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Induced systemic resistance by *Beauveria bassiana* in soybean plants and the effects on *Spodoptera frugiperda*

Carla Mariane Marassatto

Dissertation presented to obtain the degree of Master in Science. Area: Entomology

Piracicaba
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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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“The only limit to the height of your achievements is the reach of your dreams and your willingness to work for them.” — Michelle Obama

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RESUMO

Indução do sistema de resistência por *Beauveria bassiana* em plantas de soja e efeitos em *Spodoptera frugiperda*

Beauveria bassiana é um fungo entomopatogênico e endofítico. No Brasil, é um dos principais agentes de controle biológico por meio da aplicação foliar. A herbivoria de insetos pragas em plantas colonizadas por este fungo entomopatogênico endofítico pode causar efeitos secundários e o conhecimento sobre a indução de defesa da planta contra insetos pragas resultantes da relação simbionte entre planta-fungo endofítico é limitada. Este estudo teve como objetivo avaliar os efeitos secundários da colonização endofítica de *B. bassiana* (cepa ESALQ PL63) e a ativação da indução de defesa pela herbivoria de *Spodoptera frugiperda*. Plantas de soja foram colonizadas em um trifólio e uma lagarta de *S. frugiperda* (segundo instar) foi infestada em uma folha não-inoculada por oito dias (primeiro experimento) ou até pupar (segundo experimento). A indução de defesa da planta foi avaliada por meio da expressão gênica (terceiro experimento), considerando as principais vias de defesa, ácido salicílico (*PR2*), ácido jasmônico (*MYC2* e *PR3*) e etileno (*ERF1*). O experimento incluiu os tratamentos com plantas não-inoculadas e plantas inoculadas com *B. bassiana*, com e sem a herbivoria de *S. frugiperda*. As plantas foram avaliadas 5, 15 e 30 dias após a inoculação. Oito dias após a herbivoria, as lagartas que consumiram plantas inoculadas com *B. bassiana* apresentaram menor massa corporal e consumo foliar. Além disso, o mesmo tratamento resultou em uma menor sobrevivência de lagartas e pupas, diminuição no intervalo de desenvolvimento de lagartas e menor fecundidade de fêmeas, apesar do intervalo de emergência de adultos não ter sido afetado. O peso de pupas não foi afetado após herbivoria de plantas inoculadas com *B. bassiana*; porém, algumas pupas apresentaram rigidez corporal, diminuição no tamanho e má formação. A via do etileno representada pelo gene *ERF1* após 5 e 15 dias da inoculação foi altamente expressa em plantas não-inoculadas e plantas inoculadas, ambas com herbivoria, mostrando que houve um reconhecimento dos padrões moleculares associados a herbívoros (HAMPs) e dos padrões moleculares associados aos danos (DAMPs). Houve um aumento da expressão de *MYC2* na via do ácido jasmônico 15 dias após a inoculação no tratamento *B. bassiana* com herbivoria. No tratamento em que as plantas foram inoculadas com *B. bassiana* sem a herbivoria, houve um leve aumento na regulação de *PR3* 15 dias após a inoculação, indicando que a planta produziu precursores da via do ácido jasmônico. A via do ácido salicílico representada por proteína *PR2* relacionada à patogênese teve baixa regulação em todos os períodos. O 'cross-talk' entre as vias do ácido salicílico e ácido jasmônico foi observado, com resultados baixos e altos, respectivamente. Trinta dias após a inoculação, a expressão de *MYC2* em plantas inoculadas com *B. basssiana* diminuiu quando comparado as plantas não inoculadas, ambas com herbivoria; portanto, o aumento da expressão no sistema imune com *B. bassiana* não ocorreu neste período. O presente estudo destaca a potencial ação endofítica de *B. bassiana* como proteção contra *S. frugiperda* em plantas de soja.

Palavras-chave: Fungo entomopatogênico, Relação endofítica, Expressão de genes, Lagarta do cartucho, Soja

ABSTRACT

Induced systemic resistance by *Beauveria bassiana* in soybean plants and the effects on *Spodoptera frugiperda*

Beauveria bassiana is an entomopathogenic fungus as well as an endophyte. In Brazil, it is widely used by foliar applications as a biological control agent. The pest herbivory in plants colonized by this endophytic entomopathogenic fungi could result in secondary effects, and the knowledge about plant-induced defenses against pests resulting from the symbiont relationship of plant-fungi endophytic is limited. This study aimed to evaluate the secondary effects of endophytic colonization of *B. bassiana* (strain ESALQ PL63) triggered by herbivory of *Spodoptera frugiperda*. Soybean plants were inoculated in one trifoliate, and 2nd instar *S. frugiperda* were fed on non-inoculated leaves for eight days (first experiment) or until pupation (second experiment). The induced systemic resistance was evaluated by gene expression from main pathways (third experiment), salicylic acid (*PR2*), jasmone acid (*MYC2* and *PR3*), and ethylene (*ERF1*). The treatments comprised non-inoculated plants and *B. bassiana*-inoculated plants, with or without herbivory of *S. frugiperda*. Plants were assessed 5, 15, and 30 days after inoculation. After eight days of herbivory, *S. frugiperda* larvae fed on *B. bassiana*-inoculated plants presented lower body mass and foliar area consumption. Endophytic colonization by *B. bassiana* also resulted in significant reductions in larval and pupal survival, lower larval span, and female fecundity, although the period until adult emergence was not affected. The pupal weight was not affected by herbivory in plants inoculated with *B. bassiana*; otherwise, some pupae were stiff, smaller, and malformed. At five days post-inoculation *ERF1* gene was highly expressed in non-inoculated plants with herbivory compared to the slight response in *B. bassiana*-inoculated plants. The expression from ET-signaling represented by the *ERF1* gene after 5 and 15 days post-inoculation had high response levels in the treatments of *S. frugiperda* herbivory (non-inoculated plants and inoculated plants), showing recognition of Herbivore-Associated Molecular Patterns (HAMPs) and Damage-Associated Molecular Patterns (DAMPs) after the herbivory. An increase of the *MYC2* gene from the jasmonate acid pathway was observed after 15 days; a slight rise in *PR3* was observed in *B. bassiana*-inoculated plants without herbivory, indicating a precursor production from jasmonate acid. The salicylic acid represented by the Pathogenic-related protein *PR2* had lower regulation in all periods. The cross-talk between the acid salicylic and jasmonic acid signaling was demonstrated by lower and higher expression levels, respectively. At 30 days post-inoculation, the gene *MYC2* in *B. bassiana*-inoculated plants decreased when compared with non-inoculated plants both with herbivory; thus, the induction of defense by plants inoculated with *B. bassiana* did not occur in this period. The presented study highlights the potential of *B. bassiana* as an endophytic to protect soybean plants against *S. frugiperda*.

Keywords: Entomopathogenic fungi, Endophytic relationship, Gene expression, Fall armyworm, Soybean

1. INTRODUCTION

1.1. *Beauveria bassiana* and its endophytic behavior

The fungus *Beauveria bassiana* (Bals. – Criv.) Vuill. Hypocreales: Cordycipitaceae) is an entomopathogen, used as a biological control agent against many insect species. In Brazil, *B. bassiana* is the dominant species of the genus, present in soils, infecting insects, and as an endophyte in plants (SOUZA et al., 2020). *Beauveria bassiana* can cause control in a diversity of insects and mites via foliar spraying, such as *Tetranychus urticae* (ALVES et al., 2002), *Bemisia tabaci* (MASCARIN, 2014), *Hypothenemus hampei* (MOTA et al., 2017), *Anastrepha fraterculus* (CHANEIKO et al., 2019), *Cosmopolites sordidus* (MEMBANG et al., 2021), and *Delphacodes kuscheli* (TOLEDO; BRENTASSI, 2022), many of them being important crop pests.

Currently, fungi-based products used in the field to control insect pests are generally developed with isolates of *B. bassiana* and *Metarhizium anisopliae* (MASCARIN et al., 2019). Their use is due to their cosmopolitan distribution and the ease of mass production using artificial media (MASCARIN; JARONSKI, 2016). Annually, 1.5 million ha of soybean plants are treated via aerial conidia application with *B. bassiana* to manage *B. tabaci* (MASCARIN et al., 2019). In Brazil, most commercial products based on *B. basssiana* are composed of the strains ESALQ PL63 or IBCB66.

Besides their pathogenic action, insect fungal pathogens can endophytically colonize plants, forming a symbiotic relationship that could be beneficial or neutral (BACKMAN; SIKORA, 2008); the term endophyte describes fungi or bacteria that develop within plant tissues without causing symptoms of disease in the plant (WILSON, 1995). Artificial colonization of plants by *B. bassiana* can be realized via foliar application (RONDOT; REINEKE, 2019), seed dressing (CANASSA et al., 2019), root dipping (CANASSA et al., 2020), or soil drenching (GREENFIELD et al., 2016).

Beauveria bassiana has been reported as an endophyte by artificial colonization in several economically important crops, such as coffee (VEGA et al., 2008), grapevine (RONDOT; REINEKE, 2019), strawberry (CANASSA et al., 2020), soybean (RUSSO et al., 2015), tomato (NISHI et al., 2021; WEI et al., 2020), corn (RUSSO et al., 2019), alfalfa and melon (RESQUÍN-ROMERO et al., 2016), cassava (GREENFIELD et al., 2016), tobacco (QUIN XU, et al., 2020) and lemon (BAMISILE, et al., 2019). Endophytic colonization can be influenced by strain type, colonization method, time post-inoculation, plant genotype, microbial taxon, and abiotic and biotic conditions (HARDOIM et al., 2015).

The plant-fungi symbiotic relationship can induce mechanisms of plant defense, and the benefits include antagonistic effect against diseases (OWNLEY et al., 2008; JABER; OWNLEY,

2018), plant growth promotion (JABER; ENKERLI, 2016) and resistance to drought stress (AHMAD et al., 2019). Endophytic *B. bassiana* can also affect insect biology, including physiological and behavioral changes through the reduction of herbivory, affecting larval survival, altering the average survival time from insect cycle life and reproduction rate of herbivore insects (MCKINNON et al., 2017). Grasshoppers that consumed leaves of plants inoculated with *B. bassiana* for 15 days decreased their feeding activity and laid a lower number of eggs per pod, with a mean of 17.69 eggs versus 27.25 eggs from the control (PELIZZA et al., 2017).

1.2. The use of endophytic entomopathogenic fungi on soybean plants and the perspective on the use of integrated pest management

Soybean [*Glycine max* (L.) Merr.] is one of the most important crops in the world, being an important commodity in human and animal food. Brazil is the world's biggest soybean producer, with a production of 135,409 million tons, considering a global production of 362,947 million tons for the 2020/2021 harvest (EMBRAPA, 2021). Brazil has a tropical climate and large crop area, which allows high productivity but also favors the development of diseases and pests (ZAMBOLIM, 2019).

Soybean is affected by various pests, from plant emergence to grain maturity. The use of pesticides was the primary strategy of pest control until the Green Revolution in the 1960s. The average dose of pesticides before 1969 was 1,200 g/ha; currently, it is less than 200 g/ha (NISHIMOTO, 2019). Since then, biological control has been gaining space over the years, and according to Crop Life (2021), 376 biological products are already registered in Brazil, 60.4% being mycoinsecticides.

When in contact with a plant, conidia of *B. bassiana* develop a germ tube and become hyphae over time; they enter the plant tissue through natural openings (stomas) or directly from epidermal cell walls with the action of enzymes and mechanical pressure. Afterward, the hyphae from pathogenic fungi grow between the parenchyma cells and the xylem vessels; moving on the plant systematically (QUESADA-MORAGA et al., 2009; MANTZOUKAS; ALIOPOULOS; 2020). Russo et al. (2015) studied soybean plants inoculated with *B. bassiana* by seed and root immersion. Through re-isolation methodology, it reported the fungus present in the leaf, stem, and root 7 and 14 days after inoculation.

In addition, this fungus-plant association can alter the plant metabolism, production of toxic metabolites against pests, protecting during a period, and keeping a continuous control until the final crop production according to the application method (QUESADA-MORAGA et al.,

2009). In addition, Gonzáles-Mas et al. (2021) showed that entomopathogenic fungi could contribute to a blend of compounds to attract natural enemies when cotton plants colonized with *B. bassiana*.

Entomopathogenic fungi are gaining acceptance in Integrated Pest Management due to plant growth-promoting, synergistic interactions with other tactics of control and suppressing pests and disease (MANTZOUKAS, et al. 2022). Currently, *B. bassiana* and *Cordyceps fumosoroseae* are an alternative to control *Dalbulus maidis*, an important insect vector on maize plants, considering the low efficacy of chemical control (OLIVEIRA; FRIZZAS, 2021). The additional benefits from this symbiont relationship with plant-entomopathogenic fungus are not studied yet on *D. maidis*. The knowledge about the plant-fungi association, induced systemic defense, and the effects on the primary agriculture pest will contribute to rural farmers adopting more sustainable control tactics.

