for inoculation was dipping roots for 30 minutes using a bacterial suspension (0.5 λ). Plantlets were incubated at 28º and 20ºC, with constant light during 10 days. After this period, the plants were harvested and separated into root, stem and leaves. Samples were

weighed, crushed, serially diluted and plated in MB1 to determine the bacterial count outside (epiphyte) and inside (endophyte - the surface sterilization was applied during this step) for each plant tissue. Pieces of sterile leaves and stem from the colonization experiment were placed in contact with MB1 medium to confirm if any colony will appear, supporting the results for endophyte behavior. Gene Sequencing using the primes, rec and gyrA, was also performed to confirm the presence of ALB629. Bacterial cells used came from inoculated plants and compared to ALB629.

Bean plant colonization cultivated in peat with additional inoculation

This experiment was conducted at the same temperatures of 28º and 20ºC, using pre-germinated seed and plants with 10 days old. The method employed for the inoculation was dipping roots for 30 minutes using a bacterial suspension (0.5 λ). Seed was sown within black pots of 350 mL with peat. It was irrigated periodically. After five days, an additional inoculation was done, by adding 1 mL of the bacterial suspension (0.5 λ) to the center of those pots near the plant stem. Cell counting was performed similarly to the described above.

Bean plant colonization using the dipping root method cultivated in the peat

Bean plant colonization was performed under the same conditions of previous method, but this time just the dipping root method was used, without additional bacterial inoculation. Cell counting was performed similarly to the described above.

B. amyloliquefaciens **behavior when applied on the surface of the bean leaves**

This was done to study the survival of the bacteria applied on leaves. Plants were pre-germinated in vermiculite. Upon germination, they were transferred to falcon tubes filled with cotton. The source of nutrient was Hoagland's solution (HOAGLAND & ARNON, 1950). It was incubated for 10 days. Bacterial suspension (8x106) was sprayed over aerial part until runoff. Available cells were measured 0, 24, 48 ,72, 96 and 144 hours after spraying. For the time 0, samples were collected one hour after application. Two pairs of the first trifoliate leaves were harvested for bacterial cells counting. It was inserted in the Falcon tubes with 50 mL of the capacity filled with 20 mL of distillated water. The

tubes were agitated in the vortex for 1.5 minutes to transfer the bacterial cell to the water. The counting of forming colony units was performed in the Petri dishes with MB1 media. 50 µL of the suspension was added to the media surface. It was incubated at 30ºC. After 24 hours, the cells were counted to determine the bacterial population on the leaves. Results are presented as united-forming-colony (UFC) per gramme (g) of tissue.

RESULTS

Having analyzed the sequences generated for RecA and GyrA primers, the bacterium was identified as *Bacillus amyloliquefaciens*, with 99% max identity compared to those sequences deposited in the Genbank.

Temperature and substrate influenced the amount of accumulated lipopeptide, affecting both quality and quantity (Figure 1). Among all the culture media tested, the only one that supported accumulation of all lipopeptides (surfactin, fengycin and iturin) was PDB, but interestingly there was no bacterial growth at 15º C. At 30º and 25º C, the best media for surfactin and fengycin accumulation was Luria. For iturin, the only media capable of producing it was PDB. At 15º C, surfactin and fengycin were produced in higher amounts, when bacteria were grown in MB1.

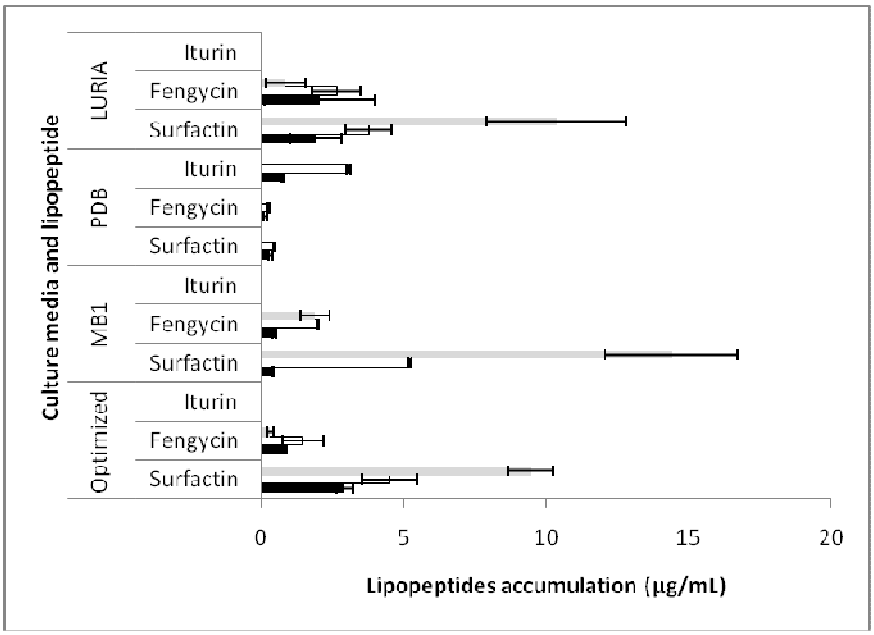

Figure 1. Lipopeptide production influenced by culture media and temperatures. *Black bars are lipopeptides accumulated at 30ºC; white bars are lipopeptides accumulated at 25ºC, and grey bars are lipopeptides accumulated at 15ºC. Error bars represent standard error.

Those lipopetides are part of the accumulated metabolites produced by the bacterium within the media. Some of those compounds inhibited the growth of plant pathogenic fungi. The inhibition zone was observed in the direct antagonism test for all challenging fungus at PDA

medium (Figure 2). *F. oxysporum* and *B. amyloliquefaciens* was affected when grown at 15º C, but even this way it was possible to observe the inhibition halo. Other temperatures did not affect any organisms. No halo inhibition was observed in other culture media.

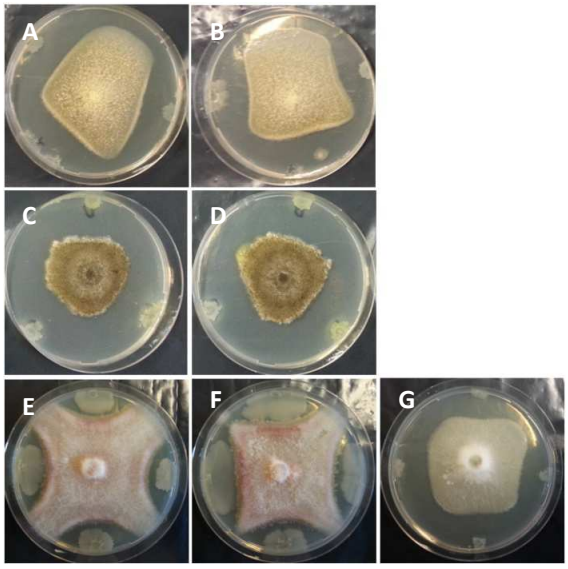

Figure 2. Direct antagonism of ALB629 against three different plant pathogens. A-B, *A. ochraceus* at 30º and 25ºC, respectively; C-D, *A. niger* at 30º and 25ºC, respectively; E-G, *Fusarium oxysporum*, at 30º, 25º and 15ºC, subsequently.

