

**University of São Paulo  
“Luiz de Queiroz” College of Agriculture**

**Quantification and monitoring of *Bacillus amyloliquefaciens* GB03  
colonization in Arugula in defense against *Plutella xylostella* and  
*Spodoptera frugiperda***

**Rafaela Cristina dos Santos**

Thesis presented to obtain the degree of Doctor in  
Science: Area: Entomology

**Piracicaba  
2021**

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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*I dedicate this thesis to my parents Valmir e Ione, to my sister Gabriela and to my husband Felipe for unconditional support and patience always. And I would like to dedicate to my niece Aurora, who brought light and happiness to our lives.*

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*“Don’t get set into one form, adapt it and build your own,  
and let it grow, be like water. Empty your mind; be formless,  
shapeless — like water. Now you put water in a cup,  
it becomes the cup; you put water into a bottle it becomes the bottle;  
you put it in a teapot it becomes the teapot.  
Water can flow or it can crash.”*

*Bruce Lee*



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## RESUMO

### **Quantificação e monitoramento da colonização de *Bacillus amyloliquefaciens* GB03 em rúcula em defesa contra *Plutella xylostella* e *Spodoptera frugiperda***

*Bacillus amyloliquefaciens* GB03 é uma rizobactéria promotora de crescimento de plantas (PGPR) que ocorre naturalmente no solo e também é encontrada disponível comercialmente, que vem demonstrando efeitos positivos na promoção de crescimento de plantas de diversas culturas, indução de resistência sistêmica a patógenos, maior tolerância a seca e salinidade e mais recentemente estudos tem demonstrado sua eficiência na defesa das plantas contra o ataque de insetos em *Arabidopsis*. No entanto, um dos grandes obstáculos da utilização de PGPR hoje é o seu monitoramento no campo. Nesse sentido, os objetivos desse estudo foram: buscar um método eficiente de monitoramento de *B. amyloliquefaciens* GB03 no solo, verificar o efeito do dano do inseto na parte aérea da planta na colonização da PGPR na rizosfera, analisar se a associação com GB03 pode afetar a interação ou desenvolvimento de insetos herbívoros acima do solo e elucidar quais mecanismos estão envolvidos nessas interações, em adição, buscou-se investigar se o grau de especialidade do inseto pode influenciar na resposta dele à interação com a planta inoculada. Todos os parâmetros analisados referentes a promoção de crescimento de plantas apresentaram resultados significativamente maiores em plantas de rúcula inoculadas com a rizobactéria em relação a não inoculada. Quanto ao monitoramento, o primer correspondente à região do gene *gyrA* foi o que demonstrou maior especificidade a cepa GB03. Observou-se que a partir da segunda semana a população da rizobactéria começou a diminuir no solo e que o dano do inseto acelerou esse processo. Em adição, verificou-se que a inoculação de plantas de rúcula com a cepa GB03 afeta a alimentação e o desempenho tanto de lagartas de *P. xylostella*, herbívoro especialista, quanto de lagartas de *Spodoptera frugiperda*, herbívoro generalista, bem como a preferência de oviposição de fêmeas adultas de *P. xylostella* e que tanto a inoculação com a cepa GB03 quanto o dano pelas lagartas com diferentes graus de especialidade a resposta induzida da planta medida em termos de emissão de voláteis de planta induzidos pela herbivoria (HIPVs). Outros mecanismos que podem ser aprimorados e que estão envolvidos na defesa de plantas que foram avaliados foi a indução de resistência sistêmica (ISR) e a produção de glucosinolatos. Verificou-se que quando submetidas ao dano de *P. xylostella*, plantas de rúcula inoculadas com a cepa GB03, apresentaram maiores concentrações de ácido jasmônico em relação a plantas não inoculadas. Ao mesmo tempo, notou-se que a cepa GB03 induziu a expressão gênica de glucosinolatos em plantas de rúcula.

Palavras-chave: Interações entre PGPR-planta-inseto, Monitoramento de PGPR, Defesa de plantas, Glucosinolatos, Indução de resistência sistêmica, Compostos orgânicos voláteis

## ABSTRACT

### Quantification and monitoring of *Bacillus amyloliquefaciens* GB03 colonization in Arugula in defense against *Plutella xylostella* and *Spodoptera frugiperda*

*Bacillus amyloliquefaciens* GB03 is a plant growth-promoting rhizobacterium (PGPR) that occurs naturally in soil and is also available commercially, which has been showing positive results in plant growth promoting of different crops, induced systemic resistance to pathogens, greater tolerance drought and salinity and, more recently, studies have been effective in plant defense against insects attack in *Arabidopsis*. However, one of the main gaps today for the use of PGPR is its monitoring in the field. Therefore, the aims of this study were: to seek an efficient method for monitoring *B. amyloliquefaciens* GB03 in soil, to verify the effect of insect aboveground damage on PGPR colonization in the rhizosphere, to analyze whether the association with GB03 can affect the interaction or development of aboveground herbivorous insects and elucidate which mechanisms are involved in these interactions, in addition, we sought to investigate whether the degree of insect specialty can influence their response to interaction with the inoculated plant. All parameters analysed related to plant growth promotion were higher in inoculated plants with rhizobacterium than uninoculated plants. To monitoring, the primer corresponding to *gyrA* gene region was the most specific for GB03 strain. From the second week onwards, we observed that the rhizobacterium population began to decrease in the soil and the insect damage accelerated this process. In addition, it was found that the arugula inoculation with GB03 strain affects the feeding and performance of both *P. xylostella*, specialist caterpillars, and generalist caterpillars, *Spodoptera frugiperda*, as well as the *P. xylostella* oviposition preference and also that both inoculations with the GB03 strain and damage by caterpillars with different degrees of specialty the induced plant response measured in terms of emission of herbivory-induced plant volatiles (HIPVs) Other mechanisms that can be improved and involved in plant defense are induced systemic resistance (ISR) and glucosinolate production. It was found that when submitted to *P. xylostella* damage, arugula plants inoculated with GB03 strain presented a higher concentration of jasmonic acid compared to uninoculated plants. At the same time, it was verified that GB03 strain induced the gene expression of glucosinolates in arugula plants.

Keywords: PGPR-plant-insect interactions, PGPR monitoring, Plant defense, Glucosinolates, Induced systemic resistance, Volatile organic compounds



## 1 GENERAL INTRODUCTION

The Brassicaceae family belongs to the order Brassicales (FRANZKE et al., 2011; HONG et al., 2008) is classified as a monophyletic group, comprising approximately by 338 genera and 3709 species, including economic importance such as cauliflower, broccoli, canola, cabbage, and arugula, as well as *Arabidopsis thaliana*, used as a model plant for several studies (AL-SHEHBAZ et al., 2006). The Brassicaceae family is characterized by the presence of glucosinolates in the chemical composition, which are secondary phenolic compounds, which give to this group of plants the characteristic aroma and flavor, besides being involved in the plant defense processes (CARTEA et al., 2011; DIXON, 2007; KEHR AND BUTZ, 2011).

As in most parts of crops, the occurrence of pests is an obstacle in brassica production, generating huge losses in the harvest and commercialization throughout the cycle (AHUJA et al., 2011). Among the insects which present greater risks to these vegetable production, *Plutella xylostella* occupies a prominent position, even causing losses of up to 90% of production in some regions of the world (CHARLESTON AND KFIR, 2000). *P. xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae) is a brown colored microlepidoptera, popularly named as diamond-back-moth, and considered the most important pest in the world of brassica cultivation (MORATÓ, 2000; ULMER et al., 2002; VACARI, 2009). It has high biotic potential, fast and wide dispersion and is highly adaptable to different environments (CASTELO BRANCO AND GATEHOUSE, 2001; CHAPMAN et al., 2002; METCALF, 1981). Plant damage is caused by the feeding of caterpillars, especially in the fourth development instar. Insects can harm crops throughout the development cycle, with a higher impact on the seedling phase, in addition to attacking the formation of the head in broccoli, cabbage, and cauliflower (CAPINERA, 2015; CASTELO BRANCO et al., 1997).

It is estimated that the worldwide cost of diamondback-moth management has increased directly in proportion to the cultivated area, reaching 4 to 5 billion dollars annually (ZALUCKI et al., 2012). Chemical control has been the most used strategy to control *P. xylostella*; however, resistance to several insecticides has promoted frequent management difficulties (SHELTON et al., 1993). Diamondback-moth is resistant to more than 51 active principles used globally, considered the most resistant lepidopteran to the action of insecticides (VASQUEZ, 1995). The resistance to many active ingredients may be related to its high fertility and short life cycle, providing

several generations throughout the year and drastically reducing the efficiency of its products. This fact has collaborated to encourage research aiming at more adequate management techniques and, consequently, developing strategies within the integrated pest management (TALEKAR AND SHELTON, 1993; THULER, 2006).

Plants interact in different ways with the most varied forms of life during their cycle; they can form beneficial associations with soil-borne beneficial microorganism, which perform an important role in their survival (ZAMIOUDIS AND PIETERSE, 2012). Through the roots, plants secrete root exudates which select microorganisms in the rhizosphere, forming the microbiome (PIETERSE et al., 2014). Some microorganisms can benefit plants by synthesizing phytohormones which promote plant growth (VAN LOON, 2007); they can also cause cell expansion and improve the photosynthetic area of plants (ZHANG et al., 2007).

Plant growth-promoting rhizobacteria (PGPR) participate in important plant physiological and biological processes, improving plant defense against pathogens and pests and nutrient absorption. Therefore, PGPR must be able to survive and multiply in the rhizosphere to compete with factors adverse to the plants in the minimum time necessary to express their benefits to them (MÁRTINEZ-VIVEROS et al., 2010).

The induction of systemic resistance (IRS) in plants is a defense mechanism that can be triggered by association with PGPR, especially by the genera *Pseudomonas* and *Bacillus* spp. (VAN WEES et al., 2008), that are the genera with the largest number of studies related to the effects caused to herbivorous insects (GADHAVE and GANGE, 2018). The induced resistance state is characterized by the activation of defense mechanisms expressed after the defense-priming process caused by a pathogen or by herbivore feeding; in this way, the induction of resistance is manifested in a systemic way in the plant (WALTERS et al., 2013). IRS occurs mainly by signaling the jasmonic acid (JA) and salicylic acid (SA) pathways, which are recognized as the main hormones for plant defense (BROWSE, 2009; VLOT et al., 2009). Thus, PGPR act mainly by increasing plant sensitivity to defense hormones (VAN DER ENT et al., 2009).

The induced volatile profile can be modified when plants are associated with PGPR, affecting the insect host selection and the behavior of natural enemies (PINEDA et al., 2010; SANTOS et al., 2014). Some PGPR also release their volatile organic compounds, promoting growth and improving plant development (RYU et al.,

2003). In recent studies, it has also been proven that PGPR can act by increasing the content of glucosinolates in plants, improving defense against insects once degradation of glucosinolates affect interactions between plants and insects of different trophic levels and can alter the plant flavor and odor, influencing insect feeding (AZIZ et al., 2016; HOPKINS et al., 2009).

Beneficial soil-borne microorganisms associated with the roots can trigger physiological changes in host plants that modify responses of interactions with multitrophic insects aboveground through improving the water and nutrients absorption and growth of the damaged plant tissue area, decreasing the loss of plant biomass. At the same time, plants are exposed to herbivorous insect attacks that also influence the production of exudates and soil-borne microorganisms colonization (HERMAN et al., 2008; KEMPEL et al., 2009; KULA et al., 2005; PANGESTI et al., 2013).

How specialist and generalist insects interact with plants also can be different (AGRAWAL, 2000; BOWERS and STAMP, 1993; POELMAN et al., 2008). The main hypothesis is that generally generalists are less tolerant to plant toxins than specialists (ALI and AGRAWAL, 2012), however studies have been demonstrated high variability in how insects with different degree of specialization respond to plant defense (MEWIS, I. et al., 2006; REYMOND et al., 2004; VOGEL et al., 2007).

The genus *Bacillus* is composed of gram-positive, aerobic, rod-shaped bacteria and usually has a single oval endospore (HOLT et al., 1994). Some species belonging to this genus can interfere in the development of plant pathogens and pests through the production of metabolites with antibiotic action and stimulate the plant defense before infection and absorption of nutrients (GARDENER, 2004).

The ability to form endospores, ensuring a long shelf life under adverse conditions, makes *Bacillus*-based biological products, such as bio-insecticides, bio-fungicides, and bio-fertilizers, the most important class of commercially available microbial products (GARDENER AND FRAVEL, 2002; GARDENER, 2004). In this way, commercial products such as Quantum®, Kodiak®, BioYield®, Epic®, Rhizo-Plus®, Serenade®, Subtilex® and System 3®, are available for commercialization and are already recommended for use in different cultures in several countries (GARDENER, 2004). However, one barrier encountered in *Bacillus*-based commercial product development is the lack of knowledge about the interactions between plants and rhizobacteria, in addition to the lengthy process for registering the product (BORRISS, 2011).



*Bacillus subtilis* GB03 recently renamed as *B. amyloliquefaciens* GB03 (CHOI et al., 2014) is a PGPR of several crops, which can be found naturally occurring in the soil or can be introduced into the soil via seed treatment with a commercial product (CHOI et al., 2014; RYU et al., 2003; 2005). Volatiles organic compounds (VOCs) released by GB03 were responsible for stimulating the *Arabidopsis* growth (RYU et al., 2004) and promoting the improvement of plant development through the mediation and signaling of auxin, providing an increase in photosynthetic capacity (ZHANG et al., 2008a) and salinity tolerance (ZHANG et al., 2008b). Likewise, GB03 has already been proven to promote better iron absorption in cassava plants in the field (FREITAS et al., 2015).

In addition, the ability of GB03 to compete and inhibit the development of other microorganisms has also been confirmed, being highly effective in suppressing root pathogens, such as *Fusarium* spp. and *Rhizoctonia solani* (BACKMAN et al., 1994; TURNER AND BACKMAN, 1991). Therefore, it has been used commercially for more than two decades as a biofertilizer and biological fungicide in various cultures and against multiple pathogens (CHOI et al., 2014). However, studies on the effects of inoculation of plants with GB03 on insect behavior and development are still recent (AZIZ et al., 2016), which requires further studies in this area.