1.3. Biology of *Spodoptera frugiperda* and the endophytic action of *Beauveria bassiana* against the pest

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is a crucial polyphagous pest in many crops worldwide. Although native to the Americas (SPARKS, 1979), several outbreaks since 2016 caused the caterpillar to spread to different regions of the world, being reported in the West with initial occurrence in the countries Nigeria, Benin, and Togo and currently spread to the Sahara desert to the North (GOERGEN et al., 2016; FELDMANN et al., 2019), in the state of Karnataka, India (GANIGER et al., 2018), and the province of Yunnam, China (SUN et al., 2021). The insect species has 353 host plants belonging to 76 families (MONTEZANO et al., 2018), including cotton, coffee, potato, tomato, corn, different kinds of grass, sugarcane, rice, soybean, cassava, banana, peanut, pepper, cabbage, broccoli, mango, garlic, onion, and other economically important crops. In Brazil, armyworm control is expensive: the country spends US$ 600 million every year, according to the Food and Agriculture Organization of the United Nations (FAO) (WILD, 2017).

Spodoptera frugiperda undergoes holometabolous metamorphosis, going through the stages of egg (2-3 days), larvae (six instars; 13-14 days), pupae (9-12 days), and adult (7-21 days) (WAN et al., 2021). The larvae are recognized through the front, which has an inverted 'Y' line (SHARANABASAPPA, 2018). In soybean plants the life cycle (egg – adult) of *S. frugiperda* lasts 45 days on average, depending on the environmental conditions; one female can oviposit 1,844 eggs (BARROS et al., 2010).

The most used control strategy adopted against *S. frugiperda* is genetically modified plants with pesticide action containing a protein from the bacterial *Bacillus thuringiensis*. In Brazil, due to incorrect use, such as a shortage of refuge areas, year-round cropping system, and similar proteins in the same field area; *S. frugiperda* developed resistance (FATORETTO et al., 2017). Other factors contributing to efficiency losses include a large number of insect generations (due to the short time to complete the cycle life) and migratory capacity. Another vital strategy adopted is the application of chemical pesticides. Still, due to their continuous use, and lack of rotation of active ingredients, *S. frugiperda* developed resistance to organophosphate, pyrethroid, and diamide insecticides due to their intensively used (GUAN et al., 2020).

In this way, it is essential to explore sustainable and different strategies to control *S. frugiperda*, one of them being the use of entomopathogenic fungi. It has been previously demonstrated that *B. bassiana* and *M. anisopliae* have the potential to control fall armyworms (GUO et al., 2020). A screening assay with 43 *B. bassiana* isolates showed that some isolates caused 98% mortality when larvae had direct contact with conidia (WRAIGHT et al., 2010).

Previous studies have evaluated the secondary effects of endophytic entomopathogenic fungi against *Spodoptera* spp.. Garrido-Jurado et al. (2020) offered discs of melon leaves inoculated with *Metarhizium brunneum* to *Spodoptera litorallis* to complete the cycle life (3rd instar - adult). Then the authors evaluated the oviposition of couples that consumed inoculated and non-inoculated (control) leaves. The fecundity of the non-inoculated was 221.3 eggs per female versus 135.3 eggs per female in the treatment with *M. brunneum* (GARRIDO-JURADO et al., 2020). In another study, wheat leaves were sprayed with *B. bassiana* and offered to *S. litorallis* to evaluate the secondary effects of the endophytic fungus, and larval mortality of 56.7% was observed on treated plants versus 0% mortality in the control (SÁNCHEZ-RODRÍGUEZ et al., 2018).

There are few studies in planta that evaluated the colonization of crop plants by entomopathogenic fungi and their effect on insect pests. Garrido-Jurado et al. (2020) studied the herbivory of *S. litorallis* larvae on plants inoculated with *M. brunneum* and non-inoculated plants for 48 hours, later allowing the larvae to complete the cycle life (3rd instar - pupae) on artificial diet. The authors then compared the results with those of the previous experiment with the discs of melon leaves. The larval mortality in the *in planta* experiment was higher than in the leaf discs experiment, due to the secretion of compounds such as phenols or saponins, toxic to herbivores.

1.4. Induction resistance in plants by endophytic microorganisms

When herbivore insects attack a plant with their mandibles, they deposit cues from the saliva the plant recognizes. Even components of insect mandible (E.g., chitin) can be recognized and trigger the immunity. An insect or herbivore's molecular 'signatures' (such as oral secretions) are termed Herbivore Associated Molecular Patterns, or HAMPs (ACEVEDO et al., 2015). These patterns can also be released during oviposition, through pheromones, cuticles, and feces (REYMOND, 2013; SCHMELZ, 2015). The damage from herbivory releases several plant components and consists of a different kind of signaling. These molecules are called Damage-Associated Molecular Patterns (DAMPs). Among them are fragments of the cell wall and cell content (ALJBORY; CHEN, 2018).

The capacity of the plant to recognize the stressors (HAMPs and DAMPs) and defend itself is a form of immunity, and the reaction can be direct or indirect. They can also consist of chemical or physical barriers. In the direct defense, plants react by releasing compounds with different functions, such as repellence, toxicity or antinutritive effects (HOWE; JANDER, 2008). Regarding indirect effects of herbivory-induced defense, plants can attract natural enemies of the insect or communicate with other plants through volatile organic compounds to activate an alert state on the plant (YI et al., 2009; BELL et al., 2020; WONGLOM et al., 2020).

Plants have a natural defense against biotic and abiotic stressors regulated by phytohormones, and the plant could activate signaling pathways, viz., salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (PIETERSE et al., 2014). Depending on the stressor agent, plants could activate a specific induced resistance pathway. Classically, biotrophic pathogens and phloem-feeding insects induce the SA defense pathway, while necrotrophic pathogens, rhizobacteria and chewing insects induce the JA and ET pathways (GLAZEBROOK, 2005; BENEDUZI et al., 2012). Furthermore, there is a crosstalk network between the SA and the JA pathways (SMITH et al., 2009).

When plants increase the levels of SA-signaling, the Systemic Acquired Resistance is in fact, activated (PIETERSE et al., 2014). The SAR is associated with triggering Pathogenesis-related (PR) genes, which could encode PR protein. Some PR proteins have microbial activity, such as β-1,3- glucanase (*PR2*), which disrupts the mycelial wall from phytopathogenic fungi. Otherwise, some have the same function but are activated by JA-signaling, such as chitinase (*PR3*) (SHORESH, et al., 2004; ALI, et al., 2018). Otherwise, when beneficial microorganisms interact with plants, the Induced Systemic Resistance (ISR) is triggered, similar to SAR but with a connection of JA-signaling (VLOT et al., 2021).

This way, the symbiotic relationship between a plant and biocontrol agents could enhance plant immunity through ISR (HAN et al., 2000; MENGISTU, 2020, GUO et al., 2021). ISR is a specific form of defense against necrotrophic pathogens, and insect herbivores, and the activation happens with a biological inductor agent or chemical (PIETERSE et al., 2014). When activated, the ISR, the plant synthesizes secondary compounds that function as insecticides, antifungals, herbicides, or antivirals (BARELLI et al., 2016; MANTZOUKAS; ELIOPOULOS, 2020). The first report of the ISR phenomenon described carnation plants (*Dianthus caryophyllus L.*) inoculated with a symbiont strain of *Pseudomonas* sp. and infected with the plant pathogen *Fusarium oxysporum* f. sp. *dianthi* on the stem. The mutualistic interaction between plants and bacteria increased the accumulation of phytoalexins, and the disease development was significantly reduced (VAN PEER et al., 1991).

The plant-fungi symbiotic relationship triggers the ISR and activates a cascade defense-related gene. In some cases, it could enable a primed state (termed "Priming"), in which the plant can recall a previous injury stressor (GOELLNER; CONRATH, 2008). The plant prepares itself (primed state) for future infections and shows a faster and more intense defense response against stress. However, it is cost-effective, not affecting the plant productivity. Nowadays, it is known that these responses could be mediated by beneficial microorganisms (CONRATH et al., 2006; HILKER; SCHMULLING, 2019). Ahmad et al. (2019) evaluated the gene expression on maize plants and *Metarhizium robertsii*-treated plants by seed inoculation. They observed an upregulated JA pathway with the genes *lox1* and *opr7*. The upregulation in the absence of insects is due to the accumulation of JA precursors, therefore the prime state.

Several studies have revealed a reduction of damage caused by insects and pathogens when plants are inoculated with endophytic entomopathogenic fungi (AHMAD et al., 2020; MENT et al., 2020; YANG et al., 2021; GUPTA et al., 2022). Tomato plants (*Solanum lycopersicum*) inoculated with *M. brunneum* and *B. bassiana* (by foliar and root inoculation methods) showed similar or greater levels of induced genes compared to plants inoculated with *Trichoderma harzianum* or non-inoculated plants (GUPTA et al., 2022). *M. brunneum* induced the expression of the pathogenesis-related protein *PR1a* (a molecular marker *of* SA-pathway), of the gene *PI-1* marker of JA-pathway, and of the genes *Pti-5* and *ERF1* molecular marker of ET-pathway, *B. bassiana* activated the *PR1b*, *Pti-5* and *PI-1*, and *T. harzianum* induced all genes except *PR1b*. Therefore, the genes expressed can vary depending on the microorganism inoculated.

Defense-related gene expression can change depending on the used strain. Two strains of *B. bassiana* (FRh2 and BG11) were used inoculated in *Arabdopsis thaliana* plants by root dipping method and they expressed a different number of genes: 1,166 and 552 genes, respectively (RAAD

et al., 2019). Both strains increased the resistance of *A. thaliana* to *Sclerotinia sclerotiorum*, although suppression of the herbivores *Myzus persicae* and *Plutella xyllostella* was not observed. The authors also evaluated the transcriptional profile 15 days after inoculation of *B. bassiana*, and they did not observe increased SA and JA expression levels. However, the induced defense hormone pathway could differ by strain. In their survey, the authors demonstrated that *B. bassiana* strains FRh2 and BG11 caused different responses in hormone plant regulation. The SA, JA, and ET pathways were upregulated only in the presence of BG11 inoculated on the root.

Another critical issue is the time until activation of induced resistance after inoculation of endophytic entomopathogenic fungi. Tobacco plants (*Nicotiana benthamiana*) inoculated with *B. bassiana* by seed inoculation method were offered to *M. persicae* 15 and 35 days after inoculation (QIN XU et al., 2020). Four of six strains tested resulted in decreased larval survival in treated plants compared to non-inoculated plants. Plants with 15 and 35 days post-inoculation treated with Bb062 and Bb02 strains showed *PR1* and *NPR1* genes upregulated from SA-signaling. Nevertheless, the increased expression of *PR1* and *NPR1* genes induced by the Bb08, Bb025, and Bb029 strains were significantly higher than the non-inoculated treatment for the same period. The gene *PDF1.2* (a JA defense-related gene) was upregulated on plants treated by Bb07 and Bb029, with similar results in 15 and 35 days post-inoculation.

A recent study evaluated the expression of defense related-genes in *Vicia faba* (L.) by inoculating seeds with spores of *B. bassiana* during 24, 48, and 72 hours post infestation of aphids, and it was observed to increase the expression of the *PR1,* a marker of SA-pathway after 72 hours and an increased expression of the gene *ERF-1* from ET-pathway after 24 and 48 hours, when compared to the control (JENSEN et al., 2020). According to the authors, the plant interaction with entomopathogenic fungi alters the defense responses, which could influence the volatile production by plants and change the pest perception.

2. OBJECTIVES AND HYPOTHESIS

Considering the importance of *B. bassiana* for biological control in Brazil, the overall objective of this study was to investigate the relationship between soybean plants and *B. bassiana* against *S. frugiperda* through induction of resistance by gene expressions.

Three experiments were conducted to investigate the effects of *B. bassiana* colonized on soybean plants by *S. frugiperda* herbivory. The first experiment accessed the leaf area consumed and insect growth parameters, such as mass weight and head capsule widths after eight days of insect feeding. In the second experiment, the development of *S. frugiperda* from 2^{sd} instar to adult and fecundity (average of eggs laid per female) was evaluated. In the third experiment, the mechanism of resistance induction by *B. bassiana* on soybean plants was elucidated through the expression of plant defense genes before and after herbivory by *S. frugiperda.* This is the first study with isolate ESALQ PL63 focusing on expression resistance levels after foliar inoculation with the presence and absence of a stressor agent.

We hypothesized that the endophytic activity of *B. bassiana* (ESALQ PL63) in soybean plants could affect *S. frugiperda* development and reproductivity due to the induction of resistance expression genes.

3. MATERIAL AND METHODS

3.1. Preparation of *Beauveria bassiana* inoculum

Beauveria bassiana isolate ESALQ PL63 was obtained from the Entomopathogenic Fungal Collection "Professor Sérgio Batista Alves," located in the Laboratory of Pathology and Microbial Control of Insects at the "Luiz de Queiroz" College of Agriculture, University of São Paulo, Piracicaba, State of São Paulo, Brazil. A fungal stock culture was prepared by preserving sporulating portions of mycelia into cryotubes containing a sterile solution of 10% glycerol and storing it in a freezer at -80 °C.