The diversity and abundance of accumulated lipopeptide contributed to the inhibition of different fungi, but we could not infer that production within the plant, upon bean colonization and, using the stem

sap as a nutrient source, the bacterium was able to grow and lipopeptides accumulated (Figure 3A). Root exudates only supported surfactin and iturin accumulation (Figure 3B). In both cases, there was no support for large populations (Table 1).

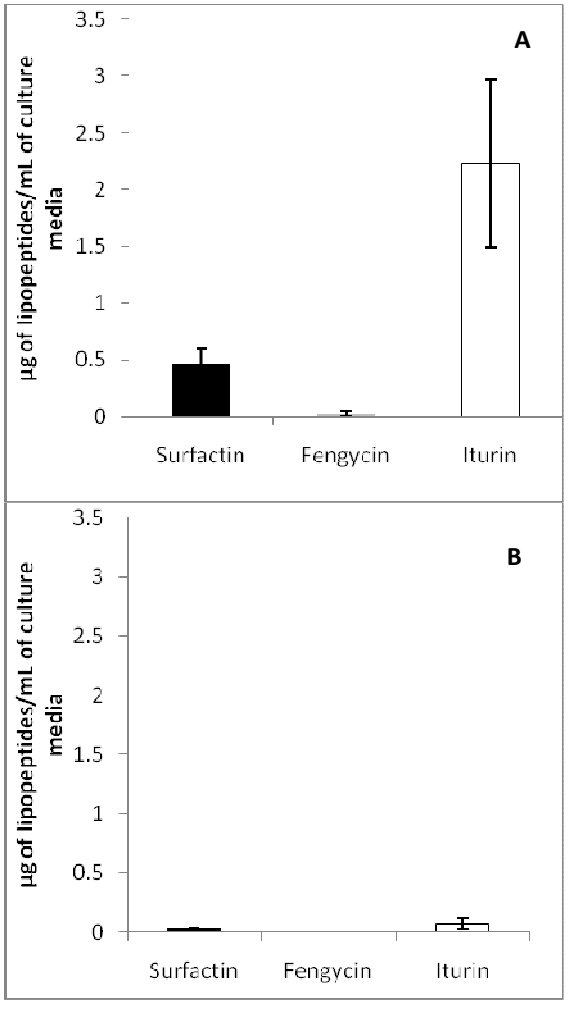

Figure 3. (A)Lipopeptides production with stem sap as a substrate and (B) lipopeptides production with root exudates as a substrate. Error bars represent standard error.

Table 1.Bacterial population on stem sap and root exudate after 48h.

	Substrates	
	Stem sap	Root exudate
Population	*17698.73	6676.05

* These values represent means of treatment.

Apart from growth conditions, pH may interfere on the dynamics of lipopeptide production. Although both pH 6 and 7 could support surfactin and fengycin accumulation, the maximum production was observed at pH 6 (Figure 4). In regard to bacterial growth at different pH, the bacterial population observed at all values of pH allowed for growth (Table 2).

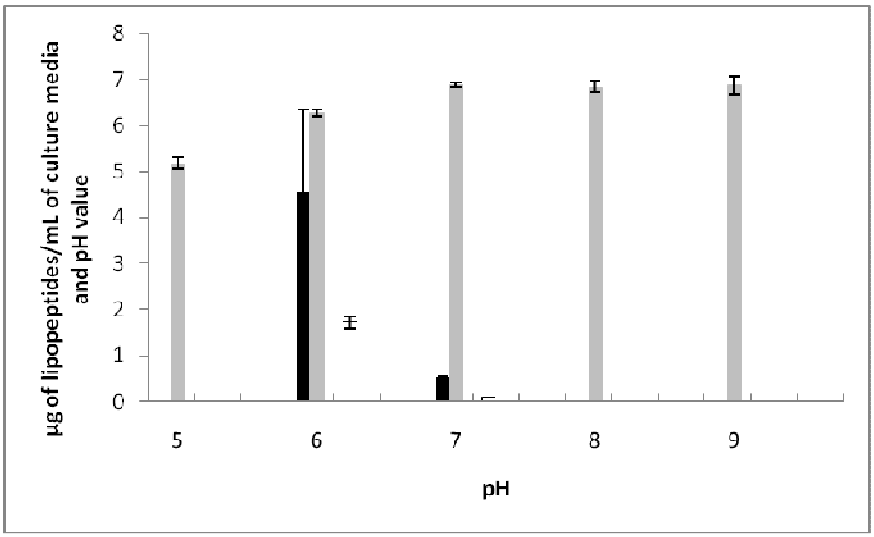

Figure 4. Lipopeptide production at MB1 media at different pH. *Black bars are surfactin; white bars are fengycin; grey bars are the pH of the experiment and the black line is the population of the experiment. Error bars represent standard error.

Table 2. Bacterial population on different pH after 48 hours.

pH	Population
5	*128277.3
6	37750.62
7	20043.98
8	54401.9
9	63352.94

* These values represent means of treatment.

Finally, we investigated if the bacterium was able to colonize the plant under different growth and bacterial delivery conditions. Colonization of bean plants was done in different manners, according to the method employed to inoculate bacteria to the plant and also to the temperature in which the plants were incubated. When in vermiculite, ALB629 bacteria were recovered from all plant organs (leaves, stems and roots) at 28ºC, either endophytically or epiphytically. When at 20°C, bacteria were also recovered both from inside or outside the plant. However, at that temperature, the bacterium was not able to colonize the leaves (Figure 5).