Root colonization by rhizobacterial inoculation is a complex phenomenon influenced by many parameters. The monitoring of metabolic activity of microbial inoculants and their impact on the rhizosphere and the microbiome, are necessary to ensure safe and reliable application (COOK, 2002). The first and most important prerequisite for the effective use of PGPR is the correct identification and knowledge of its activity on the ground. The development of techniques to detect and monitor the population of rhizobacteria after inoculation is important for evaluating the effectiveness and performance of microbial inoculants in agricultural production (AHMAD et al., 2011).

The 16S rRNA region has been used as a molecular marker to characterize the relative distribution of *Bacillus* spp. between soil and plant tissues (PRIEST, 1993; PINCHUK et al., 2002). However, *B. subtilis* shows 99.3% similarity in the sequence of the 16S rRNA as *B. atrophaeus* and 98.3% for *B. licheniformis* and *B. amyloliquefaciens*, which makes the confirmation of the identification among these microorganisms difficult and laborious using 16S rRNA (ASH et al., 1991; NAKAMURA et al., 1999). On the other hand, protein-coding genes, such as *gyrA*, have a high

genetic variation, which allows their users to classify and identify microorganisms belonging to closed groups (MOLLET et al., 1997; KIM et al., 1999; YAMAMOTO et al., 1999). The sequence analysis for the comparative gene *gyrA*, encodes the subunit A of the DNA gyrase A of several representatives of *B. subtilis*. Thus, the use of this gene is a fast and precise way for the classification and identification of species close to the genus *Bacillus* (CHUN AND BAE, 2000; NAZINA et al., 2001).

For this reason, this study was based on the following hypothesis:

- Is the colonization of the root system of arugula plants by GB03 efficient?
- Are the plants volatile organic compounds different depending on the specialty of the insect and the presence of GB03?
- Does the GB03 presence influence the induction of systemic resistance (ISR) in the plant?
- Is the glucosinolate content produced by plants and insect feeding modified in the presence of GB03?
- Is the use of the *gyrA* gene an efficient method for identifying and monitoring GB03?

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## 2 MONITORING A BENEFICIAL BACTERIUM (*Bacillus amyloliquefaciens*) IN THE RHIZOSPHERE WITH ARUGULA HERBIVORY

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### Abstract

The plant growth promoting rhizobacterium (PGPR) *Bacillus amyloliquefaciens* (strain GB03) augments *Arabidopsis* glucosinolate defenses targeting the generalist herbivore *Spodoptera exigua*. With the goal to transfer such technology to an agricultural crop, bacterial-defense induction was monitored in the closely related species, *Eruca sativa* (arugula). Plant growth promotion and leaf protection against the herbivore specialist *Plutella xylostella* (diamondback-moth) was induced with GB03-seed inoculation. PGPR-induced herbivore protection correlated with greater gene induction encoding for glucosinolate biosynthesis. To monitor rhizosphere-specific GB03 proliferation, PGPR-specific primers were designed for qPCR gene amplification. Rhizosphere colonization was detected with GB03 inoculation for 28 days, while rhizosphere GB03 colonization diminished to below detection level after 21 days for insect-damaged plants. By selectively monitoring GB03 from a complex mix of soil microbes with seed inoculation, rhizosphere PGPR proliferation and foliar glucosinolate transcription for plant protection against the diamondback-moth specialist can be monitored *in vivo* under authentic field conditions.

**Keywords:** Plant growth promoting rhizobacteria (PGPR); qPCR bacterial monitoring; *Eruca sativa* (arugula); induced glucosinolate chemical defense; herbivore specialist *Plutella xylostella* (diamondback-moth)

## 2.1 Introduction

Plants rely in part on their rhizosphere microbiome for fitness traits related to nutrient acquisition, biotic and abiotic stress tolerance, and protection against soil-borne pathogens (Berendsen et al., 2012; Mendes et al., 2013). Although microorganisms are an important component for functional agricultural ecosystems (Kumari et al., 2019), simply inoculating the soil with a beneficial bacterial strain at the time of planting does not uniformly or consistently induce improved plant health. Microbial variations in community-level physiological profiles based on specific biological and biochemical properties can be monitored by Biolog EcoPlates™ (Teng et al., 2019), and quantitative PCR provides a simple and accessible method for quantitative microbiota profiling (Jian et al., 2020). However, for the genus *Bacillus*, in which the 16S rRNA gene is highly conserved can be problematic (Ash et al., 1991). In this study, to discriminate between existing and an added *Bacillus* strain, DNA primers were designed for the highly variable DNA gyrase subunit A, *gyrA* in combination with qPCR amplification to monitor proliferation of the added *Bacillus* (Borshchevskaya et al., 2013). This approach allows for introduced *Bacillus* monitoring after soil inoculation with *Bacillus*-treated seeds coupled with plant-induced growth and defense responses.

*Bacillus amyloliquefaciens* (strain GB03) is a commercial plant growth-promoting rhizobacterium (PGPR), marketed as a seed treatment against fungal pathogens for select agricultural crops (Choi et al., 2014; Ryu et al., 2005). GB03-seed inoculation also induces plant growth promotion (Ryu et al., 2003). Bacterial signals that induce *Arabidopsis* growth include acetoin and 2,3-butanediol while microarray analysis has uncovered a diverse array of plant responses activated by GB03 exposure. Specifically, GB03 triggers plant-biosynthesized auxin accumulation, augments photosynthetic capacity (Zhang et al., 2008<sup>a</sup>), increases salt tolerance (Zhang et al., 2008<sup>b</sup>), enhances iron uptake (Zhang et al., 2009) and boosts pathogen defenses (Ryu et al., 2004). PGPR rhizosphere migration and root colonization has been observed to be mediated by plant-specific root secretions. For example, *Arabidopsis* excretes malic acid recruits the beneficial rhizobacterium, *Bacillus subtilis* FB17 to roots in a dose-dependent manner (Rudrappa, et al., 2008) and *Bacillus megaterium* strain YC4 utilizes myo-inositol to promote *Arabidopsis* root colonization (Vichez et al., 2020). Moreover, epigenetic regulation has been shown to mediate myo-

inositol root secretion with active DNA demethylation antagonizing RNA-directed DNA methylation to control myo-inositol homeostasis.

PGPR can also prime plant defenses against insect herbivory (Rashid & Chung 2017). Transcriptional regulation of sulfated glucosinolate accumulation has been shown to be upregulated in *Arabidopsis* in the presence of *B. amyloliquefaciens* strain GB03. While PGPR exposure in *Arabidopsis* activates higher glucosinolate accumulation with herbivore damage than with insect feeding alone (Schoonhoven et al., 2005; Aziz et al., 2016), the role of PGPR priming in arugula has not been examined. Jasmonic acid/ethylene or much less often salicylic acid regulate induced systemic resistance (ISR), activate downstream plant defenses, and play a role in mediating tritrophic interactions in select plants (Rashid & Chung, 2017), however the implication of these plant hormones in PGPR-primed herbivore defenses in arugula has also not been reported. Monitoring growth of introduced bacteria in the soil along with downstream plant health parameter will allow for the identification of correlations between bacterial growth with soil inoculation and downstream plant responses. By monitoring rhizosphere bacteria *in vivo*, inconsistencies in bacterial-induced plant growth promotion under field conditions as well as a greater understanding of microbe-microbe and plant-microbe interactions will be possible. This in turn will facilitate the construction and maintenance of stable microbial communities in agricultural soils.

Here we report on a specific gene primer for monitoring strain GB03 in soil rich in microbial diversity. *Eruca sativa*, an agricultural herb most closely related to *Arabidopsis*, is examined. Surveillance of GB03 proliferation is linked with monitoring several bacteria-induced plant health parameters, including seed germination, growth promotion and herbivore protection against the specialist agricultural pest diamondback or cabbage moth (*Plutella xylostella*).

## 2.2 Experimental

### 2.2.1 Bacterial Suspension Cultures

*B. amyloliquefaciens* (strain GB03) was streaked onto tryptic soy agar (TSA) plates and kept in an incubator in darkness at 28°C for 24 hours. Bacterial colonies were then transferred to tryptic soy broth (TSB) and grown with shaking (150 RPM) at 28°C for 24 hours. Final bacterial concentration was adjusted by optical density (OD<sub>600</sub>)

= 0.7) to  $10^9$  CFU mL<sup>-1</sup>, as previous studies have shown this to be an effective concentration for inducing plant growth (Ryu et al., 2003).

### **2.2.2 Plant Growing Conditions**

For surface sterilization, arugula seeds were washed three times with distilled water, soaked for two minutes in sodium hypochlorite (50% aqueous solution) and then rinsed three times with autoclaved, distilled water. Sterilized seeds were then dipped in a GB03 TSB suspension or TSB solution alone for thirty minutes. To inoculate the soil directly, a bacterial suspension (300  $\mu$ L) or TSB medium alone were mixed with autoclaved soil (All-Natural Garden Soil for flowers & vegetables – Kellogg®). Each pot (100 mL) was supplemented with NPK fertilizer (Osmocote Plus® (14-14-14), 1.5 g). Seeded pots were placed under a 12-/12-h light/dark cycle with metal halide and high-pressure sodium lamps for a total light intensity of 200  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>, temperature  $25 \pm 1^\circ\text{C}$  and relative humidity  $40 \pm 10\%$ .

### **2.2.3 Insect Rearing**

*Plutella xylostella* eggs were purchased from Benzon research (Carlisle, PA, USA) and hatched within a plastic container with a vent for air circulation; the room temperature was held at  $25 \pm 2^\circ\text{C}$ , with a 12-/12-h light/dark cycle and relative humidity  $40 \pm 10\%$ . After hatching, neonate larvae were transferred to a separate container and fed on cabbage leaves until pupae. When caterpillars had reached pupae stage, they were transferred to an acrylic cage (40 cm x 30 cm x 30 cm) with cabbage plants as an oviposition substrate.

### **2.2.4 Seed Germination Assay**

Sterilized arugula seeds were germinated within a covered glass dish (diameter 15 cm) between two layers of wetted filter paper (50 seeds per plate and 15 replications). In the middle of the plate, a Petri dish (diameter 5 cm) with strain GB03 (20  $\mu$ L suspension) on TSA medium or TSA was present. Seed germination was recorded daily up to 7 days and germination rate (%) was calculated.

### 2.2.5 Plant Growth

GB03-treated plants and controls were removed from the pots and roots were rinsed with water to remove attached soil at 28 days after planting. Fifteen shoots and roots were separated, and fresh weights determined.

### 2.2.6 DNA extraction: bacterial culture and soil

*B. amyloliquefaciens* strain GB03 and *B. subtilis* Carolina Cat. n°154921 (positive control) colonies were transferred to a falcon tube with 5 mL of TSB (tryptic Soy Broth), shaken at 150 rpm, at 25°C for 24 hours. Bacterial suspensions were centrifuged, and the pellet was utilized for DNA extraction using the DNeasy Blood and Tissue Kit and following the supplier's protocol (Qiagen, USA). A soil sample (250 mg) from arugula rhizosphere was used for soil DNA extraction, following the DNeasy PowerSoil Kit (Qiagen, USA) protocol (Qiagen, USA). After DNA extractions, samples were normalized and quantified via Nanodrop.

### 2.2.7 PCR and cloning

DNA from bacterial culture were amplified by PCR reaction under the following conditions: 2 ng of DNA at the concentration of 30 ng/μl of *B. amyloliquefaciens* strain GB03 and *B. subtilis* (Carolina Cat. n°154921) added separately in PCR microtubes (0.5 mL) with 1 μL of primer (Table A. 1), GoTaq® DNA Polymerase (Promega) (12.5 μL) with buffers (50 % of glycerol with buffers, blue and yellow dyes, Taq DNA polymerase (5 ng/μL), 0.2 mM of each dNTP, 7.5 mM of MgCl<sub>2</sub>) and nuclease-free water (9.5 μL). The amplicon was obtained by a PCR reaction with the follow parameters: initial denaturation 2 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 1 minute at 50°C and 30 seconds at 72 °C, and a final extension of 5 minutes at 72°C. The amplified product was verified at 3 volts cm<sup>-1</sup> for 45 minutes in agarose gel at 1%, with ethidium bromide (5 μL/100 mL). As a molecular weight marker, GeneMate Quanti-Marker 1 Kb (BioExpress) was added (25 μL) in the agarose gel.

The PCR product was cloned following the protocol pGEM®-Te pGEM®-T Easy Vector Systems. The reaction was composed of plasmid pGEM®-T Easy (1 μL, 50 ng), 2X rapid ligation buffer (5 μL), T4 DNA ligase (1 μL) and nuclease-free water (3 μL). Each ligation reaction was streaked on LB-Agar plates containing ampicillin (100 μL/mL of medium), isopropyl β-D-1-thyogalactopyranoside (IPTG) (40 μL/mL) and X-Gal (40 μL/mL). Ligation reaction (2 μL) was mixed with *Escherichia coli* (50 μL)

(JM109 High Efficiency Competent Cells, Promega), kept in the ice for 20 minutes and incubated at 42°C for 45 minutes. SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, and 20 mM glucose; 500 µL total volume) was added to the transformed cells and shaken at 150 rpm for 1 hour at 37°C. After incubation period, 100 µL of each reaction was transferred to each Petri dish and incubated overnight at 37°C. Three transformed colonies were individually transferred to liquid LB medium (2 mL) of and shaken (150 rpm) overnight at 37°C. The bacterial suspension was then centrifuged, and DNA extracted using the QIAprep Spin Miniprep kit (Qiagen, USA) following manufacturer's instructions.