The isolate was grown on Potato Dextrose Agar – PDA (Difco®) culture medium– in photophase 12L:12D for 12 days (Figure 1). The conidial suspensions were obtained by scraping the colonies' surface with a sterile spatula and transferred into a tube (50mL) containing 30 mL of a sterile distilled solution of 0.05% Tween® 80. The concentration was adjusted to $1x10^8$ conidia mL^{-1} using a Neubauer chamber under a microscope at 400x magnification (ALVES; MORAES 1998).

Figure 1. *Beauveria bassiana* cultivation in Potato Dextrose Agar medium for conidia production.

In each experiment, the viability of the propagules was determined using the protocol described by Oliveira et al. (2015). The suspension was diluted to 1 x 10^6 conidia $mL^{-1,}$ and a volume of 200 µL was placed on a Rodac® plate containing 5 mL of PDA, 500 mg/L of Pentabiotic® (Fort Godge Animal Health® Ltd., Brazil, the antibiotic contains the substances benzatine benzypenicillin (3,000,000Ul), streptomycin (1,500,000 Ul), procaine benzypenicollin (1,500,000

Ul), potassium benzylpenicillin (1,500,000 Ul) and dihydrotreptomycin base (1.250 mg) and 10 μL/L of Derosal® (fungistatic antimycotic). The material was incubated in B.O.D. incubator (Biological Oxygen Demand) for 18 h (25°C ± 2°C, 12L:12D day/night photophase). Viable and non-viable conidia were counted under a microscope at 400x magnification. The rate of conidial germination was determined by counting 200 total conidia in a randomly selected plate area. Conidia were considered viable when germination tubes had emerged. Germination of conidia exceeded 90%.

3.2. *Spodoptera frugiperda* rearing

Eggs of *S. frugiperda* were obtained from the Laboratory of Insect Biology at the "Luiz de Queiroz" College of Agriculture. The rearing was maintained at 25 ± 2 °C, 70% ± 10% RH, and photoperiod 14:10h L:D. Within 72 h, first-instar neonate larvae hatched, being placed in plastic containers containing an artificial diet proposed by Greene et al., (1976) and modified by Parra (1998). After four days, the caterpillars were individually separated in plastic containers (50 mL) containing a 1 cm^3 piece of artificial diet to avoid cannibalism. When molted to pupae, the individuals were sexed, and ten couples (10 ♀, 10 ♂) were placed in cages made of PVC tubes (20 cm height x 15 cm diameter), and the extremities were closed with Petri dishes. Paper sheets were attached to the cage's interior to stimulate oviposition (Figure 2). Adults were fed an aqueous diet of 10% honey prepared with distilled water. Masses of eggs were collected and placed in plastic containers with moistened filter paper. When the eggs hatched, some caterpillars were placed in containers with artificial diet to maintain the rearing. Caterpillars used in the experiments were kept in plastic containers (500 mL) and fed with soybean leaves obtained from reserves soybean plants, from neonate until they reached the second instar (ca. four days). This procedure was conducted to acclimate *S. frugiperda* larvae to feed on soybean plants versus artificial diet. To select 2^{nd} instar larvae for the study, the head capsule widths were verified through a stereomicroscope glass ruler (Wild M3, Switzerland).

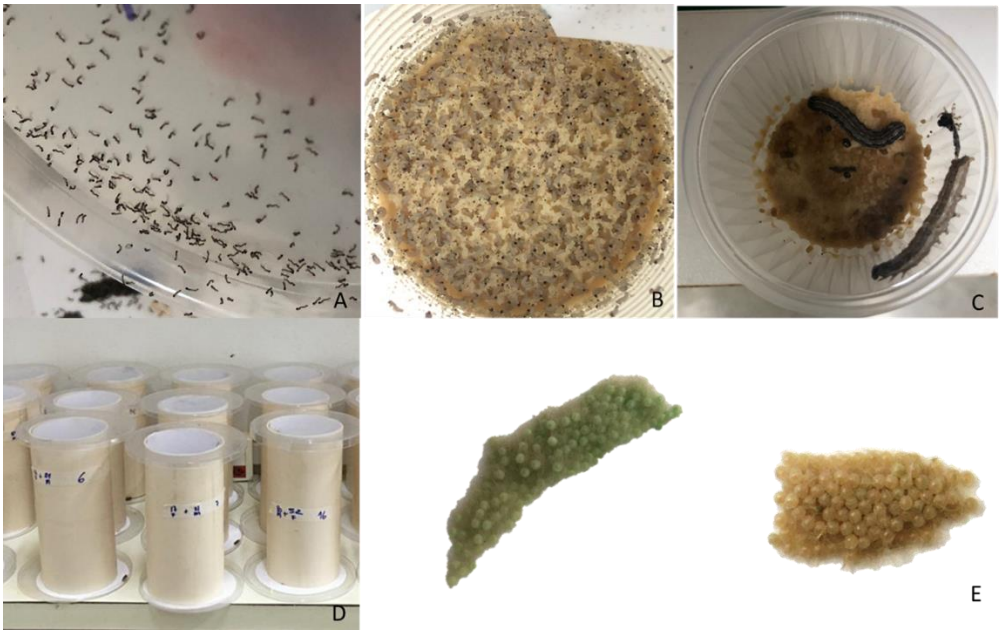

Figure 2. Overview of *Spodoptera frugiperda* rearing. After a few days, the eggs hatched and were obtained from the neonates (A). The neonates were transferred to a diet until the third instar larvae (B). Individualization of larvae of the third instar in containers with diet to avoid cannibalism (C). After the molt, the pupae stage was sexed and transferred to cages with ten couples to obtain eggs (D). Eggs obtained from cages and placed in containers with high humidity(E).

3.3. Soybean plants

Conventional soybean seeds BRS 284 were obtained from EMBRAPA Soja, State of Paraná, Brazil. All the plant experiments were carried out in a greenhouse (22°42'43.2"S 47°37'44.1"W) (Figure 3). Seeds were sown in pots containing 1 L of substrate Basaplant®. Plants were thinned to one per pot, and one plant was adopted as the experimental unit. Plants were watered and fertilized with a solution of NPK 10-10-10 and micronutrients (Dimy®) when necessary. The solution was prepared in a concentration of 10 $mL.L^{-1,}$ and 5 mL was added to each vase. Plants were fertilized every ten days.

Figure 3. Greenhouse and experiment. Greenhouse where the experiments were conducted (A). Soybean plants before thinned (B).

3.4. Foliar application of *Beauveria bassiana* on soybean plants

The plant vegetative stage for the application of *B. bassiana* was defined in each experiment. This methodology was adapted from Garrido-Jurado et al. (2017, 2020). In all the experiments, the conidial suspension was applied in the first trifoliate, which was tagged with colored adhesive tape helping the identification (Figure 4.A). Non-inoculated plants were applied with sterile 0.05% Tween® 80 solution. The application was performed using a soft paintbrush on both adaxial and abaxial leaf surfaces. To maintain humidity and avoid conidial dispersion to other parts of the plant, the trifoliate to which conidia were applied was covered with a plastic bag for the first 72 hours post-application (Figure 4.A and 4.B). The same procedure was performed with control plants. The plants were placed according to randomized blocks experimental design.

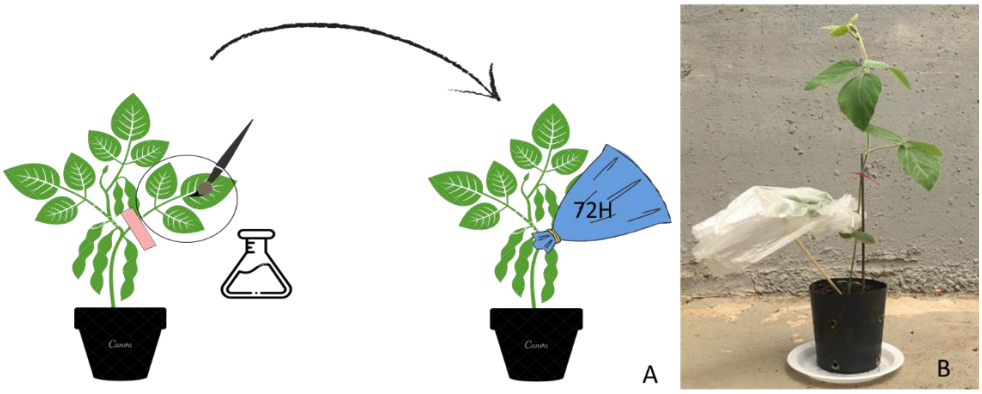

Figure 4. Application method. Experimental methodology (A). First trifoliate covered with plastic bag upon application *of Beauveria bassiana* (B).

3.5. Evaluation of *Beauveria bassiana* colonization in soybean

To quantify and detect epiphytic and endophytic colonization of soybean plants by *B. bassiana*, the plants of all experiments were submitted to a colonization confirmation procedure based on cultivation. Ten plants from the control and all the plants treated with *B. bassiana* were evaluated in both experiments (Experiment I) that was evaluated the leaf consumed area and growth parameters for eight days, and the second experiment (Experiment II) where the effects of the development of *S. frugiperda* was evaluated. The gene expression experiment (Experiment III) was evaluated in three plants on which epiphytic and endophytic colonization was confirmed.

The epiphytic evaluation was performed to confirm the presence of the fungus in the superficial foliar area. The procedures to examine the occurrence of *B. bassiana* were performed in a laminar flow cabinet, and all the materials were sterilized. Semi-selective growth medium Strasser [peptone (10 $g.L^{-1}$), dextrose (20 $g.L^{-1}$), agar (15 $g.L^{-1}$), dodine (45%) (0.3 $g.L^{-1}$), cycloheximide (0.5 $g.L^{-1}$), tetracycline (0.5 $g.L^{-1}$), streptomycin (0,5 $g.L^{-1}$) , cycloheximide (6 $g.L^{-1}$) and chloramphenicol (0.5 $g.L^{-1}$)] (KEPLER, 2015) was used to recover the fungi from plant tissues.

Both methodologies to confirm epiphytic and endophytic colonization were adapted from Borel (2018). The first and second trifoliate were evaluated. Therefore, both were collected from the plant at the end of the experiment. First, the leaves were weighed individually on an analytical scale. Then a pre-wash was conducted in the trifoliate: each one was transferred to a tube (50 mL) with 30 mL of 0.05% Tween® 80. The sample was stirred for 1 min and subsequently sonicated in an ultrasonic bath for 5 min. The epiphytic evaluation was performed by plating 150 µL of the pre-wash into a Petri dish containing Strasser culture medium and was kept in a B.O.D. incubator 25 ± 1°C and photophase 12L:12D. The number of colony-forming units (CFU) were determined after 12 days, by counting on a manual counter (CP608, Phoenix Luferco).

To evaluate the endophytic colonization, the same trifoliate was surfaces sterilized following the protocol of Greenfield et al. (2015) for bulk surface-sterilization (Figures 5A and 5B). This procedure was performed one block at a time, according to Parsa et al. (2013). The trifoliolate was surface sterilized immersed in 70% ethanol for 30 s, 0.5% sodium hypochlorite for 1 min, then rinsed three times in sterile distilled water and air-dried on sterile filter paper for 1 min (MCKINNON et al., 2017). In each block, 150 µL of water from the last rinse was plated onto a Strasser culture medium to determine the efficiency of the surface sterilization. Two replicates were made for each block.

After sterilization, the plant material was macerated with mortar and pestle and transferred to a tube (50 mL). The tubes were put in a shaker for 50 min in an ambient condition with 350 revolutions per minute (rpm). From each leaf suspension, 150 µL were spread onto two

Petri dishes using a Drigalski spatula. The endophytic samples were kept in a B.O.D. incubator 25 ± 1°C and photophase 12L:12D (Figure 6). The fungus occurrence was detected by analyzing the morphological characteristics of fungal outgrowth. The number of colonies was counted with a manual counter to determine the Colony-forming units (CFU) after 12 days (CP608, Phoenix Luferco).

Figure 5. Bulk surface-sterilization. Material to realize the bulk surface-sterilization with the sequence of 70% ethanol, sodium hypochlorite and three times of sterile water (A). Surface sterilization leaves with Falcon tubes adapted according to the methodology (B).

Figure 6. Petri dishes maintained in an incubator for 12 days to evaluate the epiphytic and endophytic occurrence of *Beauveria bassiana* from the experiments.

3.6. Experiment I: Effect of *Beauveria bassiana*-endophytically-colonized soybean plants on the development and herbivory of *Spodoptera frugiperda*

This experiment had the objective of evaluating the endophytic effect of *B. bassiana* sprayed on soybean plants on the following parameters of *S. frugiperda*: (1) larval weight, (2) head capsule width, (3) mortality, and (4) leaf consumption. The experiment was designed in

randomized blocks and was composed of two treatments: *B. bassiana*-inoculated soybean plants vs. non-inoculated plants. The experiment consisted of three blocks. Each block had ten replicates of each treatment, totalizing 30 plants per treatment.

When the soybean plants reached the V3 stage, with the third unfolded trifoliolate leaves, the suspension of *B. bassiana* was applied in the first trifoliate as described in item 3.4. After 72 h, the plastic bag was removed from the first trifoliate leaf. The second trifoliate was photographed, then one 2nd instar larva was transferred to the second trifoliate using a fine paintbrush. The larva was enclosed in the trifoliolate with a *voile* bag and was allowed to feed for eight days, with daily observations to confirm mortality and the presence of each larva. Ten larvae in the second instar were collected to measure their initial weights and head capsule widths.