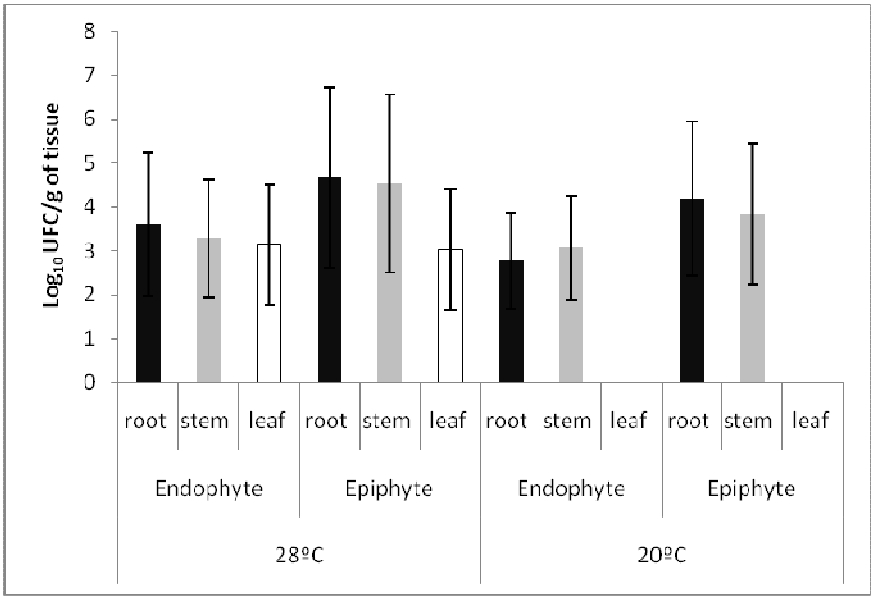

Figure 5. Bean plant colonization by *Bacillus amyloliquefaciens* cultivated in vermiculite amended with Hoagland's solution and incubated at 28º and 20ºC. * Black bars are the population at roots, grey bars are the population at stem and white bars are the population at leaves. Error bars represents standard error. When beans plants were submitted to two inoculation methods in the same experiment, bacteria was recovered at both temperatures (Figure 6).

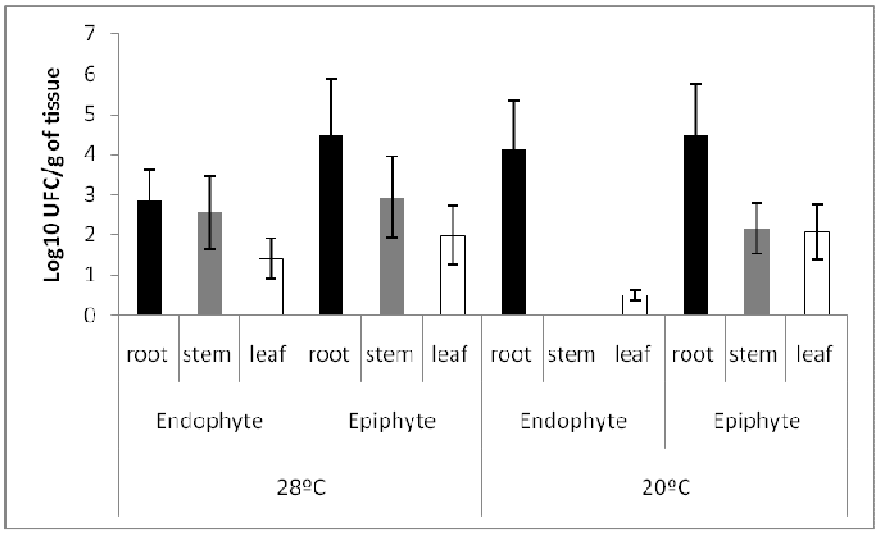

Figure 6. Bean plant colonization cultivated in peat using the dipping root method and one additional inoculation 5 days old. *White bars are epiphyte population and black bars are endophyte population. Error bars represents standard error.

Using only one method of inoculation by dipping root, the bacterial population was found only in the roots, considering endophytic and epiphytic cells (Figure 7). Epiphytic bacterial population higher than the endophytic population was observed in all experiments.

A further experiment confirmed the bacterial ability as an endophyte, noting radial colonies surrounding leaves and stem after the incubation period.

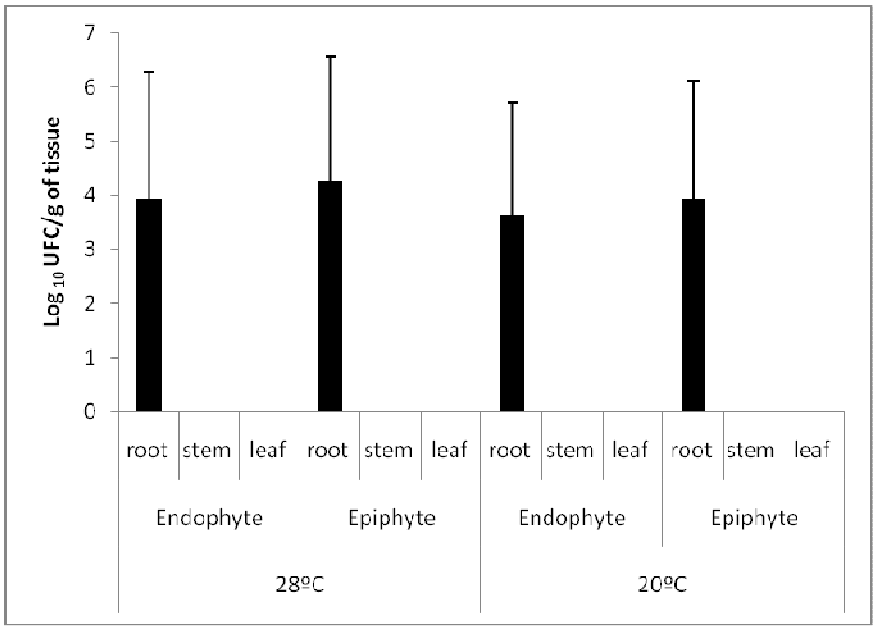

Figure 7. Bean plant colonization using the dipping root method cultivated in peat. *White bars are epiphyte population and black bars are endophyte population. Error bars represent standard error.

Since the bacterium was not recovered from leaves, when a bacterial suspension was applied to the substrates/roots or the population in leaves was kept low, another experiment was performed to demonstrate bacterial survival on the phylosphere. However, the bacterial population dropped tremendously fast in that organ, suggesting that, even at the tested temperature, leaves do not sustain bacterial growth, since the epiphytic population was reduced from 980,000 cfu/g to 600 cfu/g at 28ºC and from 240,000 cfu/g to 590 cfu/g at 20ºC within 24 hours after treatment. For later time points, the population remained low (Figure 8).