### **2.2.8 qPCR standard curve and GB03 monitoring**

To build a standard curve, five dilutions of DNA from transformed bacterial cells was prepared. The reaction was composed of DNA (1 µL), primer (2 µL), SYBR® green master mix (5 µL) (Bio-Rad) and nuclease-free water (2 µL). qPCR reactions were performed in a thermometer cycler (ABI 7300 Real-Time PCR system, SeqGen). Amplicons were generated in a thermal cycler (Applied Biosystems, 7500 Real-Time PCR System) under the following parameters: initial denaturation 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and a final extension of 1 minute at 60°C. A standard curve was generated from threshold cycle (Ct) values with a known plasmid amount; three repetitions averages with three replications each, the R<sup>2</sup> value and curve expression were then determined.

To quantify strain GB03 in the soil, the amplicons of DNA product were obtained by qPCR with the follow parameters: initial denaturation 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and a final extension of 1 minute at 60°C.

### **2.2.9 Herbivory Quantification**

Second instar *P. xylostella* caterpillars were initially weighed before being placed on GB03- or media-treated plants, 28 days after planting, fifteen plants of each treatment were covered in a breathable bag to prevent insect escape. Caterpillars were weighed daily for five days and total leaf area consumed was measured using Image J software®.



### 2.2.10 Glucosinolate Transcriptional Analysis

To examine the glucosinolate gene expression in arugula, qPCR assays were performed based on three genes that catalyze glucosinolate biosynthesis: FMO<sub>GS-OX3</sub>, CYP79F1 and SUR1. Before glucosinolate gene expression analysis, one third-instar caterpillars was placed on an arugula leaf and allowed to pre-feed for 24 h before being transferred to a 7- and 14 day-old plant either treated with strain GB03 or TSB medium. Three shoot and roots of each treatment were sampled after 0 and 24 hours feeding and kept in -80°C freezer. About 50 µl of root and shoot were macerated with Mini-Beadbeater-96 (Biospec products). RNA from these samples was extracted using RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA), following manufacturer's instructions. First strand cDNA was synthesized from 4 µL of total RNA using qScript cDNA SuperMix (5X) (Quantabio, Beverly, MA, USA). Primer sequences are listed in Table 1. The amplicons were obtained by qPCR with the following parameters: initial denaturation of 2 min at 50°C, followed by 10 min at 95°C, 40 cycles of 15 s at 95°C, and a final extension of 1 min at 60°C in a thermal cycler (Applied Biosystems, 7500 Real-Time PCR System). Relative quantification was calculated, and the gene expression was quantified using  $\Delta C_t$  and  $\Delta\Delta C_t$ .

**Table 1** – Sequence of primers employed in the semi-quantitative RT-PCR analysis.

Gene	Primer sequence (5' to 3')
FMO <sub>GS-OX3</sub>	ACCAACCTCCCGAGAGAGTGTA CGGAAAGCTTCTCATACGTATTG
CYP79F1	TCCTTGGCATCAATCACTTTGC TGACCATCAATGTTCCAACCTCT
SUR1	TCGTGCTGTTTACAGTGGTC CAATCGATGCGAACTTGGCCATT
UBQ10	CGATTACTCTTGAGGTGGAG AGACCAAGTGAAGTGTGGAC
TUB8	CGTGGATCACAGCAATACAGAGCC CCTCCTGCACTTCCACTTCGTCTTC

### 2.2.11 Phytohormone Analysis

Arugula leaves were extracted for jasmonic (JA) and salicylic acids (SA) as described previously by Schmelz et al. (2003). Tissue samples (100 µg) were ground in a bead disruptor, extracted in solvent (H<sub>2</sub>O: propanol: HCl (1:2:0.05)) (400 µL) and

then spiked with JA and SA standards (10  $\mu$ L at 10 ppm). Subsequently, samples were homogenized using a vortex and an amount of 1 mL of dichloromethane was added in each tube and centrifuged at  $-4^{\circ}\text{C}$  and 13000 RPM for one minute. 200 $\mu$ L of pellet were transferred to a glass tube (4mL) dried and to the dried material 100  $\mu$ L of methanol:diethyl ester (1:9) and 2,3  $\mu$ L of trimethylsilyldiazomethane (Sigma-Aldrich) for derivatization from carboxylic acids to methyl esters. To stop the derivatization, after 25 minutes, 2.3 $\mu$ L of hexane:acetic acid (7.3: 1) was added to the solution. After 25 minutes, glass vials were heated at  $200^{\circ}\text{C}$  for 2 minutes. Compounds were collected using filters with 30 mg of Hayesep-Q<sup>®</sup> adsorbent polymer (Alltech Associates, Deerfield, IL, USA) connected in a vacuum pump (1 L/min). Polymer columns were eluted with 150  $\mu$ L of dichloromethane and samples were stored in freezer at  $-80^{\circ}\text{C}$  until analysis.

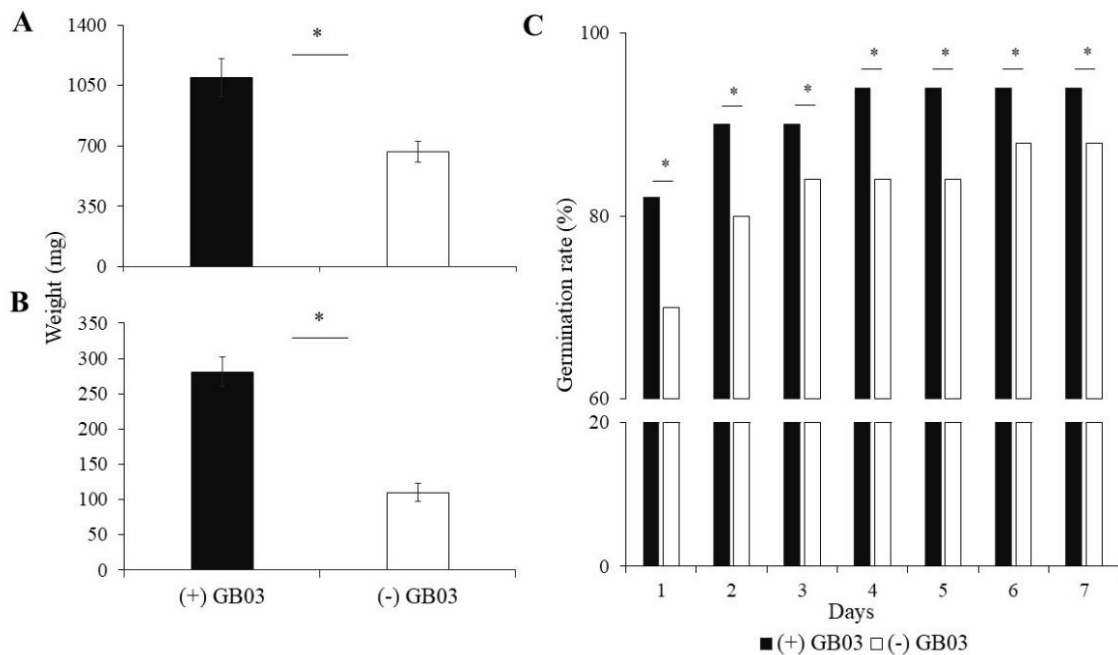
#### **2.2.12 Statistical analysis**

Normality and homogeneity of data were tested by Shapiro-Wilk and Levene tests ( $p < 0.05$ ). Seed germination, fresh weight and insect feeding were analyzed using the T test, phytohormone synthesis and GB03 monitoring in the soil and glucosinolate gene expression were analyzed using ANOVA. Statistical analyses were performed using Past 4.0 software.

### **2.3 Results**

#### **2.3.1 Strain GB03 promotes seed germination and growth**

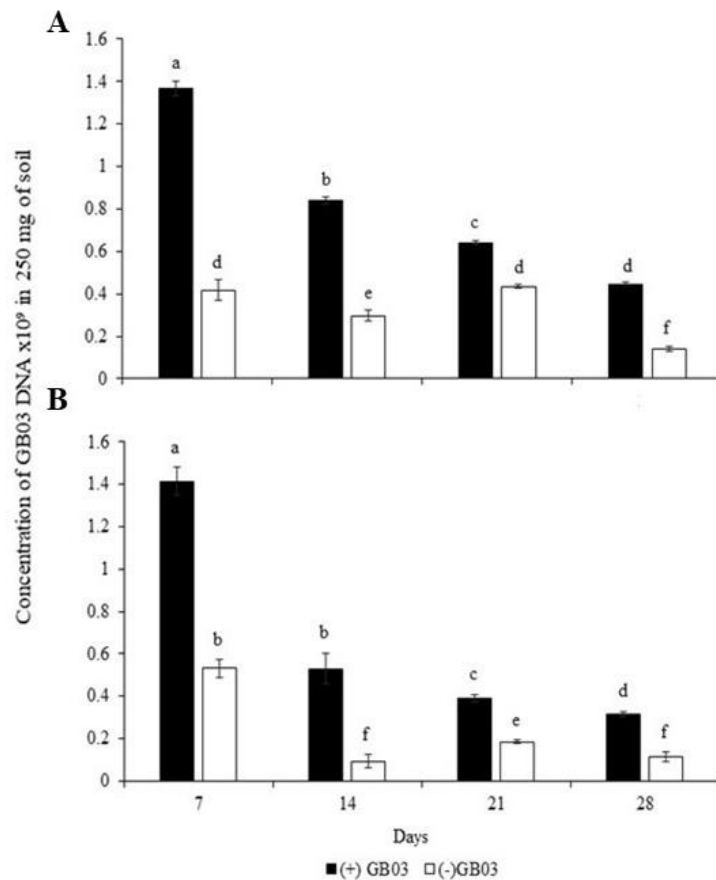
To determine the effect of GB03 seed inoculation on arugula growth, plants were harvested, and tissue weights were recorded. In plants treated with strain GB03, shoot and root fresh weight increased 154 and 67%, respectively, in comparison with untreated controls (Figure 1A and B). In addition, the germination rate of seeds exposed to GB03 volatiles was increased by 6 to 12% over a seven-day assay window (Figure 1C) ( $p < 0.05$ ).



**Figure 1:** Arugula shoot and root fresh weight was greater (A and B, respectively), and time for breaking seed dormancy faster (C) with *Bacillus amyloliquefaciens* strain GB03 inoculation than the untreated controls. Mean values are shown with bars indicating standard error (SE) (n=15); an asterisk (\*) indicates statistically significant differences between treatments ( $p < 0.05$ ; t test).

### 2.3.2 Strain GB03 rhizosphere monitoring

Strain GB03 was observed in the rhizosphere when gene specific primers, based on highly variable regions of endoglucanase and *gyrA* were employed. Gene amplified products were observed using *gyrA1* or endoglucanase specific primers and a qPCR expression curve was calculated with cDNA dilutions (up to 0.1 ng) (Fig. A2). Primer specificity was examined by qPCR and a primer corresponding to a region of *gyrA1* was found to be highly specific for strain GB03 compared with a closely related commercial species (*B. subtilis*) (Fig. A3). In contrast, both *Bacillus* species exhibited the same amplification curve when primers corresponding to endoglucanase and 16S region were utilized (Fig. A1).



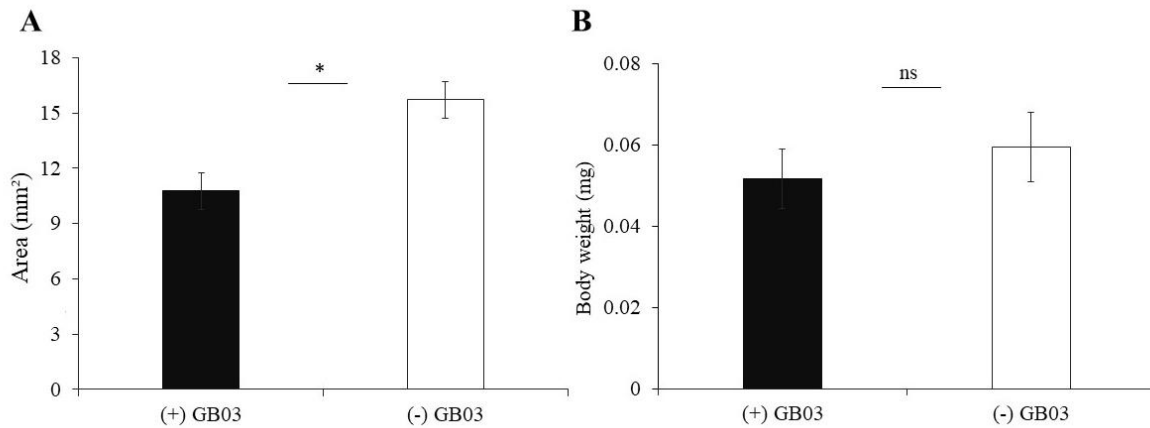
**Figure 2:** *Bacillus amyloliquefaciens* GB03 proliferation in the rhizosphere after seed inoculation: (A) plants with no insect damage and (B) plants exposed to *Plutella xylostella* larvae feeding. Mean values are shown with bars indicating standard error (SE) (n=3); Different letters indicate statistically significant differences among treatments ( $P < 0.05$ ; ANOVA).

Based on bacterial analysis using the *gyrA1* primer, rhizosphere soil within  $3 \pm 1$  mm of the inoculated arugula seeds showed the highest strain GB03 concentration seven days after planting with GB03 significantly diminishing each week after the initial sampling. Bacterial background signal was taken as the average of all soil samples without GB03 treatment (Fig. 2A - B). With herbivore damage to the aerial portions of the plant by *Plutella xylostella* larvae initiated 36 hours before sampling the plants, GB03 DNA levels decreased significantly within one week and stayed at background levels for the remaining period that was monitored.