Eight days post-*S. frugiperda* infestation, the caterpillars were individually placed in plastic containers and weighed on a high-precision semi-analytical scale. Larvae were preserved in an Eppendorf tube with 1 mL of 70% alcohol and kept in a refrigerator. Head capsule widths were measured with a stereomicroscope glass ruler (Wild M3, Switzerland). The cadavers obtained during the experiments were placed in a B.O.D. incubator 25 ± 1°C and photophase 12L:12D to provide the fungus growth conditions.

After removing the larvae, the second trifoliate was photographed again to measure the leaf area consumed by the insect. For standardization, the camera was placed on a tripod at the same height for all samples and the photography was taken with a white background and a scale. The leaf consumption was estimated using the software Image J 1.8.0 (http://imagej.nih.gov/ij/)(Figure 7).

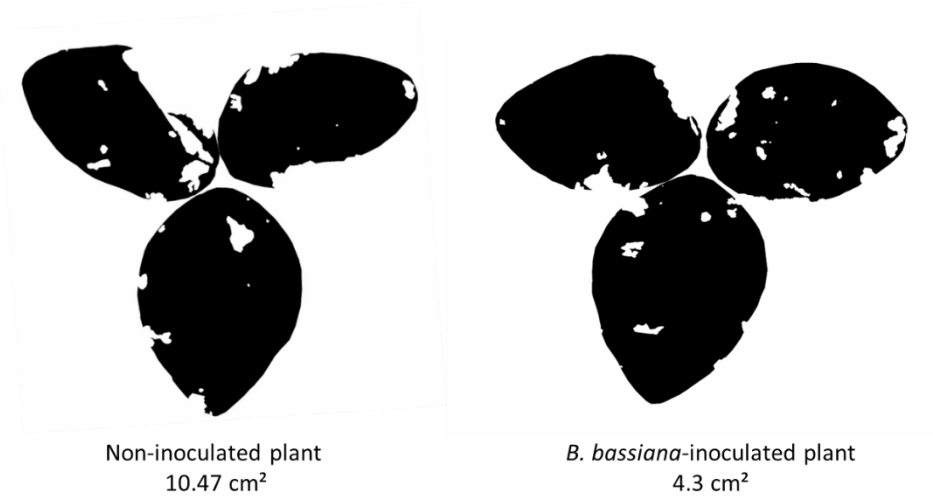

Figure 7. Second trifoliate after eight days of herbivory from *Spodoptera frugiperda* from non-inoculated plants versus *Beauveria bassiana*-inoculated plants.

To assess the endophytic colonization, the first and second trifoliolate leaves were removed from each plant and placed individually in a paper bag. To determine the *B. bassiana* colonization, the epiphytic and endophytic evaluations were performed as described in item 3.5. Two replicates in time (independent experiments) were conducted in the summer (January and February).

3.7. Experiment II: Effect of *Beauveria bassiana*-endophytically-colonized soybean plants on the development (2nd instar larva – pupa) and reproduction of *Spodoptera frugiperda*

This experiment had the objective of evaluating the endophytic effects of *B. bassiana* sprayed on soybean plants on the development of *S. frugiperda,* according to the parameters: (1) average of development (2nd instar larva – pupa) in days, (2) weight of pupa, (3) larval mortality, and (4) emergence of adults. Each experiment was designed in randomized blocks and was composed of two treatments: *B. bassiana*-inoculated plants vs. non-inoculated plants. The experiment consisted of six blocks. Each block had eight replicates of each treatment, totalizing 48 plants per treatment. The experiment was repeated twice (independent experiments) in the spring (September and October).

When the soybean plants reached the V5 stage, with five unfolded trifoliate leaves, *B. bassiana* suspension was applied in the first trifoliate as described in item 3.4. After 72 h, the plastic bag was removed from the first trifoliolate leaf. One 2nd instar larva was transferred to a second trifoliate using a fine paintbrush and enclosed with a *voile* bag (Figure 8). Daily, each larva was observed and, when necessary, was transferred to another trifoliate until molt. The first trifoliolate wasn't supplied due to the contact direct with entomopathogenic fungi during the application. The first and second trifoliolate leaves were carefully removed and put in a paper bag. Evaluation of occurrence of *B. bassiana* was performed as described in item 2.4.

Figure 8. Methodology to evaluate the development of *Spodoptera frugiperda* (2nd instar larva-pupa) in *Beauveria bassiana*-endophytically-colonized soybean plants. *Voile* bag covering leaves (A). Larvae doing herbivory (B).

Pupae were weighed on an analytic scale 24 h upon metamorphosis (MACHADO et al., 2017). To split males and females, a stereomicroscope (Wild M3, Switzerland) was used (BUTT; CANTU, 1962). The pupae were placed individually in Petri dishes with moistened filter paper; the paper was changed when necessary (Figure 8). Duration from the emergence of each adult was measured. The cadavers of larvae and pupae obtained during the experiments were placed in a humidity chamber to provide the fungus growth conditions and kept in a B.O.D. incubator (25 ± 1°C and in photophase 12L: 12D).

Figure 9. Pupa of *Spodoptera frugiperda* placed in a Petri dish with moistened filter paper until adult emergence.

To evaluate reproductive parameters of *S. frugiperda*, pupae were obtained from the previous experiment (development *in planta* from 2nd instar-pupa), and the fecundity (number of eggs per female) were evaluated. This second part of the experiment was conducted in a completely randomized design with the treatments: insects that fed *B. bassiana*-inoculated plants vs. insects that fed on non-inoculated plants. Each treatment had 7-10 replicates containing one female/male pair. The experiment was repeated twice.

As moths emerged, females and males of both treatments were joined in couples (1 ♀, 1♂) on the same day to avoid the effects of adult age on the capacity to copulate (ROGERS; MARTI, 1997). Each couple was placed in a copulation cage made with a PVC tube (20 cm height x 10 cm diameter) folded with paper to stimulate oviposition and to allow the laying egg and handling of egg masses (Figure 10). All tops and the bottoms of containers were closed with Petri dishes (15 cm in diameter). A piece of cotton was soaked in a 10% honey solution dissolved in distilled water and offered in a Rodak® dish to feed the couple. Daily the number of eggs on each cage was recorded until the female adult died. Dead adults were removed and deposited in a humidity chamber and were maintained in a B.O.D incubator (25 ± 2°C and 12L:12D) to observe the fungal growth.

Figure 10. Experiment with couples of *Spodoptera frugiperda;* after molt the pupae were transferred to Petri dishes until moths emergence and upon eight days, the adults were placed in cages with one male and one female.

3.8. Evaluation of defense genes expressed by *Beauveria bassiana*-endophytically-colonized soybean plants

3.8.1. Collecting leaf samples

Soybean plants were maintained in a greenhouse, and when they reached the V2 stage (with the second trifoliolate leaves unfolding), a suspension of *B. bassiana* (1 x 10^8 conidia mL^{-1}) was applied on the first trifoliate as described in 2.5. Non-inoculated plants were applied with sterile 0.05% Tween® 80 solution. The experiment was composed of four treatments: non-inoculated plants without *S. frugiperda* herbivory (T1), *B. bassiana*-inoculated plants without *S. frugiperda* herbivory, non-inoculated plants with *S. frugiperda* herbivory, and *B. bassiana*-inoculated plants with *S. frugiperda* herbivory. After inoculation, each treatment was sampled at 5, 15, and 30 days. Two days before being sampled, the young unfolding trifoliate was chosen for herbivory. One larva of *S. frugiperda* (4^{th} instar) was placed on the youngest trifoliate two days before the period, and the

herbivory was maintained for 48 hours. Each treatment per time had ten replicates, totalizing 120 plants in the experiment (Figure 11). From every ten replicates per time, two plants were gathered to combine one biological replicate, and each treatment had three biological replicates per time (four plants were discarded). For each biological sample, two plants were chosen and collected two leaflets from young trifoliate after herbivory.

Leaf samples were collected at 8 a.m. during each experimental time. They were carefully arranged in an aluminum paper envelope and immediately frozen in liquid nitrogen and stored in a -80 °C freezer for RNA extraction.

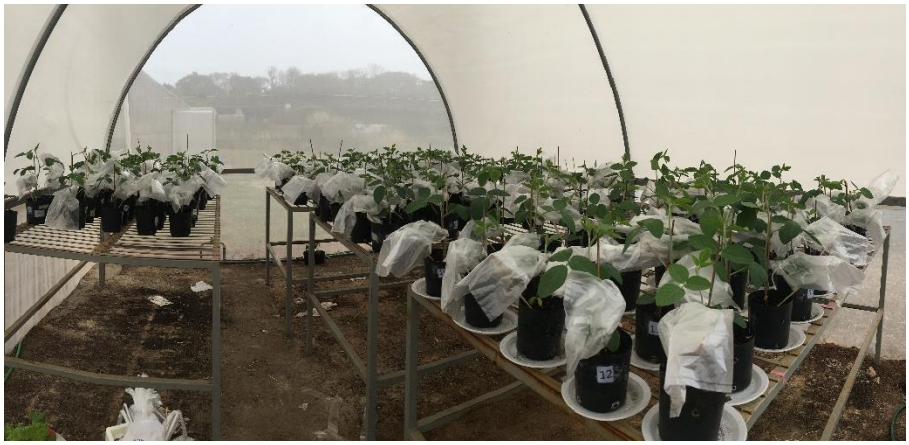

Figure 11. Experiment set up of defense gene expression in the greenhouse; all plants placed on the workbench after *Beauveria bassiana* application in the first trifoliate leaf.

3.8.2. RNA extraction

Following the manufacturer's protocol, RNA was extracted from the leaves using the PureLink® RNA Kit (Ambion® Life Technologies). One hundred milligrams of foliar tissues were submitted to cell lysis with liquid nitrogen and then transferred to an Eppendorf tube. In each sample, it was added 600 µL of Lysis Buffer and 6 µl of 2-mercaptoethanol, followed by vortexing for 1 min. The next step was centrifugation at 12,000 g for 2 min in an Eppendorf Microcentrifuge 5415. The supernatant was transferred to another Eppendorf tube. Then 600 µL of 70% alcohol was added, and the samples were vortexed for 1 min.

The mix in the Eppendorf tube was pipetted (700 µL) and transferred to on-column Pure Link, centrifuged (12,000 g for 30 s), and after this procedure, the liquid was discarded from the collection tube. The exact process was repeated with the rest of the Eppendorf tubes to retain all the samples in the column. In the same column, 700 µL of Buffer I was added, and then the samples were centrifuged (12,000 g for 30 s). The liquid from the collection tube was discarded.

Next, 500 μL of washer Buffer II was added to the column and centrifuged (12,000 g for 30 s). This procedure was repeated a second time. Then, the column was centrifuged (12,000 g for 2 min) to dry the membrane. The column was transferred to a new collection tube, where it was pipetted 60 μl of RNAse-Free in the center of the membrane, and the material was left at room temperature for 1 min.

After this, the material was centrifuged for two minutes. The quantity and purity of RNAs were analyzed on a plate reader and measured based on the 260/280 nm absorbance ratio at Epoch (BioTek Instruments Inc.).

According to the manufacturer's instructions, RNA samples were treated with DNAse I (Thermo Scientific™) to eliminate any contaminant DNA. DNA was removed by digestion with 1 μL of DNAse I RNase-free, 1 μL of solution 10x Reaction Buffer with $MgCl_2$, 1 μg of RNA sample, and ultra-pure water to complete the volume of 10μL. The reaction was incubated in a thermocycler (Applied Biosystems) at 37 °C for 30 min. Next, it was added 1 μL of EDTA (50 mM), and the samples were incubated in a thermocycler at 65 °C for 10 min. The quality and purity were evaluated at Epoch (BioTek Instruments Inc.). The RNA integrity was assessed on a 1.0% UltraPure™ Agarose and visualized in an ultraviolet transilluminator connected with a photodocumentary (BioDoc-It® Imaging System) (Figure 12).