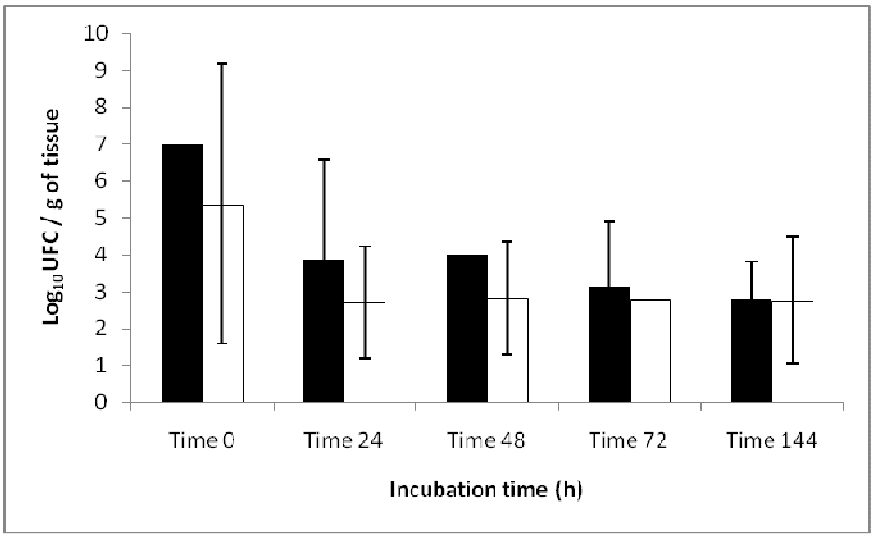

Figure 8. Bacterial spray over leaves. *Black bars are bacteria population from plants incubated at 28º C and white bars are bacteria population incubated at 20º C. Error bars represent standard error.

Discussion

All organisms have a limited ability to produce substances based on information contained in the genome. According to Stein (2005), the *Bacillus subtilis*, closely related to B. amyloliquefaciens, has an average of 4-5% of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial compounds. Bacteria have units known as operons,

responsible for the activation of several genes together. These operons are activated only in the presence of certain substances that require the action of enzymes to be used as a source of carbon and other nutrients. In the case of lipopeptide produced by *B. amyloliquefaciens* ALB629, only the PDB culture supported the production of iturin. The same antibiotic was also produced when the bacterium was grown in the bean stem sap, suggesting that a plant-born compound would be necessary to trigger the iturin-related operon. Apart from the nutrient source, lipopeptide accumulation may be influenced by temperature. Actually, interaction between temperature and the necessary substances probably interact for an optimum lipopeptide accumulation rather than nutrient or temperature contribution alone. As shown in Figure 1, this was particularly true for surfactin. Different levels of lipopepeptide production according to the nutrient source and temperature may explain inconsistencies of biocontrol efficacy in the field. The wider the range of temperature to which a specific strain works, higher the chance for expected benefits in the field, where sudden changes in temperature and nutrient availability frequently occur.

As for fungus inhibition, once in contact with pathogens the bacteria could act efficiently as a biological control agent. Considering those three types of lipopeptides, the compound that is probably more active is iturin, because biological control was observed only in a media which support iturin synthesis.

Similarly to PDB, stem sap supported ALB629 growth and the accumulation of iturin and surfactin. Previous work, performed by Minchin & Thorpe (1983) found sucrose in the apoplast of bean stems at a concentration of 25-60mM. Since this nutrient is also found in PDB, this nutrient is likely an essential nutrient for iturin biosynthesis. Other molecules found in the bean sap are polyamines, putrescine and spermidine (FRIEDMAN et al., 1986); β-D-glucose, 3-O-methyl β -D-glucose, 2-deoxy β -D-glucose, α-D-mannose and β -D-fructose (Lalonde, et al., 1999).

The molecules recovered in the root exudates were 10 fold lower in concentration, indicating that the stem sap is richer in substances that support both surfactin and iturin production. Comparing both, the stem sap seems more appropriate for lipopeptide accumulation than the root exudates. Interestingly, the lipopeptide production and bacteria growth on

stem sap or root exudates are not related to a more active metabolism or to higher bacteria population, since a small population was observed with considerable lipopeptide accumulation. A lot of factors can influence the rhizosphere colonization. According to Ric de Vos et al. (1986), rhizosphere acidification has a pivotal role in iron uptake and is induced in response to iron deficiency in bean plants. These changes can allow for generation of a range of different compounds. Dakora et al. (1993), working with bean plants root exudate noted that roots inoculated with a symbiotic *Rhizobium leguminosarum* bv. *phaseoli* contained more coumestrol phytoalexin and its isoflavoinoid precursor daidzein than exudates from plants not inoculated. This can illustrate that lipopeptide production in nature may depend on many factors and the interactions. Uren (2000), also supporting this idea, reported that root exudation includes the release of ions, oxygen and water, and mainly consists of carbon-containing compounds.

In the experiment in which different pH values were tested, although the pH was adjusted at the beginning of the experiment, the bacteria probably release substances to the supernatant that alter the pH to a likely suitable condition to grow, as all treatments had a pH surrounding

7 at the end of the incubation time. As production of lipopeptides was only observed at pH 6, and at a lesser extent at 7, the optimum lipopeptide production seems to be within the range of the optimum pH in the soil for the plant, which is, in average, 6. This finding strengthens the fact that you can have considerable bacterial population without lipopeptide production.

By using different methods for studying the colonization, significant differences were observed in endophytic and epiphytic behavior. But when it comes to colonization studies, variability between plants was high and many factors need to be investigated as regulators of endophitic colonization. Future experiments will study other biotic and abiotic factors that may govern endophitic colonization and the dynamics of lipopeptide production within the plant.

Analyzing the colonization experiment, it was seen that the bacterium can easily colonize roots and stems of bean plants, as an epiphytic and endophytic agent. In performing this experiment and others related to bacterial colonization, it was possible to observe a clear variation of the bacterial population according to nutrient source, temperature and pH. According to Bais (2006), plants create a specific

environment for microbes to develop in the rhizosphere, because they can release a wide variety of low-molecular weight compounds and macromolecules from their roots that recruit the suitable microbes. Bertin et al. (2003) reported relevant information on these compounds. According to this review, the low-molecular weight exudates are polysaccharides (such as arabinose, fructose, glucose, maltose, mannose, oligosaccharides), amino acids (such as arginine, asparagine, aspartic, cysteine, cystine, glutamine), organic acids (such as acetic, ascorbic, benzoic, ferulic, malic acids) and phenolic compounds. Higher-molecular-weight compounds can also be released as flavonoids, enzymes, fatty acids, growth regulators, nucleotides, tannins, carbohydrates, steroids, terpenoids, alkaloids, polyacetylenes and vitamins. Therefore, compounds like those could support at least a small population and lipopeptide accumulation.