### 2.3.3 GB03 seed inoculation lowers herbivory

In addition to rhizosphere strain GB03 abundance being down regulated by herbivore damage to the leaves, GB03 seed inoculation reduced herbivore foliar

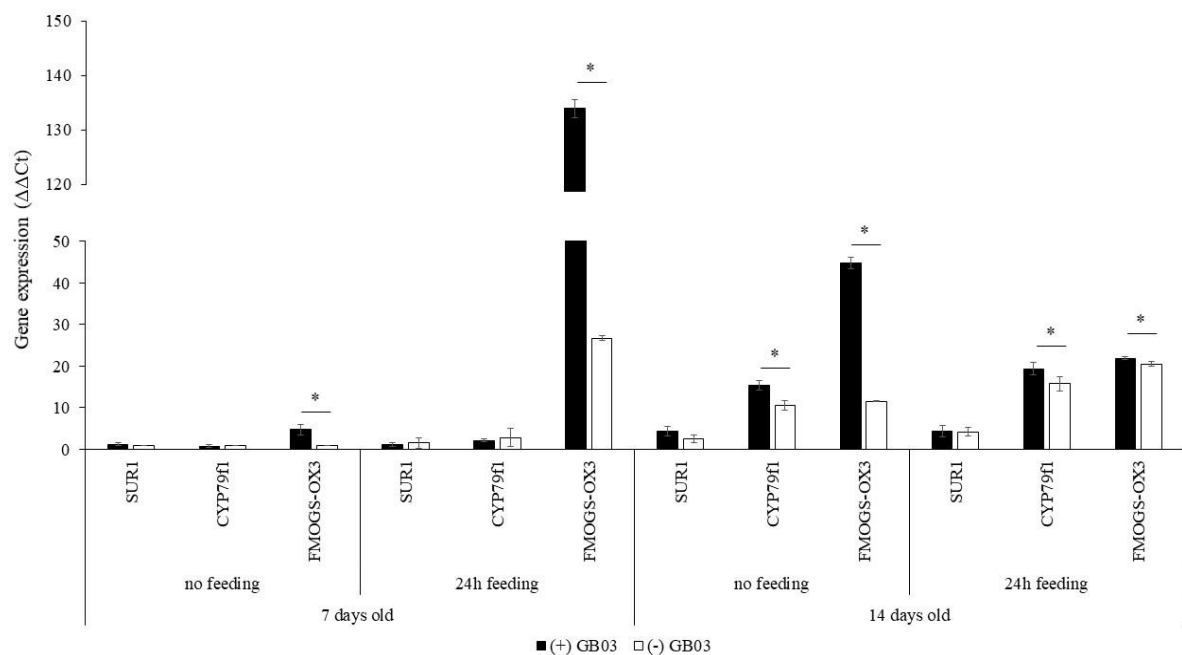
damage; specifically, GB03-treated plants had approximately 57% less leaf area consumed than untreated plants ( $p < 0,05$ ) (Fig. 3A). Although larvae feeding by *P. xylostella* was lower for strain GB03 treated plants than untreated controls, no statistical difference was observed between treatments ( $p > 0.05$ ) (Figure 3B) in terms of body weight for larvae that fed on GB03-treated or non-inoculated arugula plants.



**Figure 3:** Eating parameters for *Plutella xylostella* larvae feeding on 28-day-old *Bacillus amyloliquefaciens* strain GB03-inoculated and non-inoculated arugula plants: (A) leaf consumption and (B) *P. xylostella* weight gain. Mean values are shown with bars indicating standard error (SE) (n=15); an asterisk (\*) indicates statistically significant differences between treatments ( $P < 0.05$ ; t test).

#### 2.3.4 Strain GB03 Induces Glucosinolate Gene Expression

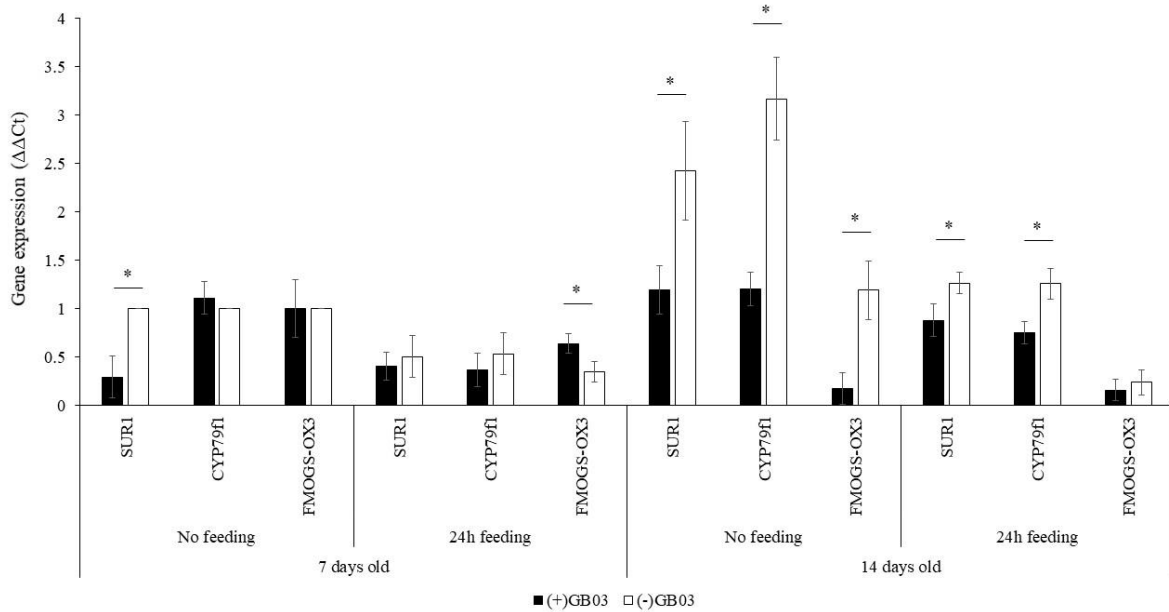
Genes that encode for glucosinolate biosynthesis including FMOGS-OX3, a flavin-monooxygenase that catalyzes the first step in chain modification of aliphatic glucosinolates (Li et al., 2008), CYP79F1 a cytochrome-P450-dependent monooxygenase, that catalyzes core structure amino acid conversion to aldoximes (Mikkelsen et al., 2000) and SUR1 a C-S glucosinolate lyase that cleaves and converts S-alkyl thiohydroximates to thiohydroxamic acids (Mikkelsen et al., 2003) were found to be differentially regulated by qPCR analysis in leaves and roots at seven and fourteen days with and without a 24 hr. herbivory by *P. xylostella* to the leaves.



**Figure 4:** Leaf glucosinolate gene expression with marker genes SUR1, CYP79F1 and FMO<sub>GS-OX3</sub>, for seven- and fourteen-day-old plants, inoculated and uninoculated with *Bacillus amyloliquefaciens* strain GB03 and with or without insect feeding. One asterisk (\*) indicates significant difference between treatments based on ANOVA ( $p < 0.05$ ).

In shoots, marker glucosinolate genes were unchanged or upregulated with GB03 seed treatment for both 7 and 14 day-old-plants. For 7-day-old plants with GB03 treatment, FMO<sub>GS-OX3</sub> is induced 4.9-fold with insect feeding than for undamaged leaves. For 14 day-old-plants with strain GB03 the trend reverses with FMO<sub>GS-OX3</sub> showing slightly greater expression in leaves without insect damage. CYP79F1 is expressed 41 and 18% higher in GB03 treated versus untreated 14 day-old-plants with and without insect feeding, respectively. SUR1 gene expression was also higher in GB03 treated versus untreated 14 day-old-plants with similar expression levels with and without herbivory (Fig. 4).

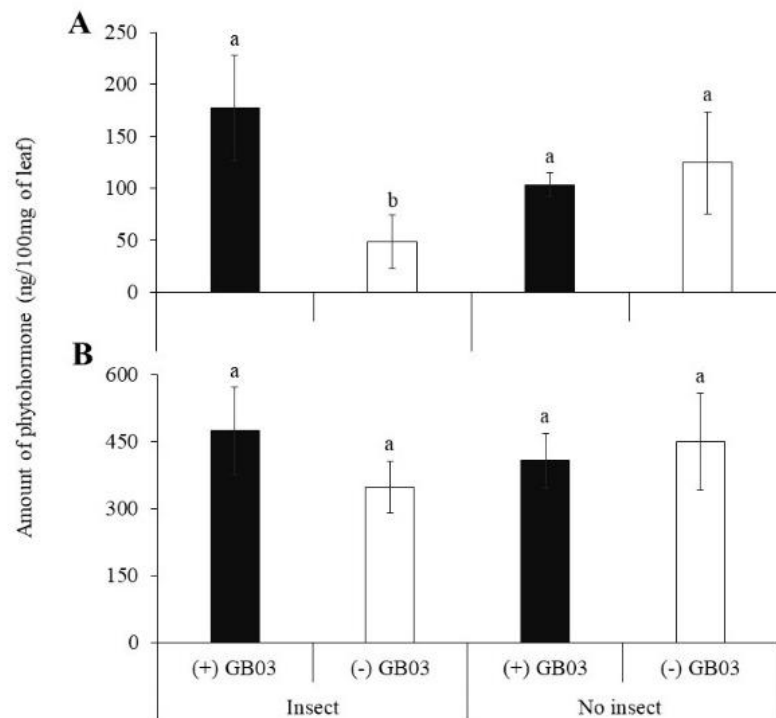
In roots, marker glucosinolate genes were expressed at much lower levels than shoots with gene expression mostly unchanged or downregulated with GB03 seed treatment for both 7 and 14 day-old-plants. The one exception was FMO<sub>GS-OX3</sub> that was upregulated approximately two-fold for 7 day-old-plants with strain GB03 combined with herbivory (Fig. 5).



**Figure 5:** Root glucosinolate gene expression with marker genes SUR1, CYP79F1 and FMOGS-OX3, for seven- and fourteen-day-old plants, inoculated and uninoculated with *Bacillus amyloliquefaciens* strain GB03 and with or without insect feeding. One asterisk (\*) indicates significant difference between treatments based on ANOVA ( $P < 0.05$ ).

### 2.3.5 Strain GB03 regulation of MeJA signaling

To probe the role of signaling pathways that mediate plant defense, the plant hormones methyl jasmonate (MeJA) and methyl salicylate (MeSA) were monitored with and without herbivory and strain GB03 seed treatment. MeJA was present at higher levels in leaves with both GB03 and insect feeding versus herbivory without strain GB03 ( $p < 0.05$ ). In the absence of herbivory, MeJA levels in the leaves were the same both with and without strain GB03 (Fig. 6A). In monitoring MeSA, no differences were observed either with or without insect damage and with or without strain GB03 (Figure 6B).



**Figure 6:** Phytohormone accumulation with indicated treatment for 28-day-old plants; (A) methyl jasmonate, (B) methyl salicylate. Values are mean, and bars indicate standard error (SE) (n=10). Different letters indicate a statistically significant difference among treatments ( $P < 0.05$ ; ANOVA).

## 2.4 Discussion

With *in vitro*-grown *Arabidopsis*, many responses are induced by the PGPR strain GB03 including enhanced photosynthetic efficiency (Zhang et al., 2008), increased glucosinolate defenses (Aziz et al., 2016) and elevated reproductive success (Xie et al., 2009), however the ability of this microbial inoculant to colonize and proliferate with seed inoculation in variable soil conditions (*e.g.*, moisture, nutrients, pH, or temperature) or plant-stress (*e.g.*, biotic, or abiotic) is less clear. By utilizing GB03-specific and glucosinolate-based primers, qPCR gene amplification allowed for monitoring bacteria proliferation directly in the rhizosphere, as well as concurrently monitor plant-defense induction. *Eruca sativa* (arugula) was chosen as an *Arabidopsis* alternative as it is the closest relative to this model plant, and as a salad crop, arugula is cultivated throughout the world. Both accelerated seed germination and greater biomass were observed in arugula with GB03 treatment.

To understand spatiotemporal dynamics of a microbial community, full-length 16S rRNA gene sequences allows for comparing and resolving bacteria taxa at the species-level. (Numberger et al., 2019; Hongxia et al., 2021). While 16S rRNA



sequencing provides extensive and in-depth information about microbial communities without culturing, biases can occur associated with sequencing-preparation steps. In comparing shotgun metagenome with 16S rRNA sequencing datasets, while the estimated number of operational taxonomic units (OTUs) may be concordant, up to ten times as many genera can be identified in metagenome analyses (Poretsky et al., 2014). While targeting of 16S variable regions with short-read sequencing platforms cannot achieve the taxonomic resolution afforded by whole gene sequencing (~1500 bp) (Johnson et al., 2019), we tested GB03 specific gene primers developed for two specific genes with higher variability in DNA sequence, endoglucanase and *gyrA*. After qPCR amplification, primers designed for *gyrA* region were able to discriminate two closely related *Bacillus* species in contrast to endoglucanase primers. Other studies have also shown *gyrA* gene primers effective at discriminating closed-related bacteria with high genetic similarity (Chun and Bae, 2000; Borshchevskaya et al., 2013). qPCR-based quantitative microbiome profiling was selected based on several conceptual and practical benefits over full-length 16S rRNA gene sequencing: [i] cost-effective and accessible in many laboratory settings; [ii] relatively simple to perform (especially compared to analysis by flow cytometry that requires considerable expertise for reproducible results); and [iii] qPCR-based quantitative microbiome profiling is applicable for samples containing significant amounts of host or non-bacterial DNA, in which bacterial density cannot be reliably estimated by total DNA yield (Poretsky et al., 2014).