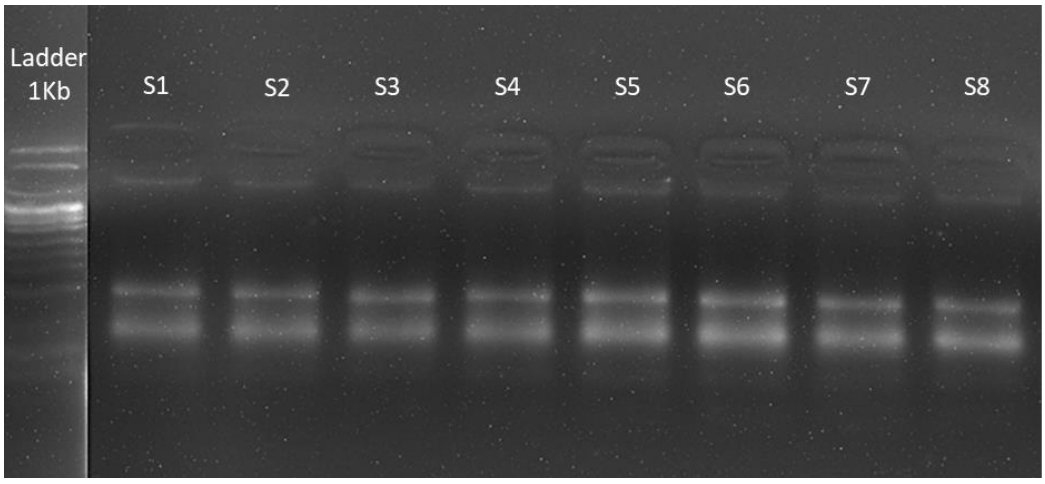

Figure 12. Evaluation of RNA integrity from RNA samples of leaf tissue from soybean plants. Each lane was loaded with 1 μL of total RNA, 4 μL with ultra-pure water and 5 μL of loading buffer previously prepared (totalizing a volume of 10 μL). The image was obtained from agarose gel (1.0 %) electrophoresis and visualized in an ultraviolet transilluminator connected with a photodocumentary (BioDoc-It® Imaging System). Ladder 1 Kb shows molecular weight markers.

3.8.3. cDNA synthesis

Complementary DNA (cDNA) was synthesized for each sample using the Kit High-Capacity cDNA Reverse Transcription (Applied Biosystems), following the manufacturer's instructions. In a tube, it was pipetted 1 μg of DNAse-treated RNA, 2 μL of Buffer Solution for Reverse Transcriptase (RT), 0.8 μl of dNTP mix (25 mM), 1 μL of multiScribe reverse transcriptase, 2 μL of random primer solution, and ultra-pure water to complete the volume of 20 μL. The reaction mix was incubated in a thermocycler (Applied Biosystems) with the following cycle: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min (this last step to inactivate the reverse transcriptase). All the cDNA samples were stored at -80 °C.

3.8.4. RT-qPCR

Gene expression levels associated with defense responses to herbivores (Table 1) were evaluated using quantitative reverse transcription polymerase chain reaction (RT-qPCR). Each gene expression was selected by the relevance according to the hormone pathway based on Pieterse et al. (2014). The housekeeping gene *ACT II* was selected based on Gao et al. (2017). Primers for the genes *PR2* and *PR3* were obtained from Takeuchi et al. (1990) and Thomma (2002). Primers for *MYC2* and *ERF1* were designed using GENEINOUS v. 6.1.7 software (Biomatters). Criteria to design the primers were: stringent set, melting temperature 57°C – 63°C, primer length of 18-25 nucleotides, G-C content of 40-60%, and PCR amplicon of 80-150 nucleotides. Table 1 contains the list of reference genes, target genes and PCR efficiency; and Table 2 contains information about the target genes.

Table 1. Oligonucleotide primers used for quantitative real-time PCR.

Gene	Primer	Sequence (5'-3') and (3'-5')	Amplicom length (bp)	PCR efficiency (%)
		Target gene		
PR2	Forward	GTCTCCTTCGGTGGTAGTG	104	105.36
	Reverse	ACCCTCCTCCTGCTTTCTC	104	
PR3	Forward	GCACTTGGTCTGGATTTG	115	90.88
	Reverse	GGCTTGATGGCTTGTTTC	115	
MYC2	Forward	ACCGAGTACCGGATGAACCT	135	106.63
	Reverse	TGCCGTGCCTGGAGTTGT	135	
ERF1	Forward	CCACCGTGGATCACTCCTTC	132	93.74
	Reverse	GACGCGTGGAGTCCCTTATC	132	
		Reference gene		
ACTII	Forward	CGGTGGTTCTATCTTGGCATC	142	94
	Reverse	GTCTTTCGCTTCAATAACCCTA	142	

Table 2. Genes used in the study and their function and signaling pathway.

Genes	Name	Function	Signaling
PR2	Pathogenesis-related (PR) protein - β-1,3-glucanase	Disrupt the fungal mycelial wall (SHORESH et al., 2004).	SA
PR3	Pathogenesis-related (PR) protein – Chitinase	Disrupt the fungal mycelial wall (SHORESH et al., 2004).	JA
MYC2	MYC proto-oncogene	Transcriptional activator that includes JA signaling response (KAZAN; MANNERS; 2013)	JA
ERF1	Ethylene response factor 1	Transcriptional factor genes in the regulation of JA/ET regulated genes (PIETERSE et al., 2014)	ET

Quantification of gene expression was performed using the Applied Biosystems 7500 real-time PCR Systems. The cDNA was diluted to 1/10, each treatment had three biological replicates, and each sample had three technical replicates. Reactions were performed in a volume of 10 μL, consisting of 1 μL of cDNA, 5 μL of SYBR Green, 0.2 μL of each primer, and 3.6 μL of nuclease-free water. The thermal conditions were a holding stage with one step (95 °C for 2 min); a cycling stage with two steps with 40 cycles, the first step was 95 °C for 3 s, and the second step 60 °C for 30 s. The melting curve stage had four steps, first 95 °C for 15 s; second 60 °C for 1 min; third 95 °C for 15 s and the fourth 60 °C for 15 s.

The relative gene expression data were calculated with the Pfaffl method (PFAFFL, 2001), which requires the primer efficiencies for the gene of interest and the housekeeping gene. First, the primer efficiency percentage was covert according to the formula $Coverted\ primer\ efficiency = (Primer\ efficiency(\%) \div 100) + 1$. The next step was calculating the average of Ct values from the technical replicates and selecting the calibrator samples, considered non-inoculated plants (control average) for each time. Subsequently, the ΔC_T was calculated separately for each gene in each

sample by $\Delta C_T = Control\ average - \text{Average Ct}$ and the gene expression ratio into the Pfaffl equation was calculated by:

$$Gene\ expression\ ratio = \frac{Converted\ primer\ efficiency^{\Delta Ct\ gene\ of\ interest}}{Converted\ primer\ efficiency^{\Delta Ct\ housekeeping\ gene}}$$

The threshold cycle value is defined as the PCR cycle number in which an arbitrarily placed threshold line was highlighted.

3.9. Statistical analysis

Data on the consumed foliar area, the larvae mass weight, pupae proportion, adult emergence proportion, and gene expression ratio, were analyzed by one-way ANOVA, a linear model was used. The normality and homogeneity of variances were confirmed with Bartlett's. Before any data analyses, the residuals were evaluated by the Shapiro-Wilk Normality test, homogeneity through the half-normal plot (MORAL et al. 2017), and standardized residual normality qqplot (LINN et al. 2015). When necessary, was realized a logarithmic transformation (y with log x). Differences significative was determined by multiple comparison test by Tukey's honestly significant difference (HSD) FDR-adjusted P-values ($\alpha = 0.05$).

Life-cycle length according to the time (days) and proportion of individuals from pupae and adults, was computed using the Kaplan-Meier method using the function 'survfit ()' from the package 'survival' (THERNEAU, 2021) and the curves were generated with the function (KALBFLEISCH; PRENTICE, 2002). Survival curves were compared with the long-rank test performed with the function 'survreg ()' from package 'survminer'.

Generalized linear models (GLMs) were used for the analyses that assumed binominal error, with the data of mortality, larvae to molted and adult emergence. A correction through the quasibinomial distribution was realized. When significative, the interaction was considered, and the quality adjustment was realized by half-normal plot (MORAL et al., 2017). Adult longevity (the total number of days lived as adults from emergence to death) was analyzed by two-way ANOVA (with treatment and sex as main factors). When significative, the comparison was performed by a Tukey HSD test through function "cld" of the package 'multcompView' (GRAVES et al., 2015).

Generalized linear models (GLMs) were used for the analyses that assumed Poisson distribution on the number of eggs per female. A correction was realized through the quasipoisson distribution. When significative, the interaction was considered, and quality adjustment was realized by a half-normal plot (MORAL et al., 2017).

Epiphytic and endophytic colonization were expressed as colony-forming unity per gram of leaf. The value was obtained using the formula:

$$\frac{(Leaf\ volume \times 2) \times (mean\ of\ colony\ forming\ unity)}{0.15}.$$

Leaf volume was the weight of each trifoliate, and the number 0.15 referred to the volume plated (150 μL). Values are expressed as means ± standard deviation (SD). The statistical analyses were conducted in the R program (version 4.2.1).

4. RESULTS

4.1. Experiment I: Effect of *Beauveria bassiana*-endophytically-colonized soybean plants on the development and herbivory of *Spodoptera frugiperda*

4.1.1. Epiphytic fungus occurrence on soybean plants

The epiphyte occurrence of *B. bassiana* was evaluated to confirm the application's success in the first trifoliate. Ten plants from each treatment (*B. bassiana*-inoculated or non-inoculated) were assessed in each replicate. The fungus was detected on *B. bassiana*-inoculated plants at 4.91 ± 1.66 x 10^3 CFU g^{-1}, and it was not seen in non-inoculated plants (Figure 13).

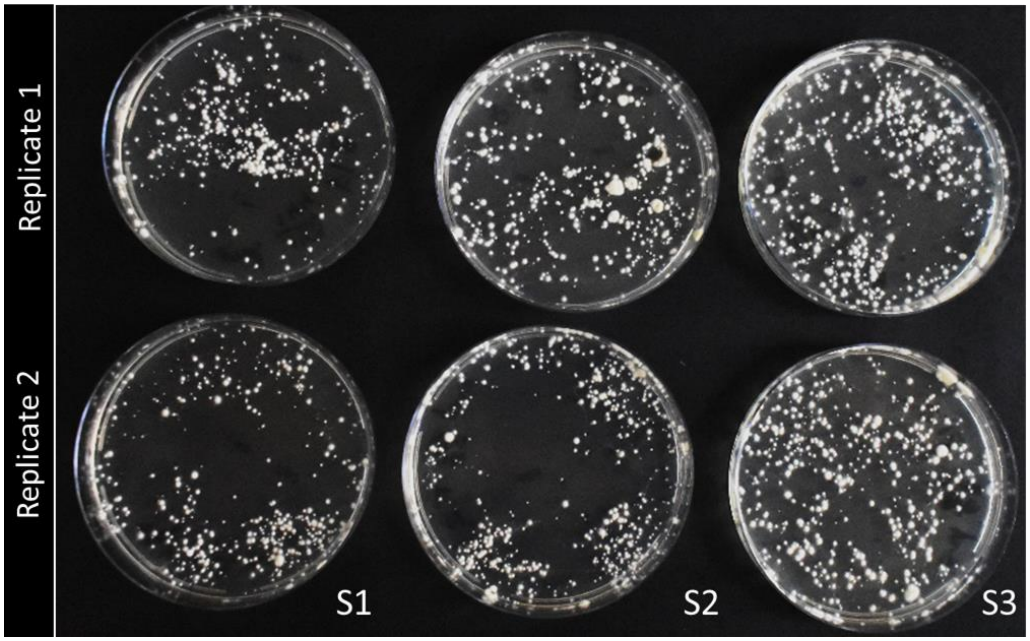

Figure 13. Epiphytic occurrence of *Beauveria bassiana* on the first trifoliate of three samples of soybean plants (S1, S2 and S3).

The second trifoliate was also evaluated epiphytically to assess the absence of *B. bassiana* on leaves consumed by *S. frugiperda*. In both treatments, the lack of the fungus was confirmed, ensuring that larvae had no direct contact with conidia of *B. bassiana*.

4.1.2. Endophytic fungus occurrence on soybean plants

The endophytic colonization of *B. bassiana* in soybean plants was evaluated in the first and second trifoliates. The surface sterilization proved successful in controlling the epiphytic presence

of *B. bassiana* because no fungi were detected. Thus, any colony *of B. bassiana* growing in the Petri dishes would have been re-isolated from plant tissues.

In non-inoculated plants, no entomopathogenic fungi growth was observed in the first and second trifoliates. The endophytic occurrence of *B. bassiana* in inoculated plants was confirmed by the presence of growing fungi on Petri dishes inoculated with suspensions of the first and second trifoliates (Figure 14). A high quantity of CFU was observed in the first trifoliate, with an average of 107.63 ± 71.72 CFU g^{-1}, while in the second trifoliolate (non-inoculated leaves) the average was 0.08 ± 0.24 CFU g^{-1}. Despite this lower occurrence, the results show that *B. bassiana* was transported within the plant by the vascular vases.