The factors that allow for bacterial survival on leaves and stem are not known. Neither is it known how the bacteria goes from roots to the leaves or stem. Probably it moves systemically through the vessels (xylem or phloem). Sterile leaves and stems allowed confirming the bacterium presence after 24 hours after plated at MB1 medium.

Comparing the sequencing of the bacteria isolated from pieces of sterile leaves and stem with our original strain, it was placed in the same group (99% of max identity with B amyloliquefaciens FZB42), confirming the higher motility of the B. amyloliquefaciens, even being considered an endophytic agent.

By applying the bacteria on the surface of leaves, we observed that leaf environment does not sustain ALB629 population for very long. Poor nutrient availability and/or the presence of UV radiation are factors that may be hostile to the bacterium colonization. According to Mercier & Lindow (2000) the population size of epiphytic Pseudomonas fluorescens on plants under environmentally favorable conditions is limited by the abundance of carbon sources on the leaf surface. This can explain at least part of the bacterial dynamic. However, even if the bacterium does not reach the leaves, it has already been shown to protect leaves from a foliar pathogen and activate defense-related responses (Martins et al., 2013) and the MAMP necessary for that could be the surfactin, already reported as triggering ISR.

The dynamics of lipopeptide accumulation within the different plant organs over time is still unknown. Initial trials were unsuccessful in

detecting any lipopeptide over time in bean plants. However, we have seen that root exudates and stem bean sap supports both bacterial growth and iturin and fengycin accumulation. Further setup experiments are necessary to remove eventual plant-borne inhibitors of detection or eventually detect a wider range of forms of lipopeptides that would eventually be bound or accumulated as precursors within the plant. Or even detect the lipopeptides in a later moment.

CONCLUSION

Lipopeptide accumulation was both qualitatively and quantitatively affected by the substrates, temperatures and pH. The stem sap and root exudate support both bacterial growth and accumulation of iturin and surfactin. *Bacillus amyloliquefaciens* ALB629 colonized plants differently according to the delivery method and temperature but, in all cases, it was recovered in higher population associated to the roots.

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* VERSÃO PRELIMINAR

ARTICLE 2 ***Bacillus amyloliquefaciens*** **as a biological agent against** ***Botrytis cinerea*** **and its protection in postharvest strawberry**

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Bacillus amyloliquefaciens as a biological agent against *Botrytis cinerea* and its protection in postharvest strawberry

Abstract

Grey mold is the most important strawberry postharvest disease and its control can be achieved by using antibiotic-producing microorganisms. Among those microorganisms, Bacillus produces a diversity of antifungal compounds, but little is still understood about the contribution of the bacterial growth conditions on the diversity of the metabolites produced. The strawberry protection was immersed in the bacterial suspension for 10 minutes and inoculated with a pathogen-spore suspension. To understand the mode of action, antagonism directly through inhibition zone and indirectly by volatile compounds was checked by streaking the antagonist and pathogen simultaneously on a regular plate or on separated compartments for direct or indirect inhibition. Antagonism was experimented at three different temperatures (15º, 25º and 30ºC) and three different culture media (PDA, MB1 and Optimized). The volatiles experiment was performed at 30ºC in five different culture media (PCA, Optimized, Luria, MB1 and NAA). The conidial germination experiment was performed under influence of bacterial supernatant at different

concentrations (0, 25, 50, 75 and 100%). Bacterium was able to stop fungal growth with apparent halo inhibition. Volatiles were also effective in controlling the hyphal growth and it was possible to identify different compounds. Conidia inhibition was inversely proportional to metabolite concentration. Protected strawberries showed no apparent sporulation.

RESUMO

O mofo cinzento é uma doença frequente em pós-colheita de morango. Seu controle pode ser conseguir usando microrganismos produtores de substâncias antifúngicas. Entre os microrganismos, Bacillus produz uma diversidade de compostos anti-fúngicos, mas ainda, pouco se sabe sobre a contribuição das condições de crescimento bacteriano na diversidade dos metabolitos produzidos. Para a proteção de morangos, este foram imersos na suspensão de bactérias durante 10 minutos e inoculados com uma suspensão de esporos do patógeno. Para entender o modo de ação, o antagonismo direto através da zona de inibição e indiretamente por compostos voláteis utilizando compartimentos separados para a inibição direta ou indireta. O antagonismo direto foi testado em três temperaturas

diferentes (15º, 25º e 30º C) e três diferentes meios de cultura (BDA, MB1 e Otimizado). O experimento com voláteis foi realizado a 30º C em cinco meios de cultura diferentes (PCA, otimizado, Luria, MB1 e NAA). O ensaio da germinação de esporos foi realizado sob a influência de sobrenadante bacteriano em diferentes concentrações (0, 25, 50, 75 e 100 %). A bactéria foi capaz de impedir o crescimento de fungos com halo de inibição aparente. Os voláteis foram também eficazes no controle do crescimento de hifas e foi possível identificar diferentes compostos. A inibição de conídios foi inversamente proporcional à concentração de metabólitos. Os morangos protegidas não mostraram nenhuma esporulação aparente.

Introduction

Strawberry is a perishable fruit with high respiratory rate, consequently it has a short shelf-life. Mechanical damages, wounds and beats during the harvest, transport or commercialization make the fruits susceptible to pathogens (NUNES & MORAIS, 1995). Rot incited by pathogen activity causes serious losses, mainly when it is cultivated far

away from the markets (Silveira et al., 2005). Among those rots, grey mold stands out as a serious disease. *Botryts cinerea* Pers.:Fr. related to this disease is a widespread plant pathogen that causes problems to hundreds of dicot plants, including cultivated species (Elad et al., 2004). Considering the symptoms on different organs and plants urged by this ascomycete, a large 'arsenal of weapons' to attack its hosts may be proposed to fight it (Choquer et al., 2007). According to Williamson et al. (2007), it produces a range of cell-wall-degrading enzymes, toxins and other low-molecular-weight substances, for instance, oxalic acid, which are relevant in the pathogenesis. Losses in strawberries due to gray mold are significant as post-harvest disease. Its postharvest life is often determined by mechanical injuries that subsequently result in fruit decay (JIANG et al., 2001; Droby & Lichter, 2007). Among all agricultural methods to manage the disease, biological control is playing an important role in the agricultural field. A great number of microorganisms are applied, attempting to control this pathogen while it remains in the soil, specially fungus and bacteria.