The successful colonization of PGPR in the rhizosphere is an initial and compulsory step in promoting plant growth and reducing herbivory. However, prior to root colonization, chemotaxis of PGPR towards roots is essential (Sood, 2003). With up to 40% of photosynthate channeled to below ground release, root exudates are a major driver of soil microbiota composition. Moreover, specific signaling molecules selectively attract individual PGPR strains. For example, in *Arabidopsis* root secretion of myo-inositol and subsequent plant growth promotion is triggered by *Bacillus megaterium* strain YC4 (Vílchez, et al., 2020). Root-secreted myo-inositol is critical for YC4 colonization, preferentially attracts *B. megaterium* among the examined bacteria species and is mediated by active DNA demethylation. With a second type of chemical signaling, both *Arabidopsis* and banana root exudates that contain organic acids that induce both chemotaxis and biofilm formation (Yuan et al., 2015; Rudrappa et al., 2008). More specifically, malic acid shows the greatest chemotactic response whereas

fumaric acid favors biofilm formation. Since foliar stress with herbivory can channel carbon away from root exudates (Bezemer & van Dam, 2005), reduced bacterial retention and/or proliferation in the rhizosphere has been proposed with above ground herbivory (Bais et al., 2006). Indeed, with rhizosphere seed inoculation in arugula, GB03 concentrations in the rhizosphere decrease to below detection levels within three weeks with insect damage in contrast to four weeks without insect herbivory (Fig. 2). Based on tracking fluorescent *Pseudomonads* that readily colonize roots in nature and are frequently the most common taxon of microorganisms, bacteria proliferation has been observed to increase in the rhizosphere up to ca. 48 hours after inoculation before bacterial populations of the microbial inoculum in the soil begins to decline (Kluepfel, 1993).

Induced systemic resistance (ISR) is activated by select PGPR that prime plants for enhanced defense against a broad range of insect herbivores and is often mediated by JA and ethylene. To establish that JA participates in the regulation of an SA-independent systemic immunity conferred by GB03, extracted JA and SA, (derivatized to MeJA and MeSA, respectively, to facilitate chemical analysis) were quantified by GC. MeJA alone was induced with GB03 treatment (Fig. 6). Indeed, the observed decrease in leaf damage with *Plutella xylostella* larval feeding (Fig. 3) is consistent with other plant priming responses that exhibit better protection via faster and/or more robust cellular-defense activation with insect attack. (Conrath et al., 2006). While sulfated glucosinolates are known to be induced as a plant defense induced in response to herbivore attack (Mewis et al., 2006; Hopkins et al., 2009), less is known as to how PGPR prime glucosinolate induction (van de Mortel et al., 2012). In *Arabidopsis*, GB03 induces mRNAs that encode for the catalysis of sulfur-rich aliphatic- and indolic-glucosinolates (Aziz et al., 2016). Moreover, *Arabidopsis* plants with elevated glucosinolates exhibit greater protection against the generalist herbivore, *Spodoptera exigua* (beet armyworm). As confirmation of inducible plant defense, a glucosinolate triple mutant compromised in the production of both aliphatic and indolic glucosinolates was found to also be compromised in terms of GB03-induced protection against insect herbivory. While glucosinolate accumulation is not measured in this study, transcription of a key enzyme in the glucosinolate core structure as well as secondary modifications, CYP79F1 and FMO<sub>GS-OX3</sub>, respectively are also upregulated in arugula with GB03 exposure. CYP79F1 specifically catalyzes the first committed step in biosynthesis of the aliphatic glucosinolate core structure that involves

conversion of amino acids to corresponding aldoximes (a rate-limiting step in glucosinolates biosynthesis; Mikkelsen and Halkier, 2003) while FMOGS-OX3a is responsible for encoding one of the five flavin monooxygenases involved in S-oxygenation of aliphatic glucosinolates resulting in conversion of methylthioalkyl glucosinolates to methylsulfinylalkyl glucosinolates (Sønderby et al., 2010). While specialist and generalist herbivores have been shown to exert opposing selection on chemical defenses (Lankau, 2007), with induction of glucosinolate accumulation, herbivory by the generalist beet armyworm as well as the specialist, diamondback-moth, positively correlated with transcriptional induction encoding for glucosinolate biosynthesis.

In *Arabidopsis*, GB03 transcriptionally induces sulfate assimilation and coordinates this process with enhanced sulfate uptake, as well as elevated sulfur, cysteine, and glucosinolate accumulation (Aziz et al., 2016). Besides glucosinolates functioning in plant defense, select isothiocyanates derived from the hydrolysis of methylsulfinylalkyl glucosinolates are potent cancer-preventive agents (Hansen et al., 2007; Li et al., 2008). With current agriculture strategies including integrated pest management, advances in PGPR soil monitoring may well lead to rational-PGPR applications, lower growth-promotion variability, reduced environmental impacts, and a greater comprehensive understanding of bacterial stability and proliferation in the rhizosphere.

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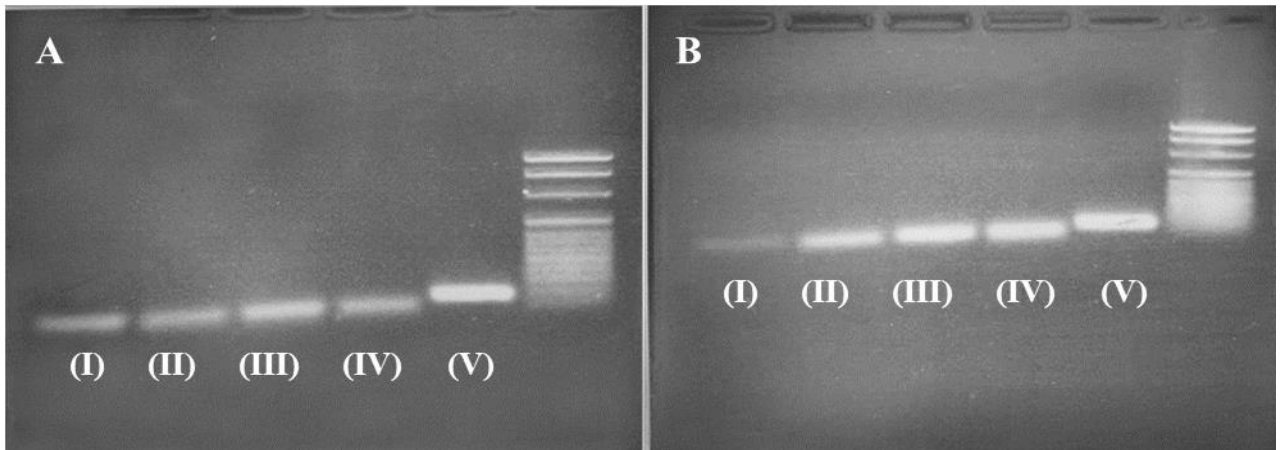
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## Appendices

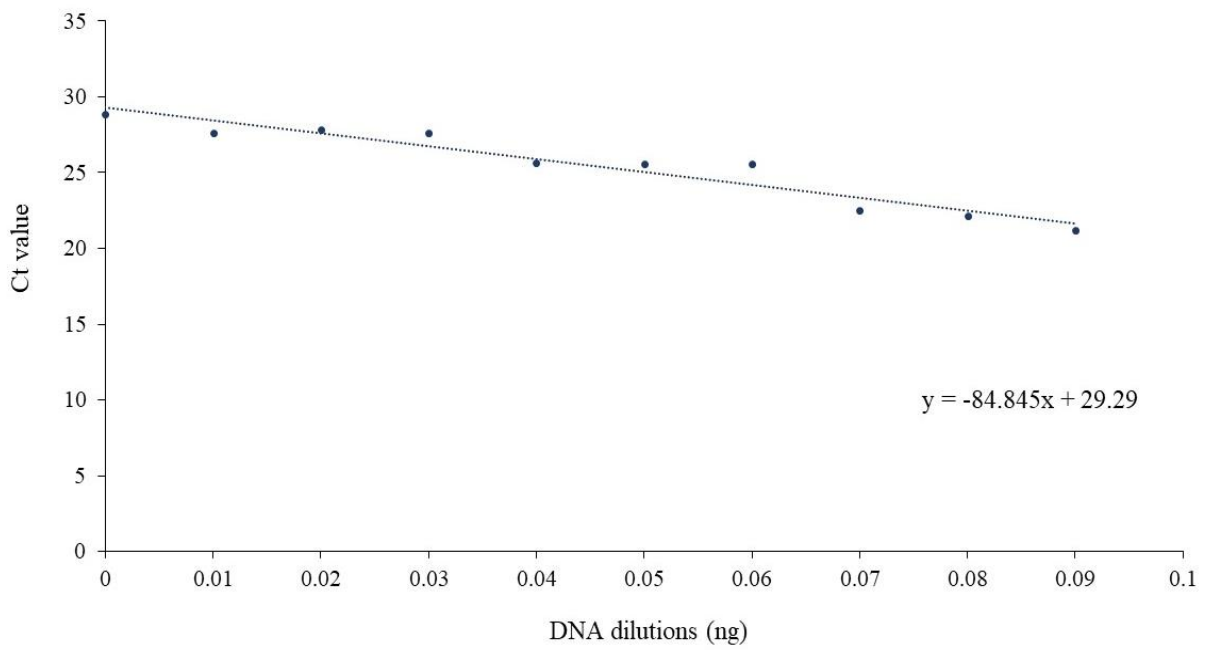
**Table A. 1** – Specific primers for *Bacillus amyloliquefaciens* strain GB03

<b>Gene</b>	<b>Primer name</b>	<b>Sequence (5'-3')</b>
16S	<i>B. subtilis</i> 16S -F	TCGCGGTTTCGCTGCCCTTT
	<i>B. subtilis</i> 16S -R	AAGTCCCGCAACGAGCGCAA
gyrA	gyrA 1 - F	CCGTATCGTCGGTGAAGTTATC
	gyrA 1 - R	AACAAGCATGTAGCGGTAGTTA
gyrA	gyrA 2 - F	CGGTTCACAGGCGGATTT
	gyrA 2 - R	CCGATAACTTCACCGACGATAC
Endoglucanase	Endoglucanase 1 - F	CTCATCCAATCTGCGTGTAGTC
	Endoglucanase 1 - R	CGGGAATACCACGGTTGATTTA
Endoglucanase	Endoglucanase 2 - F	ACCCAGTTCCAGGTAGGTAT
	Endoglucanase 2 - R	GACTGTGACTACACGCAGATT

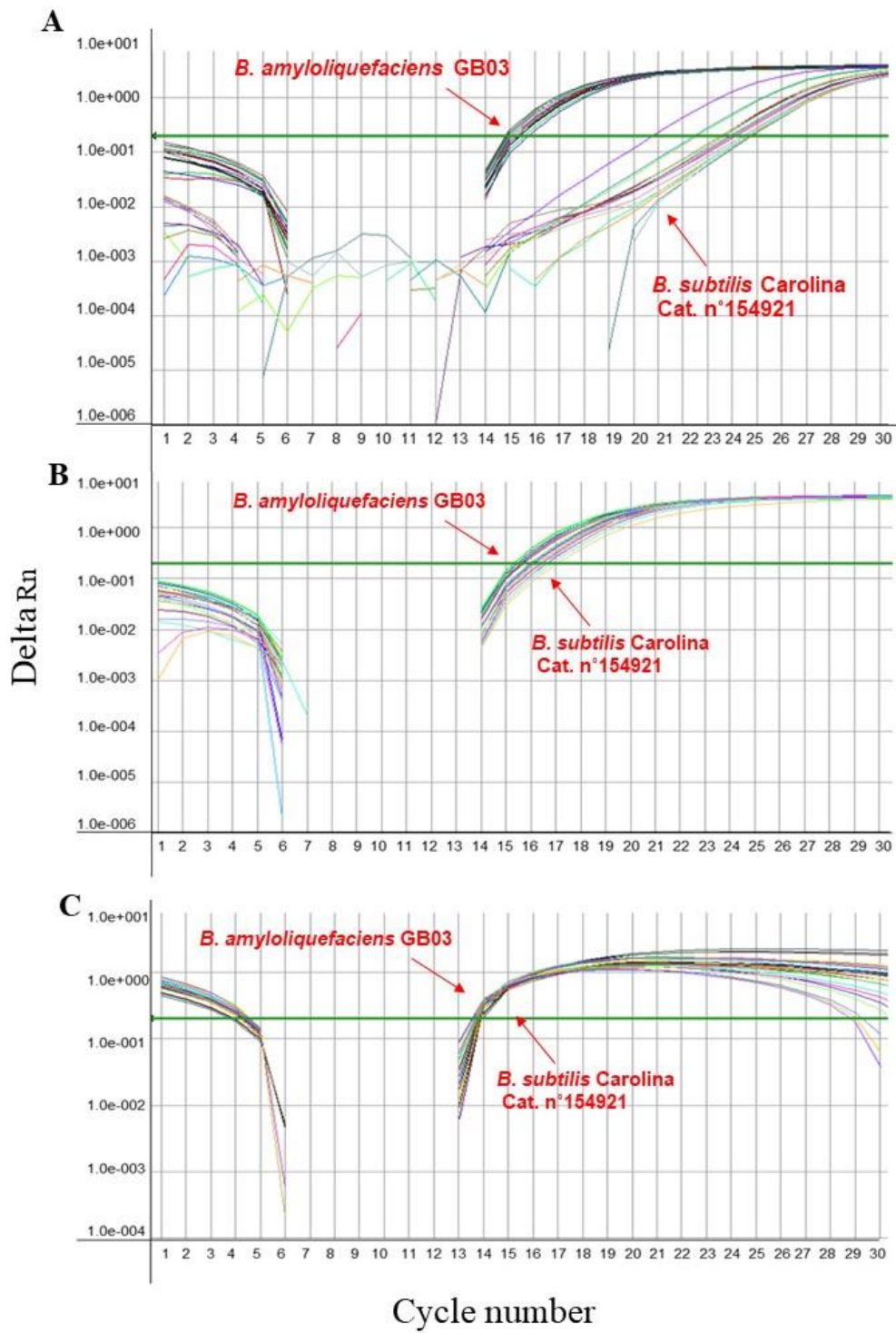


**Figure A. 1** **A** *Bacillus amyloliquefaciens* strain GB03 **B** *Bacillus subtilis* Carolina Cat. n°154921. Agarose gel with primers: (I) gyrA 1, (II) gyrA 2, (III) endoglucanase 1, (IV) endoglucanase 2 and (V) 16S.





**Figure A. 2** DNA from transformed bacterial cells used to build the standard curve and curve expression for qPCR.



**Figure A. 3** RT-PCR amplification curve of the following *Bacillus amyloliquefaciens* GB03 genes: (A) *GyrA* gene; (B) Endoglucanase gene and (C) 16S gene.