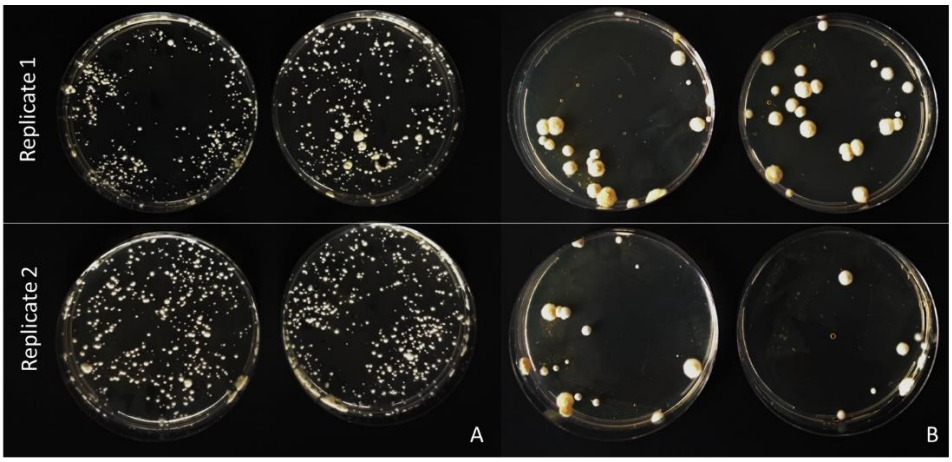

Figure 14. Endophytic occurrence of *Beauveria bassiana* on the first and second trifoliates of *B. bassiana*-inoculated soybean plants (1x10^8 conidia mL^{-1} applied on the first trifoliate). A) Petri dishes showing a high quantity of colony-forming units (CFU) of *B. bassiana* re-isolated from the first trifoliate. B) Petri dishes showing a low quantity of CFU of *B. bassiana* re-isolated from the second trifoliate.

4.1.3. Secondary effects on *Spodoptera frugiperda* after eight days

The proportion of larvae alive after feeding on *B. bassiana*-inoculated plants was 90%, significantly lower than larvae fed on non-inoculated plants 95% (P = 0.02). Cadavers obtained during the experiment did not show signs of fungal sporulation.

The mean leaf area consumed by *S. frugiperda* on non-inoculated plants was significantly higher (12.10 ± 5.48 cm^2) than the leaf area consumed on *B. bassiana*-inoculated plants (8.18 ± 4.75 cm^2) ($F_{1,81}$ = 12.32, P = 0.007). The mean body weight of larvae in *B. bassiana*-inoculated plants (0.041 ± 0.023 cm^2) was significantly lower compared to non-inoculated plants (0.054 ± 0.032 g) ($F_{1,81}$ = 4.29, P = 0.041).

The mean head capsule width of *S. frugiperda* in non-inoculated plants and *B. bassiana*-inoculated plants were 1.19 ± 0.62 mm and 1.09 ± 0.66 mm, respectively. According to Parra

(1984), the width of fourth-instar larvae head capsules varies between 1.07 and 1.27 mm, indicating that the larvae evaluated from both treatments were in the fourth instar and there was no instar difference between the treatments based on the head capsule width of *S. frugiperda*.

4.2. Experiment 2: Effect of *Beauveria bassiana*-endophytically-colonized soybean plants on the development and reproduction of *Spodoptera frugiperda*

4.2.1. Epiphytic fungus occurrence on soybean plants

The epiphytic occurrence of *B. bassiana* was evaluated to confirm the application's success on the first trifoliate. Ten plants from each treatment (*B. bassiana*-inoculated or non-inoculated) were assessed in each replicate. The fungus was detected on inoculated plants at $5.39 \pm 0.76 \times 10^5$ CFU g^{-1}, while it was absent on the non-inoculated plants. In both treatments, no fungus was observed on the second trifoliate, ensuring the larvae had no direct contact with *B. bassiana* conidia.

4.2.2. Endophytic fungus occurrence on soybean plants

Endophytic colonization was evaluated in the first and second trifoliate. Colonies of *B. bassiana* were observed in non-inoculated plants. On the other side, the average of $2.5 \pm 1.75 \times 10^3$ CFU g^{-1} of *B. bassiana* was detected in the first trifoliate of inoculated plants, whereas only 3.9 ± 9.78 CFU g^{-1} were seen in the second trifoliate.

4.2.3. Insect feeding assay and reproduction of *Spodoptera frugiperda*

The proportion of larvae alive after feeding on *B. bassiana*-inoculated plants was 59.0%, significantly lower than the proportion of 89.6% of larvae fed on non-inoculated plants (control) ($F_{1,22} = 17.88$, $P < 0.001$). Cadavers obtained during the experiment did not show any signs of fungal sporulation.

The time required for larvae of *S. frugiperda* to molt was affected by the treatment ($\chi^2 = 24.74$, df =1, $P < 0.001$), being higher in non-inoculated plants (26.2 ± 0.7 days) than in *B. basssiana*-inoculated plants (23.0 ± 0.5 days) (Figure 15). Control insects lived longer than those treated with entomopathogenic fungi. Cadavers obtained during the experiment did not show any signs of fungal sporulation.

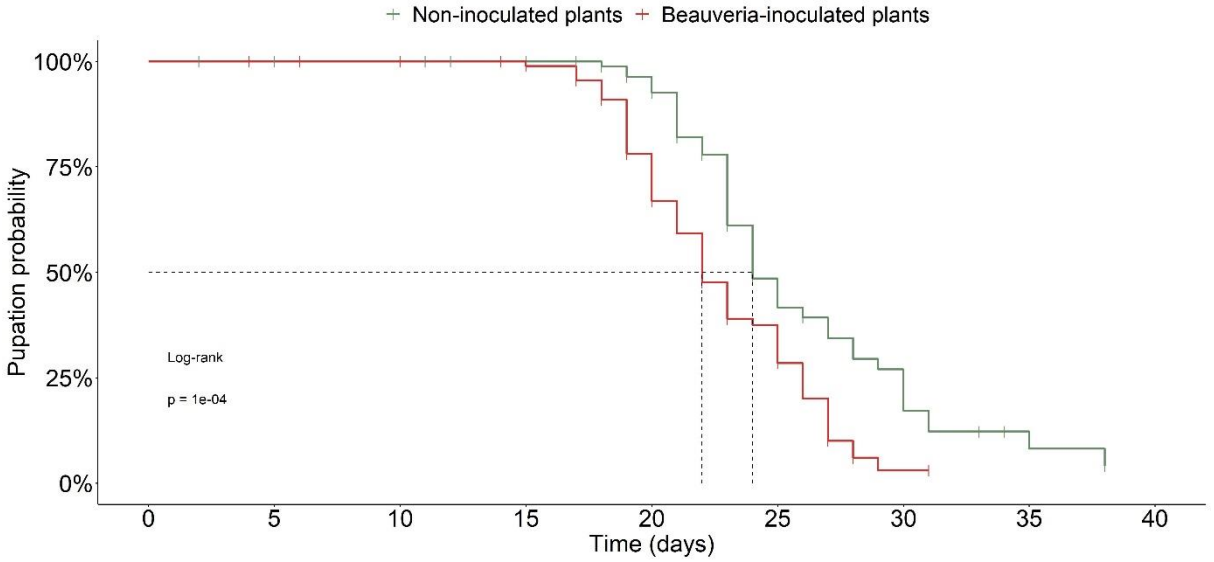

Figure 15. Assessment of the probability of larvae to molt to pupae according to the time (days) when *Spodoptera frugiperda* (2nd instar larvae) were fed on *Beauveria bassiana*-inoculated soybean plants (1x10^8 conidia mL^{-1} applied on the first trifoliate) and non-inoculated soybean plants (solution of 0.05% Tween® 80 applied on the first trifoliate). Larvae from *Beauveria bassiana*-inoculated plants are shown in the red line; larvae from non-inoculated are shown in the green line (Kaplan-Meier survival analyses).

Feeding on plants inoculated with *B. bassiana* did not affect the probability of emergence of adults ($\chi^2 = 0.2$, df = 1, $P = 0.079$). The adult emergence from *B. bassiana*-inoculated plants and non-inoculated plants had an average of 12.84 ± 0.524 and 12.54 ± 0.46 days, respectively (Figure 16).

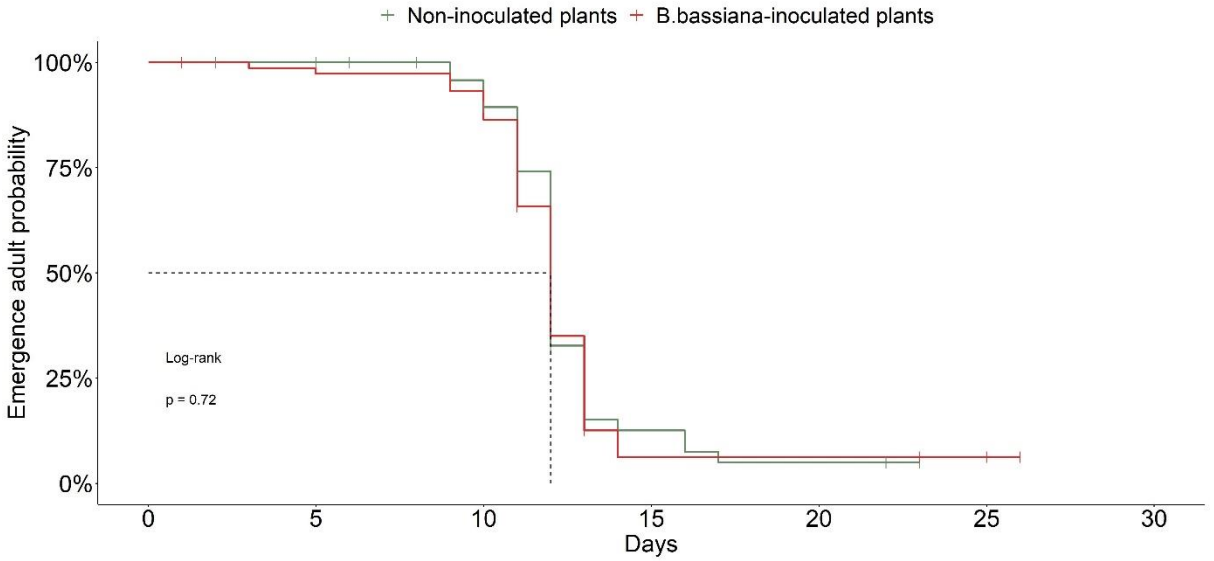

Figure 16. Assessment of the time for pupae to get adult emergence (days) when *Spodoptera frugiperda* (2nd instar larvae) were fed on *Beauveria bassiana*–inoculated soybean plants (1x10^8 conidia mL^{-1} applied on the first trifoliate) and non-inoculated (a solution of 0.05% Tween® 80 applied on the first trifoliate). The adult's emergence from *B. bassiana*–inoculated plants are show in red line; adults emergence from non-inoculated (control) is show in the green line (Kaplan-Meier survival analyses).

The mean weight of female and male pupae bodies (measured 24 h after larvae molted to pupae) from *B. bassiana*-inoculated plants was not significantly different compared to pupae from non-inoculated plants on both analyses ($F_{1,58} = 0.20$, $P = 0.65$ and $F_{1,58} = 0.30$, $P = 0.58$), respectively (Figure 17). Otherwise, some pupae from *B. bassiana*-inoculated plants were smaller and had deformation than the non-inoculated treatment.

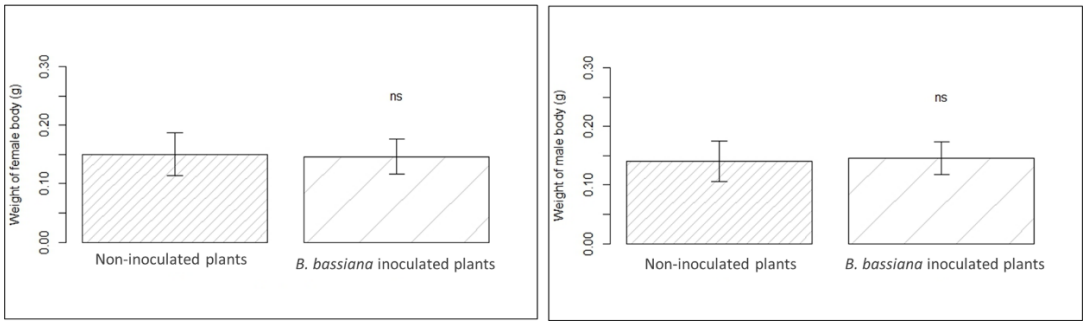

Figure 17. Weight of female and male pupae from both treatments, non-inoculated plants vs. *B. bassiana*-inoculated plants. The bar is represented by mean and standard deviations (±SD). The bar graph was obtained by R program (version 4.2.1).

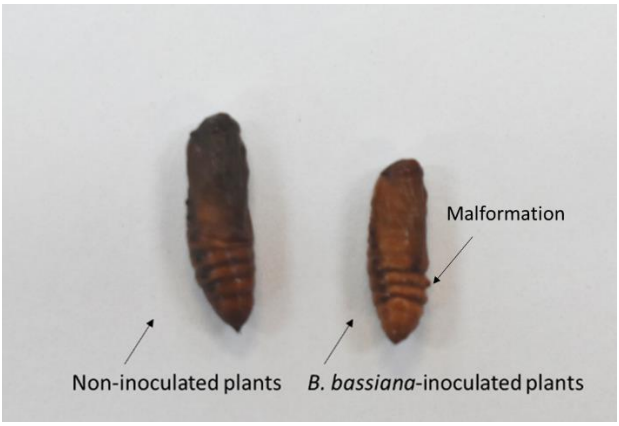

Figure 18. Difference visual from pupae in the treatment of non-inoculated plants vs. *B. bassiana* inoculated plants.

The proportion of larvae to become pupae and had adult emergence on *B. bassiana*-inoculated plants was 52.7%, significantly lower than the proportion of 74.7% of larvae fed on non-inoculated plants (control) ($F_{1,22} = 7.31$, $P < 0.001$).