Many technologies have been employed with the purpose of reducing post harvest rot, such as chemical control applying maturation

inhibitors, systemic and protective fungicides; biological control with antagonists; physical control by cooling, heat treatment, radiation, controlled and modified atmosphere; and resistance induction with biotic and abiotic elicitors (Barkai-Golan, 2001).Some commercial products have been used in post-harvest as Agro-Mos® (based in Saccharomyces cerevisae Meyen ex Hansen 1026), Aspire® (based on Candida oleophila), MessengerTM (harpin protein hrpN from Erwinia amylovora) (Oliveira et al., 2004), but all those are classified as resistance inductors. Experimentally, *Gliocladium roseum, Penicillium* sp.; *Trichoderma viride* and *Trichothecium roseum* appeared to be the most effective organisms, suppressing the incidence of *B. cinerea* on fruits by 48-76% (PENG & SUTTON, 1991). Hang et al. (2005) reports the success of *Bacillus subtilis* S1-0210 using a wet powder formulation with 85 to 89% effective control of gray mold incidences on strawberry fruits. Positive results such as those support the practical application of a biological control agent as part of the disease management.

The reason that it is successful as a powerful biological control agent is due to an efficient production of antifungal compounds. The substances responsible for the control are largely known as Bacisubin

(Liu et al., 2006), Chitinases (Huang et al., 2005), Subtilosin (Stein, 2004), Ericin (Stein, 2002b) and lipopeptides surfactin, fengycin and iturin (Plaza et al., 2013; Ongena & Jacques, 2008).

The volatile substance can also provide an important protection system against phytopathogens (Chen et al., 2008). According to Owens et al. (1997), the main compounds produced were 2-hydroxi-3-butanone (acetoin), 2,3-butanediol, acetic acid, propanoic acid, 2-methylpropanoic acid and 2-methylbutanoic acid. A wide range of pyrazines was identifyied in cultures of B. subtilis (Yamaguchi et al., 1993). Additionally, Weisskopf (2013) published a book chapter exposing the potential of bacterial volatiles for crop protection against phytopathogenic fungi.

Even if the bacteria have genes to produce a molecule, it will still be subject to temperature and substrate changes. Temperatures may modify the efficiency of biological species and its metabolite production (Moita et al., 2005). For volatiles, temperatures also play a relevant role in determining the efficiency of biological control. Fiddaman & Rossall (1993), studying the *Bacillus subtilis* showed that at 30ºC it controlled *Rhizoctonia solani* and *Pythium ultimum*, while temperatures below the

25ºC were not efficient. Mukherjee & Das (2006), comparing two *Bacillus subtilis* strains showed that they had distinct preferences for the carbon and nitrogen substrates. Volatiles in vitro antifungical activity of B. subtilis on nutrient agar is enhanced with the addition of D-glucose, complex carbohydrates and peptones (FIDDAMAN & ROSSALL, 2008).

This study will demonstrate how temperature and substrate changes can influence the efficiency of disease control by producing different active compounds. Further, this paper explores the qualitative response to the fungistatic action according to the substrates.

Material and methods

Fungal and bacterial isolation

B. cinerea was isolated from infected strawberries obtained from market, identified based on the colony morphology according to Agrios (2005). The fungal culture was deposited at the Mycological Collection of Lavras (CML) as CML 2317. The bacterial strain, *Bacillus amyloliquefaciens* ALB629, employed as biological control agent, was obtained from the bacterial collection of Universidade Federal do Reconcavo da Bahia.

Direct antagonism

The experiment to attest antagonism against *B. cinerea* was performed using three different media (PDA, Optimized and MB1) and also three different temperatures (15, 25 and 30ºC). Fungus was placed in the middle of each Petri dish containing different culture media. At the same time, ALB629 was streaked at four spots at equal distance from the pathogen and crosswise. Plates were incubated at three different temperatures. The experiment was finished when the first inhibition halo appeared. The experiment was performed in a randomized design with four replicates. Part of these treatments was analyzed for lipopeptide accumulation. Initially, a standard antibiosis experiment was performed in dishes with three different culture media (PDA - at 23ºC, Optimized and MB1 - 15ºC). After appearance of the first halo formation in dishes with PDA, three disc of 9mm was collected from halo zones to identify the harmful substance. These discs were inserted in plastic tubes (1.5mL) with acetonitrile (50%) and formic acid (0.1%).The samples werefixed on a rotary shaker and agitated for 2 hours. This was performed with UPLC (Acquity Hclass; Waters s.a., Zellik, Belgium) coupled to a single mass spectrometer (Waters SQD mass analyzer) on an ACQUITY UPLC BEH

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C18 1.7 μm column. Elution was performed at 40°C with a constant flow rate of 0.6 mL/min using a gradient of ACN in water acidified with 0.1% formic acid as follows: one min at 30 %, from 30 to 95% in 3.4 min, and maintained at 95% for 1 min. Compounds were detected in electrospray positive ion mode by setting SQD parameters as follows: source temperature: 130°C; desolvation temperature: 400°C, and nitrogen flow: 1000 $L.h^{-1}$.

Volatile compounds

The diversity of produced volatiles was evaluated by streaking the bacterium in five different media (PCA, Optimized, Luria, MB1 and NAA). The same medium was poured both in the plate and in its lid. The fungus was inserted in the center of the plate and bacterium spread on the lid to evaluate volatile produced by the bacteria over time (24h and 96h incubation) and the inhibition of the pathogen growth. The technique used to analyze volatiles was SPME (VALENTE & AUGUSTO, 2000). The chromatograph used in the volatile separation was GC-MS. The SPME fiber employed was DVB/CAR/PDMS (Divinylbenzene, Carboxen,

Polydimethylsiloxane). Time extraction was 30 minutes and the fiber was exposed in the chromatograph injector for 10 minutes. The injector and detector temperature was 260ºC and 280ºC, respectively. The injector was operated on splitless and split mode in all the injections. Helium was used as carrier gas. Volatile identification (quantitative analyses) was performed with the software *Automated Mass Spectral Deconvolution and Identification System* (AMDIS) equipped with Mass Spectral Search Program (NIST) library and also by the retention time. As a complementary part, the growth was measured when the fungus took over all of the dish in the treatment control. The experiment was performed at random design with five replicates.