### **3 *Bacillus amyloliquefaciens* GB03 AFFECTS *Spodoptera frugiperda* PERFORMANCE AND *Plutella xylostella* HOST SEARCH**

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#### **Abstract**

*Bacillus amyloliquefaciens* GB03 is a plant growth promoting rhizobacteria (PGPR) which has provided several benefits to plants. Recently, it has begun to explore how GB03 can influence the plant defense against herbivorous insect damage. The aim of this study was evaluate the ability of GB03 to promote arugula growth by measuring root growth parameters, identify arugula volatile organic compounds released by plants with and without GB03 inoculation and the effects in *Spodoptera frugiperda* larval development and *Plutella xylostella* behavior. At the same time, we verified whether inoculated and uninoculated plants with GB03 released different blend of VOCs when they were under attack by the insects tested, as plant defense response. We observed that GB03 strongly increased the arugula root development. Both, *S. frugiperda* gain of weight and *P. xylostella* host search were affected by the GB03 inoculation. We observed that there were quantitative and qualitative difference in plant volatile profile released according to inoculation and uninoculation with GB03 and the herbivore specialization.

**Keywords:** Volatile organic compounds; *Eruca sativa*; diamondback-moth; plant defense; beneficial soil borne microorganisms; fall armyworm.

#### **3.1 Introduction**

Although world population growth is slowing, UN estimates an increase of around 2 billion people worldwide by 2050 (UN DESA, 2019). Supplying food to the entire world population is becoming a huge challenge for agriculture, where the aim is to obtain more productive crops without increasing the area under cultivation. At the same time, it is notable that both producers and consumers are increasingly concerned

with the quality of the food and the impacts caused to the environment (Shaikh and Sayyed, 2015).

In the seventh decade in the past century, the green revolution was essential for the evolution of food production globally. However, with the advance in studies that report the functions and the importance of beneficial soil-borne microorganisms for agriculture, a new green revolution is emerging, where the valorization of beneficial soil-borne microorganisms and their use in agriculture has been a central pillar (Ansari and Mahmood, 2017).

Within this context, plant growth-promoting rhizobacterium (PGPR) is a group of beneficial soil-borne microorganisms widely studied because their ability to improve plant growth and development by producing phytohormones and providing a better nutrient uptake (Sharifi and Ryu, 2017). Among the PGPR group, species belonging to the genera *Bacillus* and *Pseudomonas* are the ones which have been reported the largest number of studies related to the effects caused to herbivorous insects, presenting great potential for use in pest management (Gadhawe and Gange, 2018).

In order to have an interaction between insects aboveground and PGPR belowground, plants perform as a linking element, triggering a bidirectional interaction between these organisms (Pineda et al., 2010). Researchers have been suggested that PGPR can manipulate aboveground interactions by enhancing tolerance of plants under insect attack through biochemical changes that alter the production and emission of secondary metabolites (Zehnder et al., 1997; Aziz et al., 2016; Zebelo et al., 2016).

Plants naturally release a range of specific volatile organic compounds (VOCs), secondary metabolites that act as a universal language in plant-to-plant interactions and between plants and other organisms (Mithofer and Boland, 2016; Simpraga et al., 2016). However, the quality and concentration of VOCs released by plants can be affected by several factors as soil microbiome composition and insect herbivory, and its degree of specialization (Portillo-Estrada et al., 2015; Jiang et al., 2016).

Specialist and generalist herbivores interact with plants in different ways (Ali and Agrawal, 2012). In general, is observed that specialists are less impacted by plant defense than generalist insects, because they have a closer relationship with host plants and can develop specific defense to these plants toxins, while generalist develop a general mechanisms that allow to feed a wider range of plant species (Cornell and Hawkins, 2003; Whittaker and Feeny, 1971)

Most the studies about the influence of PGPR-plant association in plant volatiles blend are related to *Bacillus* and *Pseudomonas* genera and involve qualitative and quantitative modification in both constitutive and induced compounds (Zebelo et al., 2016). *Bacillus amyloliquefaciens* GB03 is a commercially available PGPR that has been well documented to promote plant growth (Freitas et al., 2015; Wu et al., 2016). The ability of GB03 to release a bouquet of VOCs and how they can influence in plant development has also been studied (Xie et al., 2009; Ryu et al., 2003, Zhang et al., 2007; 2008).

*Arabidopsis thaliana*, when exposed to GB03 volatiles, has been shown to increase plant growth promotion and development. The mechanisms associated to plant growth-promoting by GB03 volatiles have been reported and by increasing photosynthetic efficiency and chlorophyll content (Zhang et al., 2008) and by cell wall histological changes (Ryu et al., 2003). Molecular and physiological researchers have been described that the plant growth in *Arabidopsis* is triggered by regulating auxin homeostasis and cell expansion (Zhang et al., 2007). In addition, *Arabidopsis* seedlings exposed to the bacterial volatiles activated the induced systemic resistance against the bacterial pathogen *Erwinia carotovora* subsp. *carotovora* (Ryu et al., 2004). Arugula plants exposed to GB03 volatiles presented an augmenting plant growth and VOCs released (Chou et al., 2013).

All the above reported studies suggest the importance of GB03 in plant growth promoting and in induced systemic resistance. However, there are gaps that we need to understand, as well as how GB03 inoculation in seeds can influence plant volatiles, how the association with GB03 can affect insects aboveground, how insects and GB03 can modify the composition of VOCs blend and how the degree of specialization of the insect can affect the insect-plant-PGPR interactions. For this reason, the aim of this study was to identify arugula VOCs released by plants with and without GB03 inoculation and the effects in *Spodoptera frugiperda* (generalist) larval development and *Plutella xylostella* (specialist) behavior. At the same time, we verified whether inoculated, and uninoculated plants with GB03 released different blends of VOCs when they were under attack by specialist and generalist insects.

## 3.2 Methods

### 3.2.1 *Bacillus amyloliquefaciens* GB03 Culture

*B. amyloliquefaciens* GB03 was streaked onto tryptic soy agar (TSA) plates and kept in an incubator in darkness at 28°C for 24 hours. Bacterial colonies were then transferred to tryptic soy broth (TSB) and grown with shaking (150 RPM) at 28°C for 24 hours. Final bacterial concentration was adjusted by optical density ( $OD_{600} = 0.7$ ) to  $10^9$  CFU mL<sup>-1</sup>.

### 3.2.2 Plant Growing Conditions

For surface sterilization, arugula seeds were washed three times with distilled water, soaked for two minutes in sodium hypochlorite (50% aqueous solution), and then rinsed three times with autoclaved purified water. Sterilized seeds were then dipped in a GB03 TSB suspension or TSB solution alone for thirty minutes. To inoculate the soil directly, a bacterial suspension (750 µL) or TSB medium alone were mixed with autoclaved soil (Basaplant®). Each pot (250 mL) was supplemented with NPK fertilizer (Osmocote Plus® (14-14-14), 1.5 g). Seeded pots were placed in a greenhouse for 21 days under a temperature of 28±5°C and humidity of 40±10%.

### 3.2.3 *Plutella xylostella* rearing

*P. xylostella* pupae were provided from Laboratory of Biology and Insect Rearing – UNESP (Jaboticabal, SP, Brazil). Pupae were kept in an acrylic cage (40cm x30cm x 30cm) with cabbage plants as oviposition substrate. After hatching, neonate larvae were transferred to a separate container and fed on cabbage leaves until pupae. *P. xylostella* rearing was maintained in an incubator chamber at 25±2°C, with a 12-/12-h light/dark cycle and relative humidity of 40±10%.

### 3.2.4 *Spodoptera frugiperda* rearing

To establish *S. frugiperda* rearing, eggs were collected in cabbage field (Piracicaba, SP, Brazil). Eggs were placed separately into plastic containers (4 cm high x 7 cm diameter) with cabbage leaves to feed, which were changed daily. When insects reached the pupa stage, they were placed into cages made with PVC tubes (20 cm in height x 9 cm in diameter) lined with sulfite paper to oviposition. Small plastic containers with 10% honey solution were provided inside the cages to feed the moths. After hatching, neonate larvae were transferred separately into plastic containers (4

cm in height x 7 cm in diameter) with cabbage leaves. *S. frugiperda* rearing was kept in an incubator chamber at  $25\pm 2^{\circ}\text{C}$ , with a 12-/12-h light/dark cycle and relative humidity of  $60\pm 10\%$ .

### 3.2.5 Root growth measurement

Arugula roots from 21 days old plants, previously inoculated and uninoculated with GB03, were extracted and carefully rinsed with water to remove soil particles before being stored individually into Falcon tubes with a 30% ethanol solution for preservation. Images of each root were obtained using the equipment Epson LA2400 scanner (2,400 dpi resolution) and then were analyzed by the WinRHIZO software (Reagent Instruments Inc., Quebec, Canada). This software provided measures of root surface area ( $\text{mm}^2$ ), root volume ( $\text{mm}^3$ ), and average root hair length (mm). All measurements were recorded individually by plant, thereby resulting in five replications per treatment.

### 3.2.6 Plant volatile collection and analysis

The plant volatile collection system was based on the methodology described by Turlings et al. (1998). Arugula plants were placed in glass chambers (10 cm in diameter x 5 cm in height) previously washed with acetone and hexane and coupled to the 'ARS Volatile Collection System' (ARS, Gainesville, FLA, 19 USA). The inlet and outlet airflow was 0.6 L/min which was controlled by a vacuum pump connected to filters with 30 mg of Haysep Q® adsorbent polymer linked to the glass chambers. The assay was conducted in the laboratory under controlled conditions ( $25\pm 1^{\circ}\text{C}$ ,  $70\pm 10\%$  RH), and volatiles were collected for eight hours (8:00 AM – 4:00 PM).

We collected volatile organic compounds of 21 days old arugula plants, under the following conditions: I) arugula plant ((-) GB03); II) arugula plant + GB03 inoculation ((+) GB03); III) arugula plant + *P. xylostella* damage ((-) GB03 + *P. xylostella*); IV) arugula plant + GB03 + *P. xylostella* damage ((+) GB03 + *P. xylostella*); V) arugula plant + *S. frugiperda* damage ((-) GB03 + *S. frugiperda*); VI) arugula damage + GB03 + *S. frugiperda* ((+) GB03 + *S. frugiperda*). Each treatment consisted of ten replications.

Filters were eluted with 150  $\mu\text{L}$  of hexane mixed with 5 $\mu\text{L}$  of nonyl acetate (internal standard solution at 100 ng/ $\mu\text{L}$ ) and stored at  $-30^{\circ}\text{C}$  until analysis. For compounds quantification, a 2- $\mu\text{L}$  aliquot of each sample was injected in splitless into

a gas chromatograph coupled to a mass spectrometer (GC-FID) equipped with HP5 capillary column (JeW Scientific, Folsom, CA; 30 m; 0.25 mm; 0.25  $\mu\text{m}$ ) using helium as carrier gas.

For compound identification, a 1- $\mu\text{l}$  aliquot of each sample was injected into a gas chromatograph coupled to a mass spectrometer (GC-MS-2010 Plus) equipped with HP1 MS column (30 m; 0.25 mm; 0.25  $\mu\text{m}$ ). The column temperature was held at 40°C for 1 minute, increased to 150°C (5°C/min) and then raised until it reach 250°C (20°C/min). Compounds were identified by comparing their mass spectra with NIST08 library and calculating Kovats Index (KI) using n-alkane standards (C7-C30). Thereafter, the retention time of each compound was compared with the spectra of respective synthetic compounds.

### **3.2.7 *Plutella xylostella* Y tube olfactometer test**

To evaluate olfactory response to arugula odors, two-day-old adult females, fertilized and without previous contact with the plant, were individualized in test tubes. Then, a “Y” olfactometry system was connected to an “ARS volatile collection system” (ARS, Gainesville, FLA, 19 USA). In this system, the equipment's airflow passes through charcoal filters for cleaning, then it is pushed into the glass vats containing the treatment plants and reaches the side arms of the olfactometer through flexible hoses of Teflon®. The olfactometer consisted of the main tube with two side arms (10 cm long x 2.5 cm in diameter) and was positioned vertically. The incoming airflow was adjusted to 0.350 L/min, releasing one female at a time in the main arm and keeping her under observation for a maximum of 10 minutes, recording the choice between one of the treatments when the insect traveled at least half the length of the side arms. At each trial, the sides of the olfactometer were inverted and for every ten, the vats containing the plants also were changed. The tests were always conducted between 6 pm and 9 pm.

The combinations of arugula plants tested in this system were: i) white (no plant) x control (arugula plant) and ii) Control x plant + *B. amyloliquefaciens* GB03, with each combination consisting of at least thirty replications, each female was observed just one time, thus each replication was composed by a different female. Only replications with females answer were considered for the results, considering a non-answer when, after ten minutes of observation, the insect did not exceed half the length of any of the lateral arms.



### **3.2.8 *Spodoptera frugiperda* performance**

To evaluate the effect of GB03 inoculation in arugula plants, on *S. frugiperda* performance, second instar caterpillars were individually weighed on a precision scale and placed on 21 days old arugula plants inoculated and uninoculated with GB03 (one caterpillar/plant). Plants were involved with a voile tissue to prevent the caterpillars from escaping. *S. frugiperda* caterpillars were weighed separately daily for ten consecutive days. Arugula plants were replaced every two days. Each treatment was composed of fifteen replications.

### **3.2.9 Statistical analysis**

The data from root parameters and from *S. frugiperda* performance were analyzed using the T test. The data from volatile collection assays was analyzed using a repeated measures analysis of variance (ANOVA). The Chi-square test was used to analyze the olfactory answer of *P. xylostella*. Levene's and a Kolmogorov–Smirnov test were carried out to determine heteroscedasticity of error variance and normality of the data. All analyses were performed using the statistical tool NumXL in Excel.