Spodoptera frugiperda fed on *B. bassiana*-inoculated plants laid fewer eggs (285.2 ± 274.1 eggs/female) than females fed on non-inoculated plants (823.5 ± 418.2 eggs/female) ($F_{1,29} = 18.19$, $P < 0.001$). Cadavers had no signs of fungal sporulation.

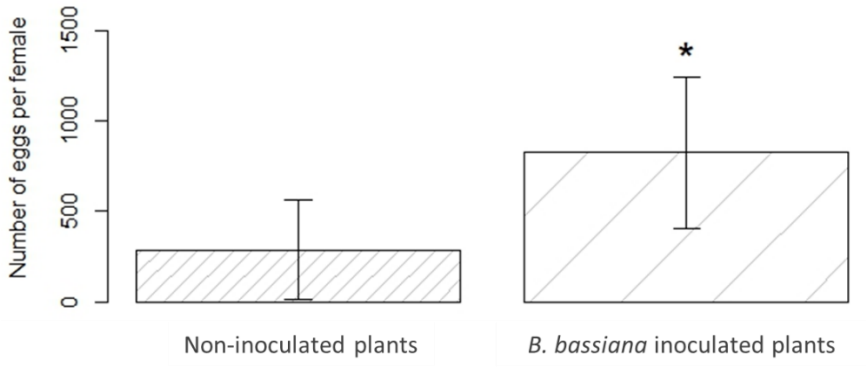

Figure 19. Mean (±SD) number of eggs per female from both treatments, non-inoculated plants and *B. bassiana*-inoculated plants. Bar with * differ significantly (Tukey's HSD, $P < 0.05$).

Figure 20. Couples experiment. Example of adult emergence of *S. frugiperda* failed (A). Female of *S. frugiperda* dead and did not complete the oviposition (B).

4.3. Evaluation of defense genes expressed by *Beauveria bassiana*-endophytically-colonized soybean plants with herbivory of *Spodoptera frugiperda*.

4.3.1. Epiphytic occurrence of *Beauveria bassiana*

The epiphytic occurrence of *B. bassiana* was evaluated to confirm the application's success on the first trifoliate. The fungus was confirmed 5, 15, and 30 days after inoculation in *B. bassiana*-inoculated plants with and without herbivory (Table 3). Non-inoculated plants (solution of 0.05% Tween® 80 applied on the first trifoliate) had no incidence of *B. bassiana*, either with or without herbivory.

Table 3. Evaluation of epiphytic occurrence (means ±standard error) on the first trifoliate (leaf inoculated) in non-inoculated plants (solution of 0.05% Tween® 80) with or without herbivory and *Beauveria bassiana*–inoculated plants ($1x10^8$ conidia mL^{-1}) with or without herbivory. One *Spodoptera frugiperda* larvae fed on the youngest trifoliate.

Treatments	CFU g^{-1} (5 days)	CFU g^{-1} (15 days)	CFU g^{-1} (30 days)
Non-inoculated plants without herbivory	0	0	0
Beauveria bassiana-inoculated plants without herbivory	$1.57 \pm 0.4 \times 10^5$	$2.19 \pm 0.6 \times 10^5$	$1.34 \pm 1.31 \times 10^4$
Non-inoculated plants with herbivory	0	0	0
Beauveria bassiana-inoculated plants with herbivory	$1.39 \pm 0.3 \times 10^5$	$1.71 \pm 0.4 \times 10^5$	$1.11 \pm 1.39 \times 10^4$

4.3.2. Endophytic occurrence of *Beauveria bassiana*

The endophytic occurrence was evaluated in the first trifoliate, and the youngest trifoliate wholly expanded. Before the endophytic confirmation, the leaves were surface sterilized, and no fungus was detected epiphytically. Therefore, no colonies of *B. bassiana* were re-isolated from plant tissues.

In non-inoculated plants with and without herbivory, no *B. bassiana* growth was observed. On the other hand, the presence of *B. bassiana* in inoculated plants, with or without herbivory, was confirmed in the first trifoliate 5, 15 and 30 days after inoculation (Table 4).

The youngest trifoliate was evaluated to confirm fungal translocation in the plant. The fungus was not observed in plants where *B. bassiana* was not applied. On the other hand, *B. bassiana* was re-isolated from inoculated plants with and without herbivory after 5, 15, and 30 days. The fungus concentration was higher after 15 days compared to 5 days post-inoculation, but after 30 days, a decrease in concentration was observed.

Table 4. Evaluation of endophytic colonization (means ± standard error) on the first trifoliate (leaf inoculated) in: non-inoculated plants (solution of 0.05% Tween® 80) with or without herbivory and *Beauveria bassiana*–inoculated plants ($1x10^8$ conidia mL^{-1}) with or without herbivory (one *Spodoptera frugiperda* larvae on the youngest trifoliate).

First trifoliate			
Treatments	**CFU g^{-1} (5 days)**	**CFU g^{-1} (15 days)**	**CFU g^{-1} (30 days)**
Non-inoculated plants without herbivory	0	0	0
Beauveria bassiana-inoculated plants without herbivory	$1.79 \pm 0.6 \times 10^3$	$3.9 \pm 0.7 \times 10^3$	$7.1 \pm 5.4 \times 10^2$
Non-inoculated plants with herbivory	0	0	0
Beauveria bassiana-inoculated plants with herbivory	$1.87 \pm 0.5 \times 10^3$	$4.6 \pm 1.2 \times 10^3$	$1.29 \pm 0.8 \times 10^2$
Youngest trifoliate			
Non-inoculated plants without herbivory	0	0	0
Beauveria bassiana-inoculated plants without herbivory	5.3 ± 5.57	168 ±169.53	34.66 ±63.66
Non-inoculated plants with herbivory	0	0	0
Beauveria bassiana-inoculated plants with herbivory	21.3 ± 22.3	124 ± 87.67	5.33 ± 11.92

4.3.3. Evaluation of gene expression

As detailed in the Method section, the effect of *B. bassiana* colonization on the alteration of immunity-involved genes of soybean plants was investigated by RT-qPCR. The amplification efficiencies were obtained for each primer set; it was accepted only as detailed in primers that lied between 90% - 110% efficient.

The transcript levels of *PR2* were downregulated in all the treatments five days after inoculation. The treatment with *B. bassiana* without herbivory (T2) slightly increased when compared with non-inoculated plants (T1), but both treatments did not differ.

Fifteen days after inoculation, *PR2* expression was increased in treatment non-inoculated plants with herbivory (T3) compared to the treatment *B. bassiana*-inoculated plant with herbivory (T4) ($P = 0.01$). At 30 days post-inoculation, the treatments *B. bassiana* (T2), non-inoculated plants with herbivory (T3), and *B. bassiana*-inoculated plants with herbivory (T4) had an up-regulation when compared with the times fifteen- and thirty-days post-inoculation. The treatment of non-inoculated plants with herbivory (T3) had high gene levels and had difference significative when compared with non-inoculated plants (T1). In this situation, it was observed that *B. bassiana* with herbivory (T4) retained the gene expression.

Five days post-inoculation, the gene *MYC2* did not differ among the treatments. Fifteen days post-inoculation, the treatment *B. bassiana*-inoculated plants with herbivory (T4) had an up-regulation gene expression, otherwise did not differ significantly from the treatment of non-

inoculated plants with herbivory (T3). Thirty days post-inoculation, all the treatments had a downregulation compared with fifteen days post-inoculation.

At five days post-inoculation, the gene *PR3* in all the treatments had no significant difference presenting the same expression levels ($P = 0.16$). After fifteen days post-inoculation, all the treatments had an up-regulation, except the non-inoculated treatment (T1) ($P = 0.0001$). Thirty days post-inoculation, all the treatments had a downregulation with low levels.

Transcript levels of the *ERF1* were upregulated only five days post-inoculation in the treatment subjected to *S. frugiperda* herbivory; thus, the presence of insects interfered with gene levels. Non-inoculated plants (T3) had a higher expression when compared with *B. bassiana*-inoculated plants (T4), both with herbivory. Fifteen days post-inoculation, all the treatments did not have difference significative with a low value from gene expression. Thirty days after *B. bassiana* inoculation, *ERF1* expression was drastically reduced in all treatments.

The graphs (Figure 19) show the induced system of resistance obtained from SA, JA/ET-signaling according to the specific gene expression.

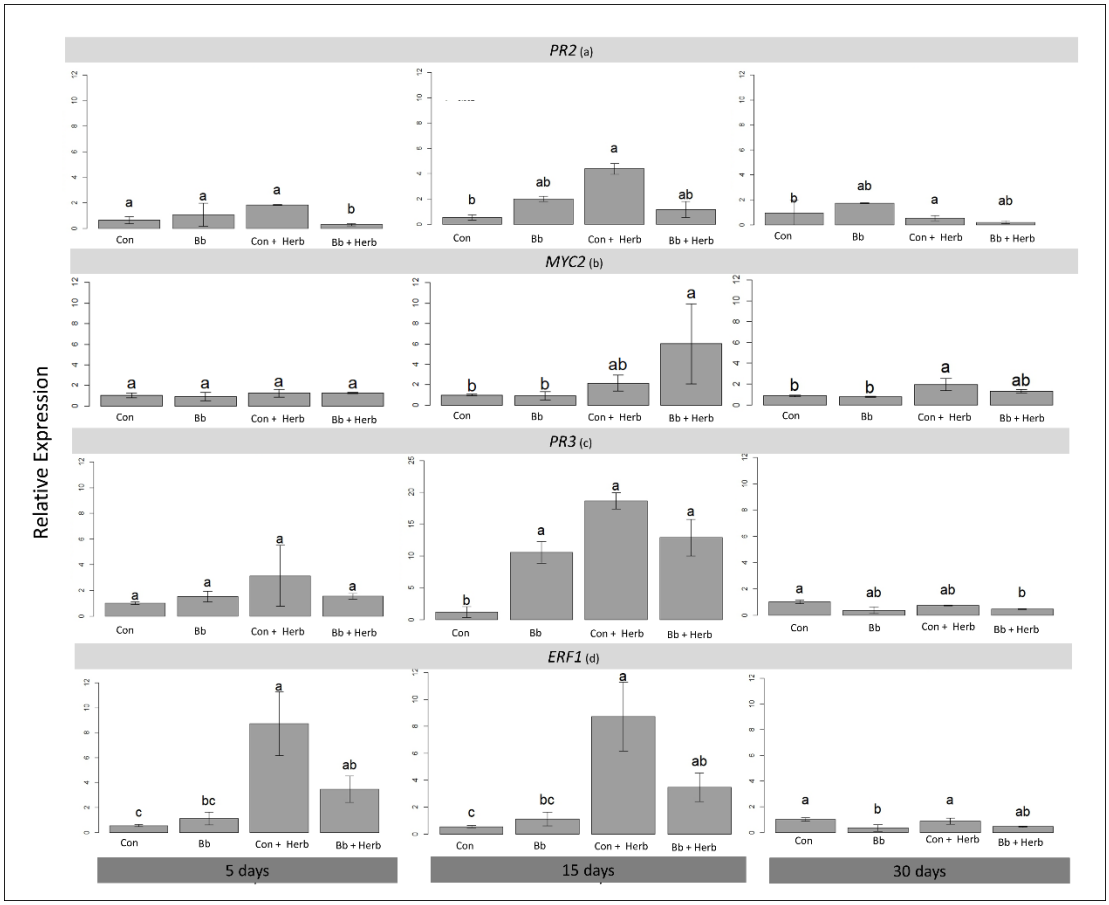

Figure 21. Each graph shows the relative expression of SA, JA/ET-signaling according to the gene in soybean plants. RNA was extracted 5, 15, and 30 days after inoculation in non-inoculated plants without *Spodoptera frugiperda* herbivory – control (Con - T1); *Beauveria bassiana*-inoculated plants without *S. frugiperda* herbivory (Bb - T2); non-inoculated plants with *S. frugiperda* herbivory– control (Con + Herb - T3), *Beauveria bassiana*-inoculated plants with *S. frugiperda* herbivory (Bb + Herb - T4). The control plants were inoculated with a solution of 0.05% Tween® 80, and *B. bassiana*-inoculated plants were applied with $1x10^8$ conidia mL^{-1}. The herbivory consisted of *S. frugiperda* larvae (4th instar) fed in the youngest trifoliate. Two leaflets from these young trifoliate were collected to evaluate the gene expression. The data were represented by mean from three independent biological replicates (each represented by two plant samples) and standard deviations (±SD). The bar graph was obtained by R program (version 4.2.1).

5. DISCUSSION

The foliar application of fungal spores in grown crops is conducted to control pests by direct contact, however, it is essential to confirm the increased pest protection by effects secondarie metabolites by the endophytic association in colonized plants. Colonization of soybean plants by *B. bassiana* affected the *S. frugiperda* biology and reproduction, as observed by reduced consumption and number of eggs per female, for example. In this study, we tried to understand the tri-trophic correlation plant-fungi-insect looking at the induction of expression of key plant immunity pathway genes involved in salicylic acid, jasmone acid, and ethylene cascades after herbivory of *S. frugiperda*.