Effect of thermo-stable metabolites on conidial germination

The effect of the metabolites produced by *B. amyloliquefaciens* on the germination of *B. cinerea* conidia was performed by incubating the pathogen conidia on the bacterial supernatant. The bacterial suspension (10^{-5} cells/mL) was inoculated in 50mL of potato-dextrose-broth and incubated at 30ºCand 110 rpm for 72h. After this period, the cultures were sterilized in the autoclave at 120ºC for 40 minutes. The autoclaved supernatant was diluted in sterile distilled water to 0%, 25%, 50%, 75%

and 100% (v/v). To check the contribution of the bacterial metabolites on the germination of *B. cinerea* conidia, µL of each dilutions of the autoclaved supernatant as well as 50 µL of a 10^{-6} conidia/mL suspension of the pathogen were pipetted into different wells of an ELISA plate. After 72 hours of incubation at 25ºC in a photoperiod of 12h,the spore germination was evaluated counting the number of conidia with germ tube then the highest diameter of the conidia. The experiment was performed at random design with seven replicates.

Biological control of grey mold

The autoclaved bacterial supernatant was prepared as described above. After the incubation period, the strawberries were immersed in the sterile culture for one minute with. The setup was four strawberries per plastic box over a layer of filter paper. 1 ml of the pathogen suspension (10^{-6}conidia/mL) was deposited over the strawberries. This experiment was performed at random design with five replicates. Fruits were placed at 25ºC (+/- 2ºC)and a photoperiod of 12 hours. Fruits were evaluated 10

days after incubation. The incidence of grey mold was determined by counting the number of strawberries with apparent fungal sporulation.

Results

Direct antagonism

Among all substrates, only PDA allowed the production of an inhibition zone. At 15ºC, the halo of inhibition was formed with 2.3 cm. For the other temperatures, the fungus acted oddly, growing abnormally. Huge differences regarding bacteria spreading at Optimized and MB1 were clearly seen, probably due to over-production of surfactant in this environment (Figure 1 and 2).Lipopeptide production was found only at surfactin at MB1 and Optimized. For PDA, surfactin and iturins were identified.

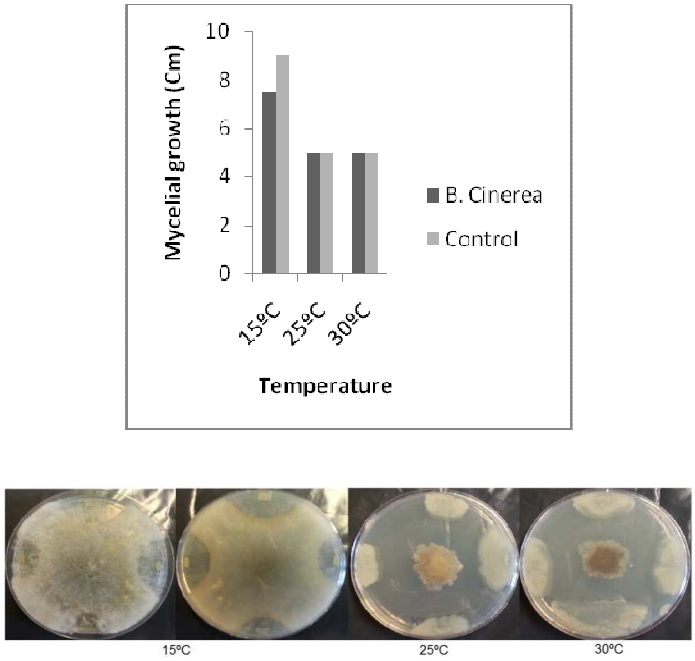


Figure 1 - *B. amyloliquefaciens* on antagonism with *B. cinerea* on the PDA media over three different temperatures (15º, 25º and 30º C).

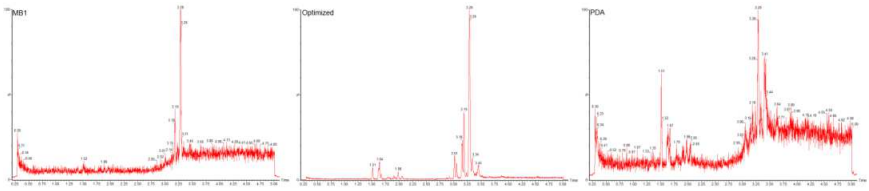
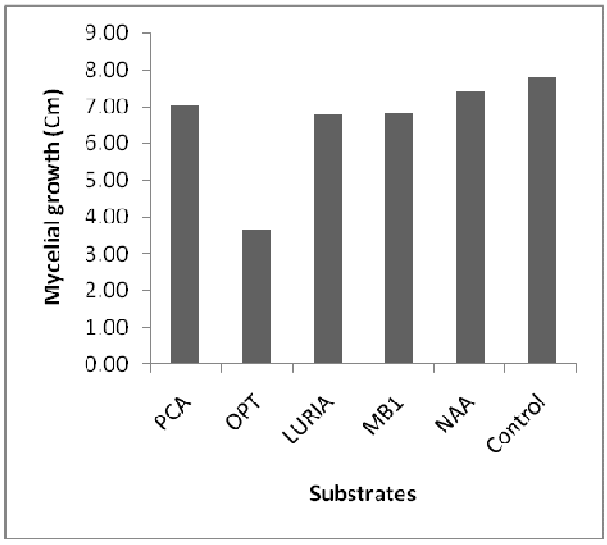


Figure 2 - *B. amyloliquefaciens* soluble substances accumulated at Petri dishes.

Volatile compounds

All culture media fostered the production of antibiotic volatile organic compounds compared to the water control. However, when the bacterium was grown on the optimized substrate, the fungus could not spread vigorously (Figure 3).



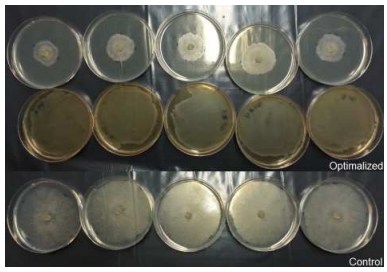


Figure 3 - *Botrytis cinerea* affected by volatiles compounds produced in five culture media.

Considering two incubation periods, no qualitative differences were obtained. The most frequently obtained molecule, regardless of the used medium, was 2,3-Butanediol and Acetoin. Among the substrates, luria could support different compounds in both incubation periods (Figure 4).