## **3.3 Results**

### **3.3.1 Root growth measurement**

To analyze the effects of GB03 inoculation on the arugula root growth and development, the surface area of the roots, the volume and the number of root hairs of plants with and without association with GB03 were analyzed. It was found that when previously inoculated with the bacteria, the surface area of the roots was approximately five times greater ( $p=0.017$ ), while the volume was almost four times greater ( $p=0.008$ ) and the presence of a greater amount of root hair was observed, being seven times greater than uninoculated treatment ( $p=0.011$ ) (Figures 1 and 2).

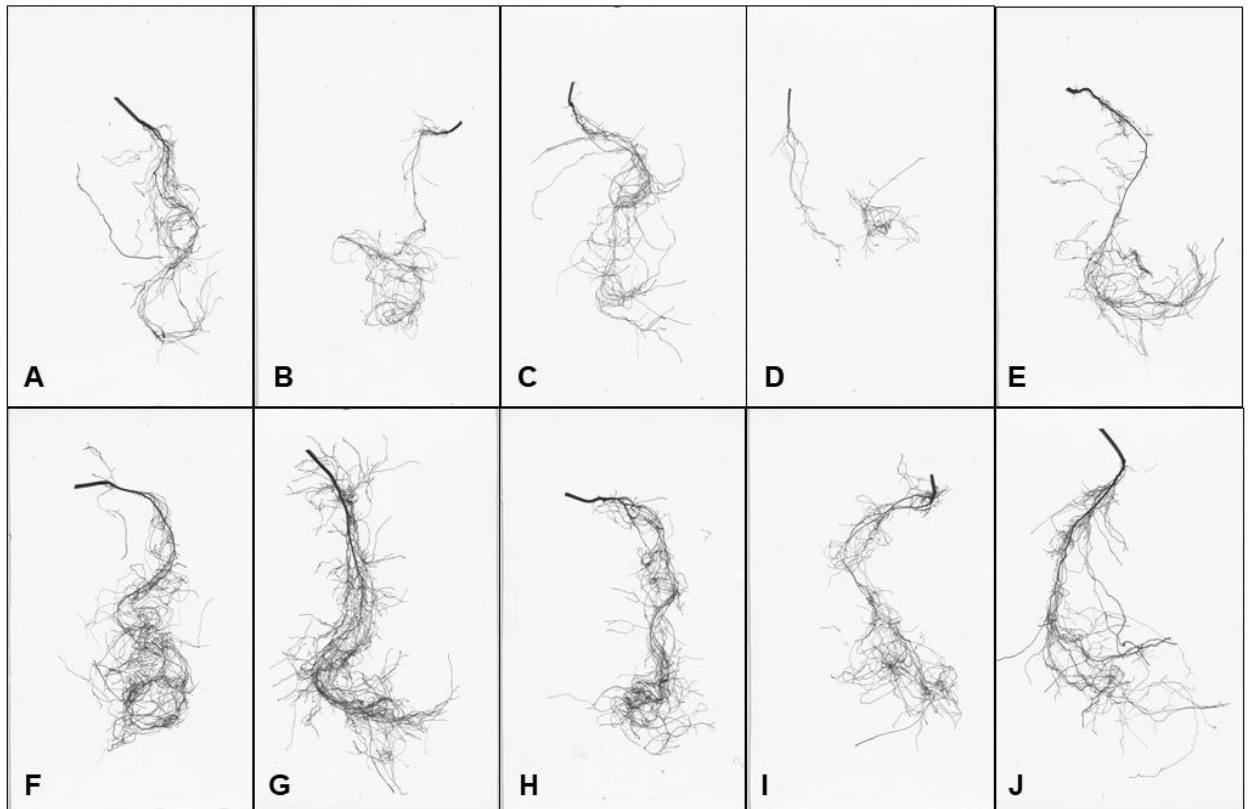


Figure 1 – Images of arugula roots obtained using the equipment Epson LA2400 scanner. A-E – Roots from uninoculated arugula plants; F-J – Roots from arugula plants inoculated with *Bacillus amyloliquefaciens* GB03.

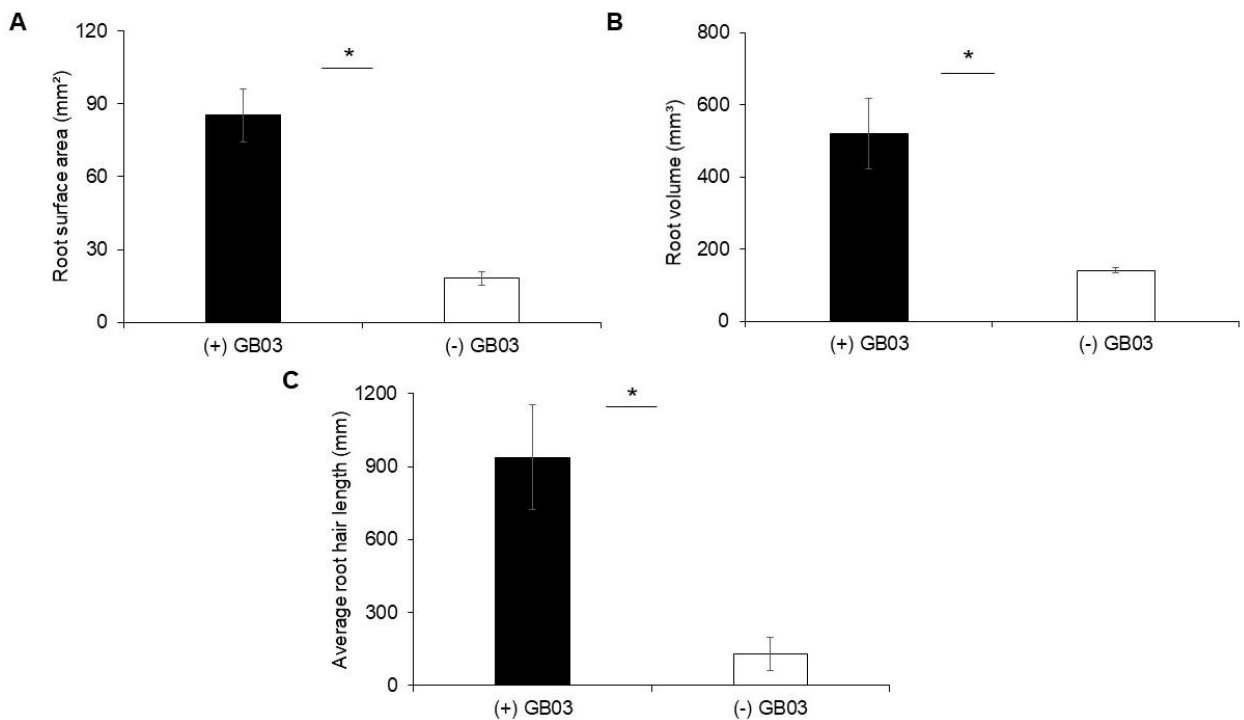


Figure 2 – WinRHIZO software analysis of root parameters of GB03 inoculated and uninoculated plants. A – Root surface area (mm<sup>2</sup>); B - Root volume (mm<sup>3</sup>); C – Average root hair length (mm). (\*) An asterisk means a significant difference at 5% according to the T-Test.

### 3.3.2 Plant volatiles

Arugula plants volatiles were collected to verify whether the association with GB03 and insect herbivory with different degrees of specialty changes the composition of the volatile profile of these plants. Thus, it was noted that there were quantitative and qualitative differences in the composition of the volatile profiles among the treatments tested.

It was found that there was a greater variety of compounds belonging to the group of monoterpenes in all treatments, with higher concentrations of compounds belonging to that group in uninoculated treatments.

A higher concentration of compounds belonging to alcohol group was observed in the treatments with GB03 inoculation both with the healthy plant and exposed to the damage of *P. xylostella* and in the treatment without GB03 inoculation with damage of *S. frugiperda* (Figure 3).

The compound 1-hexanol was not emitted only by plants of the treatment with inoculation of GB03 and damage of *S. frugiperda* and at the same time, the lowest concentration of this compound was observed in the treatment without inoculation and herbivores damage. The alcohol  $\alpha$ -terpineol was found only in treatments where there was the association of GB03 with plants, with a higher concentration in the treatment with *P. xylostella* damage.

$\beta$ -pinene was observed only in three treatments, inoculated with GB03, uninoculated, and inoculated + *P. xylostella* damage. There was no significant difference in concentration among them. The compound  $\beta$ -myrcene was observed in both treatments, plant inoculated with GB03 and inoculated + *P. xylostella* damage, with no significant difference between the treatments.

Only the uninoculated treatment did not have the emission of the monoterpene p-cymene in its volatile profile. For the other treatments, there was no significant difference in the concentration of this compound. In contrast, the emission of the monoterpene D-limonene was observed in all treatments with a significant difference without GB03 inoculation and with damage from *P. xylostella*.

Just the control treatment, that is, without insect damage and GB03 inoculation, had monoterpene  $\beta$ -ocimene in its volatile profile. The undecane hydrocarbon was observed only in the treatments inoculated with GB03, uninoculated and inoculated + *P. xylostella* damage and there was no significant difference in concentration between

them. The compounds found in each treatment and their respective concentrations can be better observed in Table 1.

Table 1 – Volatile organic compounds of arugula plants inoculated and uninoculated with *Bacillus amyloliquefaciens* GB03, submitted to *Plutella xylostella* or *Spodoptera frugiperda* herbivory

Compound	RT (min)	Treatments					
		(+) GB03	(-) GB03	(+) GB03 + <i>P. xylostella</i>	(-) GB03 + <i>P. xylostella</i>	(+)GB03 + <i>S. frugiperda</i>	(-) GB03 + <i>S. frugiperda</i>
Alcohols							
1-hexanol	7,704	5,94±1,17 <sup>a</sup>	0,95 ±0,42 <sup>b</sup>	5,27±1,40 <sup>a</sup>	10,55±1,59 <sup>a</sup>	0±0 <sup>b</sup>	9,23±1,17 <sup>a</sup>
α-terpineol	16,592	7,21±1,67 <sup>b</sup>	0±0 <sup>b</sup>	10,59±0,82 <sup>a</sup>	0±0 <sup>b</sup>	2,11±0,63 <sup>b</sup>	0±0 <sup>b</sup>
Monoterpenes							
β-pinene	10,977	0,61±0,35 <sup>a</sup>	0,19±0,24 <sup>a</sup>	0,58±0,27 <sup>a</sup>	0±0 <sup>b</sup>	0±0 <sup>b</sup>	0±0 <sup>b</sup>
β-myrcene	11,436	1,91±1,81 <sup>a</sup>	0±0 <sup>b</sup>	2,49±1,14 <sup>a</sup>	0±0 <sup>b</sup>	0±0 <sup>b</sup>	0±0 <sup>b</sup>
p-cymene	12,173	1,58±0,92 <sup>a</sup>	0±0	2,69±1,33 <sup>a</sup>	5,82±1,31 <sup>a</sup>	0,29±0,29 <sup>a</sup>	2,08±0,51 <sup>a</sup>
D-limonene	12,413	3,97±1,03 <sup>b</sup>	3,64±1,34 <sup>b</sup>	2,69±1,33 <sup>b</sup>	11,16±1,55 <sup>a</sup>	2,76±1,26 <sup>b</sup>	2,98±1,39 <sup>b</sup>
β-ocimene	12,984	0±0 <sup>b</sup>	0,49±0,23 <sup>a</sup>	0±0 <sup>b</sup>	0±0 <sup>b</sup>	0±0 <sup>b</sup>	0±0 <sup>b</sup>
Hydrocarbons							
undecane	14,471	1,37±0,95 <sup>a</sup>	1,00 ±0,73 <sup>a</sup>	2,94±0,87 <sup>a</sup>	0±0 <sup>b</sup>	0±0 <sup>b</sup>	0±0 <sup>b</sup>

\* values on the same line followed by different letters differ from each other statistically (ANOVA,  $P < 0,05$ )

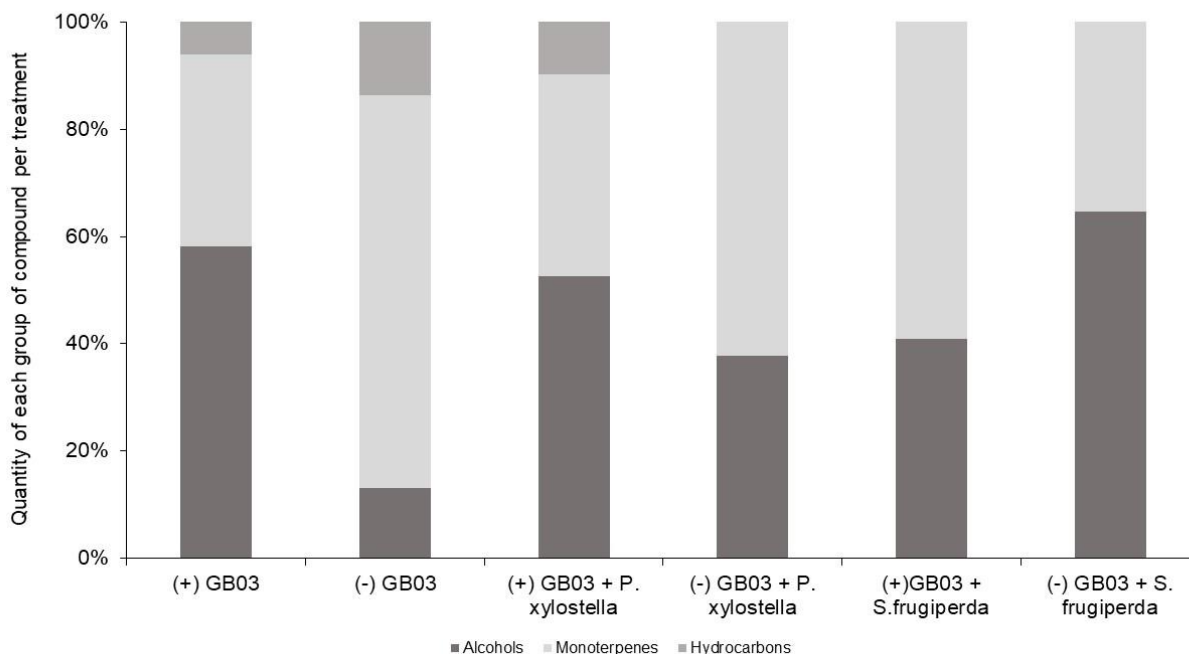


Figure 3 – Concentration of compounds belonging to different groups in each treatment.