In all the experiments, *B. bassiana* ESALQ PL63 was able to colonize epiphytically and endophytically the soybean plants via foliar application. Application of *B. bassiana* on the first trifoliate resulted in high epiphytic and endophytic colonization of this trifoliate. The occurrence of entomopathogenic fungus in the second trifoliate (not inoculated with *B. bassiana*) in treated plants was confirmed, although in lower quantities when compared with the inoculated trifoliate. A similar result was reported by Garrido-Jurado et al. (2020) with the re-isolation of *M. brunneum* from melon leaves by foliar application. The process of endophytic colonization occurs by direct invasion of the fungus through the epidermal cell wall into the leaf interior (rarely on stomates), followed by fungus translocation to the entire plant via xylem vessels (WAGNER; LEWIS, 2000); translocation of *B. bassiana* was previously reported by several studies (GARRIDO-JURADO, 2016; MCKINNON et al., 2017; RONDOT; REINEKE, 2019; RUSSO et al., 2017, NISHI et al., 2021, ZHANG et al., 2022; SUI et al.; 2022). The confirmation of ESALQ PL63 as endophytic and its persistence indeed constitute the basis for possibly improvement from plant immune system that affects *S. frugiperda* development.

Endophytic persistence was confirmed 5, 15, and 30 days after inoculation by the gene expression experiment. The occurrence was assessed in the first trifoliate (where *B. bassiana* was applied) and in the youngest trifoliate (Table 4). A high fungus concentration in the first trifoliate was confirmed, although it decreased over time. Russo et al., (2021) also observed corn leaves inoculated with 12 different *B. bassiana* strains showed endophytic growth after 7, 14, 21, and 28 days, with a decrease in fungal occurrence over time, with just four isolates present after 28 days. Other crops such as cotton, beans, wheat, corn, and pumpkin also decreased *B. bassiana* CFUs from day 1 to day 21 (GURULINGAPPA et al., 2010). The persistence of *B. bassiana* inside the plant is associated with factors such as strain, plant and fungal species, environmental conditions, secondary plant metabolites, and inoculation method (SÁNCHEZ-RODRÍGUEZ et al., 2018; RAAD et al., 2019; MANN; DAVIS, 2020). The occurrence of *B. bassiana* until 30 days post-inoculation, at the concentration of $7.1x10^2$ CFU g^{-1} in inoculated plants without herbivory and

$1.29x10^2$ CFU g^{-1} inoculated plants with herbivory indicates that the fungus can be possibly found for more extended periods. That corroborates the study of Canassa et al. (2019) that confirmed the endophytic occurrence of two isolates of *B. bassiana* in strawberry leaves 180 days post-inoculation (using the root inoculation method).

The present study shows the indirect effects of *B. bassiana* inoculation in soybean plants against *S. frugiperda*. Decreased leaf consumption, larval weight, and larvae survival were observed after eight days of herbivory, confirming that *B. bassiana* protects the plant from *S. frugiperda*. Less foliar consumption ensures better plant development and lower larvae fitness, assuring a feed deterrence and growth retardation of *S. frugiperda* fed on *B. bassiana*-inoculated plants. Several studies aligned these results with different pests, including *S. frugiperda* (RESQUIN-ROMERO et al., 2016; GARRIDO-JURADO et al., 2017; SÁNCHEZ-RODRIGES et al., 2018; RUSSO et al., 2019a; CANASSA et al., 2019; MWAMBURI. 2021; RUSSO et al., 2021; SINNO et al., 2021; QIN et al., 2021).

This feeding deterrence reported in the present study could result from antibiosis and plant immune system. Few studies detected and quantified metabolites *in planta* (JABER; OWNLEY, 2018). For example, Rassol et al. (2020) identified four flavonoids in *B. bassiana*-inoculated common bean plants (*Phaseolus vulgaris*) after aphid herbivory when compared with control plants, indicating these metabolites were responsible for the aphid feed deterrence. On this study, was increased the emphasis to understand the plant defense by gene expression from main phytohormones pathways and was observed an increased expression of JA/ET-signaling. This occurrence could be associated with production of toxic metabolites by symbiotic association by plant-endophytic fungi and consequently, reduced the herbivory area. Future studies are critical to identify and quantify these metabolites to elucidate the plant-endophytic fungi interaction.

Despite the significant secondary effects on body mass and leaf consumption by *S. frugiperd*a, no differences from instar were observed. This is probably due to the short experimental time; eight days of herbivory. Larvae were kept from the 2nd instar (period of infestation) and were removed at the beginning of the 4th instar.

The experiment which evaluated the development of *S. frugiperda* (2nd instar – pupa) had a lower average survival time (Figure 15) and a reduction in the percentage of individuals who reached the pupa and adult on *B. bassiana*-inoculated treatment when compared with non-inoculated plants. These results are aligned with Russo et al. (2021), showing a reduction in average survival in *S. frugiperd*a after herbivory on *B. bassiana*-inoculated plants. Similar results were found in Noctuidae by Lopez and Sword (2015), that observed a significant difference in survival (2nd instar – adult) of *Helicoverpa zea* feeding on non-inoculated vs. *B. bassiana*-inoculated cotton plants

(20 vs. 16.6 days). Regarding individuals who reached the pupa stage, a higher pupae percentage was obtained in non-inoculated plants (89.6%) compared to those fed on leaves of *B. bassiana*-inoculated plants (59.0%); hence, fewer individuals became adults (52.7%) due to mortality of larvae and pupae in *B. bassiana*-inoculated plants. Since none of the larvae and pupae cadavers had signs of fungi growth, it was assumed this mortality was from the indirect effect of *B. bassiana* colonization. The presence of entomopathogenic fungi as endophytic ensures a decrease in the pest infestation in the present study and as reported for *Macrosiphum euphorbiae* in tomato (SINNO et al., 2021), *S. frugiperda* in corn (RUSSO et al., 2021), *Phthorimaea operculella* in potato (*Solanum tuberosum*) (ZHANG et al., 2022).

Although *S. frugiperda* pupal weight was not statistically different from *B. bassiana*-inoculated plants vs. non-inoculated plants treatments, some pupae in *B. bassiana* treatment with stiff body, smaller and malformed (Figure 17). Thakur et al. (2013) also observed malformation in *S. litura* after herbivory of cauliflower plants inoculated with endophytic fungi *Nigrospora* sp. and *Cladosporium* sp. According to Koul and Ismon (1991), the malformation is associated with juvenile hormonal disturbs affecting the proteins deposit and fat production, which are critical to the metamorphosis, molting, and reproduction process. The secondary metabolites produced by plant-endophytic fungi association could cause malformation in the pupa stage. Following studies could focus on the influence of endophytic fungi from pupae malformation to confirm it, it is necessary.

The adult emergence span (days) was not affected by herbivory in plants inoculated with *B. bassiana* vs. non-inoculated plants; otherwise, the female fecundity was affected. This decreased fecundity observed in *B. bassiana*-inoculated plants in a field situation could lead to fewer individuals from the next generation. Russo et al. (2021) obtained similar results under laboratory conditions, with a reduction of eggs deposited per female, comparing females from *B. bassiana*-colonized plants and non-inoculated plants (495.7 vs. 848 eggs, respectively). Besides fecundity decrease, Russo et al. (2021) observed a reduction in *S. frugiperda* herbivory on plants inoculated with *B. bassiana* compared with control.

The herbivory of *S. frugiperda* in plants inoculated with *B. bassiana* can result in a slower growth rate, and the insect could become more vulnerable to the attack of natural enemies than those with a faster growth rate (CHEN; CHEN, 2018). The deterrence of herbivory, low body weight, decreased survival time and other occurrences reported in this study are due to the plant-fungus symbiotic relationship, with a "powered" induced defense providing high levels of metabolites such as alkaloids, flavonoids, phenols, and hydrogen peroxide against insect herbivory (HOMAYOONZADEH et al., 2022). Also, metabolites from *B. bassiana*, such as bassianin, beauvericin, bassacriadin, oosperein and tenellin, have insecticidal action and influence insect

development (HAMILL et al., 1969; MANYES et al., 2018, QUESADA-MORAGA; VEY, 2004; VINING et al., 2011; BARRA-BUCAREI et al., 2020). The study of KHOURY (2021) confirmed high levels of beauvericin and bassianolide recorded after two days post-inoculation of *B. bassiana*-inoculated common bean plants.

In addition, a stressor agent could trigger the plant immunity system, activating the hormone pathways that liberate a cascade of genes to produce insecticidal substances (PIETERSE et al., 2014). When a plant has a symbiotic association with a biological agent, the plant's immune system triggers, and it can defend itself with more intensity (OWNLEY et al., 2010). The central players are both hormones JA and ET, with an increase when there is a chewing insect and a decrease in SA hormone. This study evaluated the expression of genes from the principal hormone pathways when plants were inoculated with *B. bassiana* and with herbivory of *S. frugiperda*.

The defense response of plants to colonization by *B. bassiana* was examined by few study. This study considered the time, and the evaluation was realized after five, fifteen, and thirty days post-inoculation. The gene *MYC2* had an up-regulation after fifteen days post-inoculation in the treatment *B. bassiana*-inoculated plants with herbivory compared with non-inoculated plants with herbivory. This alteration was observed just in this time with *S. frugiperda* presence.

The Pathogenesis-Related protein *PR3* gene was up-regulated fifteen days after herbivory and was observed an up-regulation on the treatment of *B. bassiana* inoculated plants in contrast with non-inoculated plants. The up-regulation of JA-pathway in the absence of herbivory in *B. bassiana*-endophytic soybean plants may be due to the accumulations of jasmone acid precursors , as observed in the study of Ahmad et al. (2020). In this study, both treatments with herbivory had an up-regulation. This gene alteration was associated with the type of chewing insects, Rehrig et al. (2014) confirmed an increased expression of *PR3* when *A. thaliana* plants suffered herbivory by *S. exigua;* otherwise, the caterpillar *Pieres rapae* did not increase *PR3* expression. Consales et al. (2012) explain this suppression occurs due to the release of oral secretions from *P. rapae*, which defuses the defense, and that this process could vary according to the insect herbivory. Studies about elucidating the elicitor occurrence are interesting, with addition of mechanical damage treatment to confirm the elicitor presence wich activated the gene expression.

The Pathogenesis-Related protein *PR2* gene encodes the protein β-1,3 glucanase, an enzyme associated with SA-signaling, and are activated by biotrophic diseases and sucking insects (PIETERSE et al., 2014). The present study observed downregulation of *PR2* from five and thirty days post-inoculation and a slight alteration at fifteen days post-inoculation. In general, the gene for all the periods had low expression levels, confirming that chewing insects generally do not activate the SA-signaling. Similar results were observed in tomatoes with downregulation of two

Pathogenesis-Related genes (*PRb-1b* and *GluB*) when plants were subjected to herbivory of *S. exigua* and *Spodoptera litura* (KAWAZU et al., 2012).

Many studies have shown that chewing insects trigger ET-signaling of several plant species (BARI; JONES, 2009; DIEZEL et al., 2009; ALJBORY; CHEN, 2018). After the herbivory, the plant rapidly triggers the ET biosystems, and this hormone functions by fine-tuning the JA-signaling (WU; BALDWIN, 2010). Five and fifteen days post-inoculation, both treatments with herbivory had an up-regulation of the ethylene pathway, confirming the plant needs a challenger to recognize the elicitors (HAMPs) or the plant-derived signals (DAMPs) and consequently activate the plant immune system (BOOLER; FELIX, 2009, DIEZEL et al., 2009). The same was observed by Gupta et al. (2022) with slight up-regulation of *ERF*1 five days post-inoculation of *B. bassiana* (foliar and soil drenched) on tomato plants without herbivory.

In this study, was observed a cross-talk from both jasmone acid and salicylic acid pathways, as expected according to the literature, which helped the plant to minimize energy costs and have a flexible signaling network with finely tuned defense response according to the stressor's agent (KOORNNEEF; PIETERSE; 2008). The same was observed in some *B. bassiana* strains tested by Qin et al. (2020). Moreover, the authors observed an increase in the quantity of both stomatal and trichome development which may contribute to protection against insect with a reduction of palatability (SATYABRATA et al., 2021).

The priming phenomenon represents a faster and more robust response to a challenger; was not observed with *S. frugiperda* herbivory in the present study,. Expression was higher in plants inoculated with *B. bassiana* compared with non-inoculated plants at 15 days post-inoculation; otherwise, the values were not expressively higher to consider a primer state. In the JA-signaling at 15 days post-inoculation, a higher upregulation of *MYC2* in *B. bassiana*-inoculated plants after herbivory was observed, confirming a defense from the plant-fungi association when compared to the response from non-inoculated plants with herbivory.

The present study demonstrated a positive interaction between the plant-endophytic fungi, *B. bassiana* (ESALQ PL63), and soybean; when applied just in one leaf, the fungus increased the immune system of soybean plants to *S. frugiperda*, decreasing the pest fitness and affecting the population increase. Field trials are needed to evaluate the impact of this induction system of resistance by the endophytic fungi along the crop cycle. These results suggest an alternative tool for pest management, showing the importance of biological control beyond pests' direct effects.

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