Literature Kovats index	Experimented Kovats index	Match	Compounds
PDA substrate 24 h			
769	781	938	2,3-Butanediol
980	988	886	Heptane, 2,2,4,6,6-pentamethyl
1193	1198	843	Octanoic acid, ethyl ester
1490	1503	839	2-Tridecanol
PDA substrate 96 h			
718	709	823	2-Butanone, 3-hydroxy- (Acetoin)
769	782	936	2,3-Butanediol
1030	1030	946	1-Hexanol, 2-ethyl-
1094	1093	933	2-Nonanone
1118	1120	920	Phenylethyl alcohol
1580	1556	895	2-Tetradecanone
LURIA substrate 24 h			
718	715	912	2-Butanone, 3-hydroxy (Acetoin)
769	800	942	2,3-Butanediol
875	866	936	Butanoic acid, 3-methyl
850	877	926	Butanoic acid, 2-methyl
1035	993	912	Benzene, 1,2,3-trimethyl-

1094	1093	873	2-Nonanone
1124	1126	936	Octanoic acid, methyl ester
-	1131	915	Benzenamine, N-ethyl
1173	1174	786	Cyclohexanol, 5-methyl-2-(1-methylethyl)
LURIA substrate 96 h			
743	749	936	2-Pentanone, 3-methyl-
819	856	930	2-Hexanone, 5-methyl-
1030	1030	949	1-Hexanol, 2-ethyl-
1094	1093	936	2-Nonanone
1291	1294	941	2-Undecanone
-	1360	874	2-Dodecanone
1499	1504	803	alpha-Muurolene
OPTIMIZED substrate 24 h			
718	712	905	2-Butanone, 3-hydroxy-(Acetoin)
736	731	908	1-Butanol, 3-methyl-
769	782	938	2,3-Butanediol
936	847	919	Butanoic acid, 3-methyl-
OPTIMIZED substrate 96 h			
718	710	825	2-Butanone, 3-hydroxy-(Acetoin)
769	806	938	2,3-Butanediol
936	868	932	Butanoic acid, 3-methyl-
926	872	896	Butanoic acid, 2-methyl-

Figure 4 - Volatiles identified by NIST library and retention time produced by *Bacillus amyloaliquefaciens* incubated.

Spore germination

The conidium germination was proportional to the metabolite concentration (Figure 5). Hyphal inhibition was clearly observed at higher

concentration (above 75%) compared to the control. No deformation on the spores or hyphal was observed.

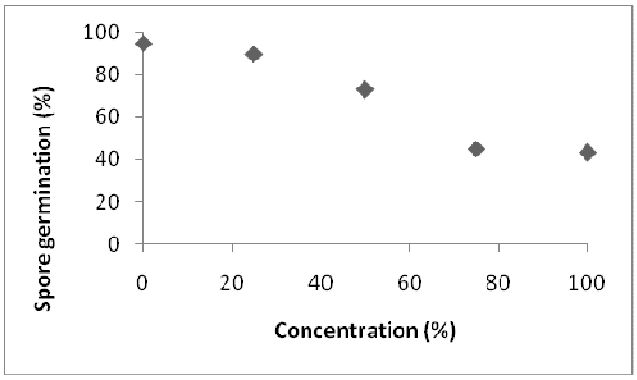


Figure 5 - Spore germination influenced by five metabolites concentration (0%, 25%, 50%, 75% and 100%).

Biocontrol in post-harvest strawberries

Treatments with the metabolites were able to protect the strawberries against grey mold, with no visible symptoms of the diseases (Figure 6).

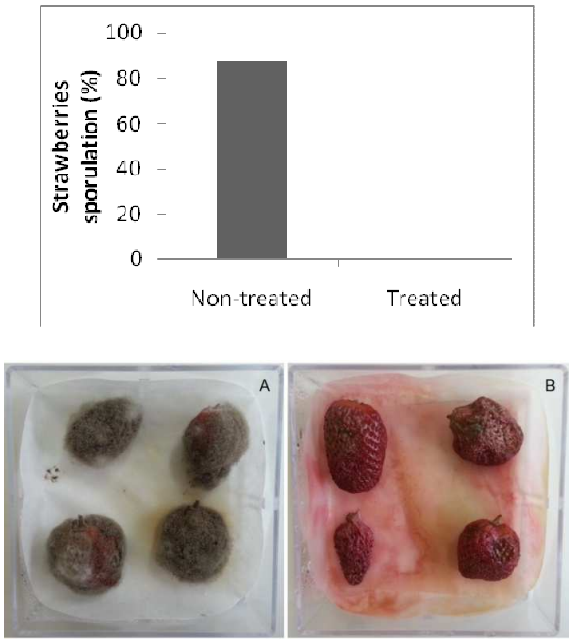


Figure 6 - Infested strawberries, storage at 25ºC, influenced by bacterial metabolites. (A) treated with the supernatant and (B) water control.

Discussion

Regarding the direct antagonism test, considering MB1 and Optimized in all temperatures, the bacteria covered the plates in 24 hours, but possibly a shorter time was required to produce active metabolites against the fungus. In this particular case, the bacterium may also have controlled the fungus by nutrient and space competition. For the PDA at

25ºC and 30ºC, the bacteria may over-produce metabolites, preventing fungus growth, and, thus, the usual halo formation was not seen. Maybe this scenario, combined with inappropriate temperatures, did not allow fungal growth as normally happens. Joshi et al. (2008) studying different carbon sources observed changes in the biosurfactant production. It can illustrate the environmental importance for the success of biological control. Once it was possible to identify mainly surfactins in solid MB1 and Optimized, this can explain why the bacteria took over all the dish surface after 24h. For PDA, surfactins and iturins were observed. Iturins are likely to be responsible for the observed control, as already shown for other post harvest diseases. Probably the same substances that act on the direct antagonism also play the same role preventing conidial germination. The Arguelles-Arias et al. (2009) works showed that different compounds from *B. amyloliquefaciens* may be involved in this control.

Additional studies of bacteria genome are required to know when an specific compound will be produced, because the substances produced by the bacteria may change from one substrate to another. The volatile study can clearly illustrate this scenario. Chen et al. (2008) working with

B. subtilis and *B. cinerea* also reported a complete growth inhibition, inhibition of spore germination and protoplast retraction. Yuan et al. (2012), studying a different pathosystem between *B.* cinerea and *Fusarium oxysporum* f. sp *cubense* also reports active volatile against this fungus. The volatile substance responsible for the control was not identified. As butanediol and acetoin were the major compounds, it was tested against the fungus, but there was no effect against fungal growth.

Apparently, the metabolites produced by the bacteria could have some harmful substances, because we noted surface modification on the treated strawberries. Although the harmful substance that caused some visible changes on the fruits was not identified, it is still interesting to note that the metabolites controlled the fungal growth and sporulation. *B. amyloliquefaciens* has shown great potential for post-harvest biocontrol of peaches against *Monilinia fructicola* (ZHOU et al., 2008). Further studies are necessary to introduce the metabolites in a practical way, keeping the shelf quality of strawberries.

Conclusion

Both temperature and substrate can interfere in pathogen control. Volatiles compounds can differ according the substrate. Spore germination was also affected by the bacterial metabolites. Strawberries were protected by the bacterial metabolites preventing the sporulation.

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