### 3.3.3 *Plutella xylostella* olfactory

To verify the effect arugula plants inoculation with GB03 in olfactory response of *P. xylostella*, the “Y” olfactometer was used, allowing insects to be exposed only to olfactory cues. Thus, it was observed that fertilized adult females of *P. xylostella* showed a greater olfactory preference for arugula plants than no plant (white) ( $n=30$ ;  $\chi^2=4.8$ ;  $p=0.001$ ), which means that the system is working.

The higher preference for uninoculated plants with GB03 strain than inoculated plants by fertilized adult females of *P. xylostella* was also observed ( $n=30$ ;  $\chi^2=1.2$ ;  $p=0.012$ ), thus it was found that inoculation with GB03 strain negatively alters the insect's choice by the plant (Figure 4).

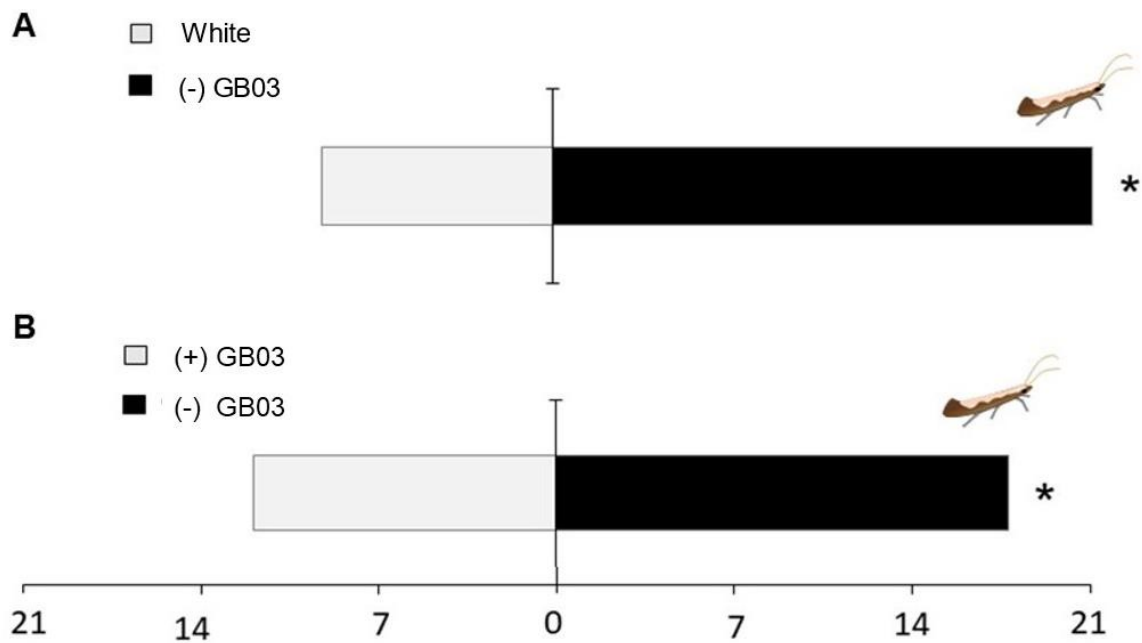


Figure 4 – Olfactory answer of adult females of *Plutella xylostella* to arugula volatiles in a “Y” olfactometer. A) White (no plant) vs. (-)GB03 (uninoculated arugula plant); B) (-) GB03 vs (+)GB03 (Inoculated arugula plants with *Bacillus amyloliquefaciens* GB03). (\*) An asterisk means a significant difference at 5% according to the Chi-square test.

### 3.3.4 *Spodoptera frugiperda* performance

To verify whether the association of arugula plants with GB03 could affect the *S. frugiperda* feeding, caterpillars were fed for ten days on both plants inoculated and uninoculated with the rhizobacteria. After daily measurement of the caterpillar's weight, it was observed that there was a 136% greater weight gain in caterpillars that were fed with noninoculated plants ( $p = 0.002$ ) than caterpillars that were fed on plants with the presence of rhizobacteria (Figure 5).

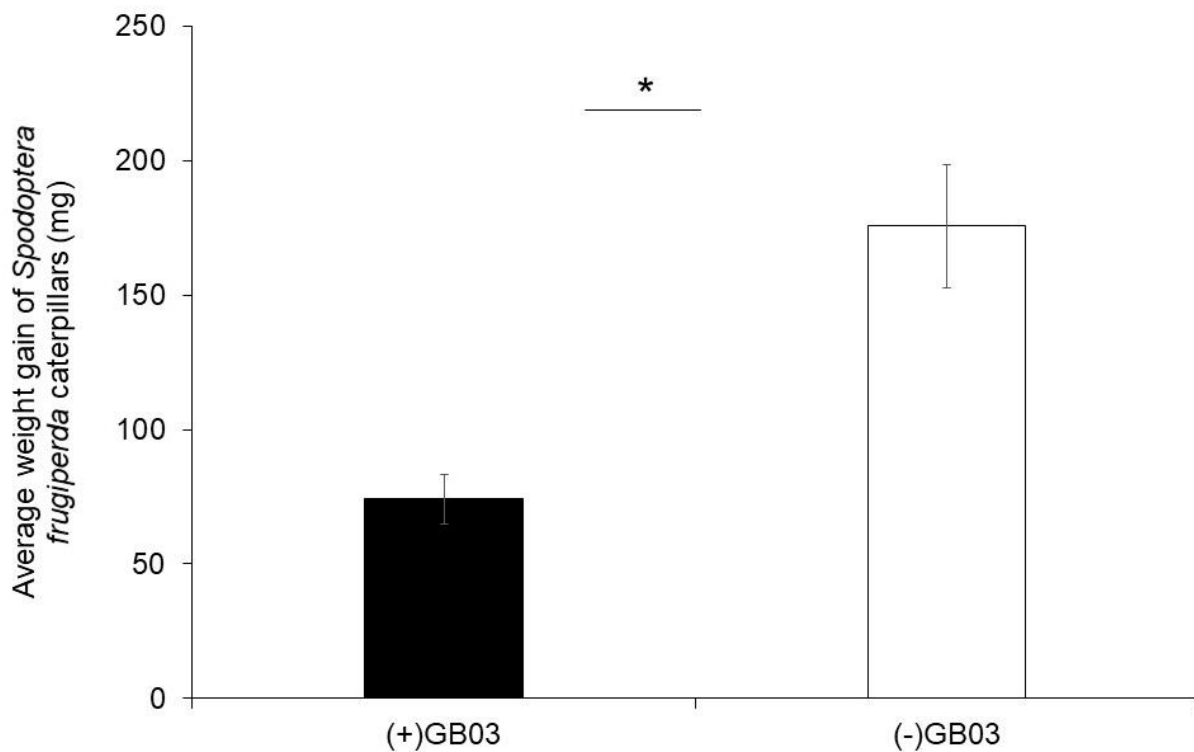


Figure 5 - Average weight gain of *Spodoptera frugiperda* caterpillars submitted to feeding on arugula plants with and without the inoculation of *B. amyloliquefaciens* GB03. (\*) An asterisk means a significant difference at 5%, according to the T-Test.

### 3.4 Discussion

The efficiency of using GB03 in plant growth-promoting has been very well documented in several, such as *Arabidopsis* (Ryu et al., 2003; Zhang et al., 2007; Xie et al., 2009), *Codonopsis pilosula* (Wu et al., 2016), wheat (Zhang et al., 2014), white clover (Han et al., 2014), sweet basil (Banchio et al., 2009) and cassava (Freitas et al., 2015). With the aim to observe the efficiency of GB03 in arugula growth promotion, parameters related to root growth were measured. For this reason, data related to the surface area, volume, and amount of root hair from roots of inoculated and uninoculated arugula plants with GB03 were collected.

It was found that inoculated treatment presented a surface area about five times greater, a volume about four times greater, and an amount of root hair seven times greater than uninoculated plants. As already verified by other researchers in different cultures, the GB03 strain helps plants promote growth through different ways. We can compare the results obtained in this study with those obtained by Han et al. (2015), where it was observed that white clover plants inoculated with GB03 showed a longer

root length than uninoculated plants, and this efficiency was maintained even in saline conditions.

During the life cycle of plants, they interact with several organisms above and below ground. The volatiles emitted by them symbolizes the universal language between them and other living creatures, such as beneficial soil microorganisms, pathogens, herbivorous insects, and their natural enemies and pollinators (Ali et al., 2012; Pangesti et al., 2016; Simpraga et al., 2016). However, many beneficial soil microorganisms, including PGPR, can emit their own compounds and when in association with plants, they can change the volatile profile emitted by plants. Sometimes the same compounds can be observed when under insect herbivory and in association with some PGPR or other beneficial microorganism (Schausberger et al., 2012; Ballhorn et al., 2013).

Plants emit a different plethora of compounds according to the conditions in which they are found and may present other compounds according to the microorganisms associated with their roots and the type of damage and degree of the specialization of herbivorous insect aboveground (Pineda et al., 2010).

Studies have shown that specialist and generalist insects interact with plants in different ways (Ali and Agrawal, 2012). In general, it is observed that specialists have a closer relationship with the host plant, these insects have a co-evolution with the plant, being less impacted by plant defense than generalist insects (Cornell and Hawkins, 2003). On the other hand, generalists should have general mechanisms to avoid defenses of a wider range of plant species (Whittaker and Feeny, 1971).

In this study, arugula plants were submitted to different conditions to verify the effects on the composition of volatiles profile. To verify the influence of PGPR on volatile emissions, plants inoculated and not inoculated with GB03 were tested and, at the same time, the influence of the degree of specificity of two chewing insects was verified by submitting separately inoculated and uninoculated plants to the damage of *P. xylostella* (specialist) and *S. frugiperda* (generalist). Thus, it was observed that there was a change in the composition of the volatile profile of plants, both quantitative and qualitatively.

According to Zhang et al. (2013), alcohols are one of the groups of compounds most produced by *Bacillus* species, and it was the group that presented the highest concentration in the composition of the volatile profile of treatments with GB03



inoculation with both the healthy plant when exposed to *Plutella xylostella* damage. On the other hand, the group of monoterpenes presented the greatest variety of compounds in general, being observed in higher concentrations in uninoculated plants, both no damaged and submitted to the *P. xylostella* damage.

$\alpha$ -terpineol was emitted only by plants that were in association with GB03 in all submitted conditions. Thus, it is believed that the emission of  $\alpha$ -terpineol is related to inoculation with rhizobacteria.

It was noted that when under *S. frugiperda* attack, both plants inoculated and uninoculated with GB03 showed low concentrations of compounds in general. This fact probably is related to the severity of herbivore damage, once *S. frugiperda* feeding was strongly affected by GB03 inoculation we could observe lower amount of herbivory-induced volatiles being released in the treatment with inoculation of GB03 and damage of *S. frugiperda*. As verified by Rieksta et al., (2020), the emission of green leaf volatiles increases as the severity of the insect's damage intensifies.

The total weight gain of *S. frugiperda* caterpillars was 136% higher in uninoculated plants. One hypothesis that can be raised in the production of proteinase inhibitors by PGPR, which can directly affect the weight gain of insects. In addition, it was observed that *P. xylostella* adult females fertilized showed a greater preference in the Y olfactometer test for uninoculated arugula plants. Once the insect chooses the plant that it considers most ideal for the development of its offspring, we can verify that plants with GB03 inoculation can threaten the development of the insect offspring in this case. It was observed that the compounds  $\alpha$ -terpineol,  $\beta$ -myrcene, and p-cymene were not present in the treatment without GB03 inoculation, so it can be hypothesized that the presence of these compounds may be one of the factors that are influencing the choice of *P. xylostella* by plants not inoculated. However, more studies must be carried out to confirm this hypothesis.

The number of studies related to how PGPR can interact with plants and improve defense against insects has grown in recent years, as well as the understanding of how the interactions that occur between PGPR, plants, and insects affect the emission of plant volatiles and how it can change the communication of plants with other organisms (Sharifi et al., 2018). *B. amyloliquefaciens* GB03 has been demonstrated a considerable potential to be used in integrated pest management, however, studies under field conditions must be conducted to confirm the potential in plant defense in the field.

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#### 4 FINAL CONSIDERATIONS

The idea that the soil is alive and the knowledge of the huge importance of beneficial soil-borne microorganisms in agriculture are increasingly evident. Beneficial soil-borne microorganisms, such as PGPR, are changing paradigms and have been considered critical factors for more sustainable and productive agriculture. For efficient use in the field, it is necessary to understand how their behavior and functions in the soil are associated with plants.

In this study, we aimed to understand how GB03 colonization occurs in the soil and how *P. xylostella* damage can affect the interactions between arugula and GB03. We have found an efficient specific primer based on the *gyrA* gene region to monitoring GB03. Also we verified that its population decreased in the soil over time, and *P. xylostella* damage could interfere in this process.

GB03 promoted plant growth, both shoot and root parameters. At the same time, we tested how GB03 could improve plant defense against two chewing insects with different degrees of specialization. It was verified that the rhizobacteria inoculation decreased the gain weight of *S. frugiperda* caterpillars and negatively influenced *P. xylostella* adult females in the host search. Both GB03 inoculation and insect damage changed the volatile arugula profile.

The inoculation with GB03 also showed activate glucosinolate genes and induced systemic resistance via the jasmonic acid pathway. Thus, in this study, we got to take a step further in the knowledge about GB03 and how it can help in integrated pest management in agriculture. However, tests carried out in the field are still necessary to consolidate the results obtained in the laboratory and greenhouse.