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Le rôle des microorganismes symbiotiques dans la modulation des interactions plantes-insectes

Vers le développement de nouveaux outils et stratégies pour la lutte contre les ravageurs

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Résumé

La réduction de l'utilisation des pesticides synthétiques pour la protection des cultures est essentielle pour améliorer la durabilité écologique et économique de l'agriculture et pour minimiser leur impact sur la santé humaine. Les champignons entomopathogènes (EPF) ont été proposés comme une alternative écologique à la lutte chimique, toutefois, leur efficacité reste limitée par leur sensibilité à des conditions environnementales extrêmes. Cependant, grâce à leur adaptabilité, les champignons entomopathogènes peuvent évoluer dans l'environnement en adoptant différents modes de vie, notamment en tant qu'endophytes des plantes. Ils peuvent ainsi établir des associations symbiotiques mutualistes avec leur plante hôte, favorisant leur croissance et leurs défenses contre divers agents de stress abiotiques et biotiques, y compris les insectes. En tant qu'endophytes, les EPF pourraient surmonter les obstacles traditionnels des champignons entomopathogènes et fournir une nouvelle alternative pour la gestion des insectes nuisibles.

Les mécanismes associés à ce réseau complexe d'interactions entre EPF, plante, insecte nuisible et antagoniste naturel des insectes sont néanmoins encore mal compris. Par conséquent, l'objectif de cette thèse est d'étudier les bases fonctionnelles et moléculaires des interactions multitrophiques impliquant l'EPF *Beauveria bassiana* colonisant les plants de tomates, et le lépidoptère ravageur *Spodoptera littoralis*, responsable d'importants dégâts sur les plants de tomates chaque année.

Dans le premier chapitre de cette étude, des essais biologiques d'alimentation ont démontré le rôle endophytique prometteur de *B. bassiana* dans la réduction de l'immunocompétence des larves de *S. littoralis*, les rendant plus sensibles à d'autres agents de biocontrôle.

Le deuxième chapitre a permis de mieux comprendre les mécanismes sous-jacents à cette interaction. Une approche métabolomique a montré l'effet de la colonisation par *B. bassiana* sur l'activation des réponses systémiques de la plante. De plus, des analyses métagénomiques et métatranscriptomiques ont révélé la contribution du microbiote de l'insecte à l'effet endophytique de *B. bassiana* sur les performances de *S. littoralis*.

Enfin, dans le troisième chapitre, le risque auquel les organismes bénéfiques peuvent être exposés en utilisant des insectes nourris sur des plantes conditionnées par la colonisation endophytique a été déterminé. Ainsi, l'effet ascendant de la colonisation endophytique de *B. bassiana* sur l'endoparasitoïde *M. pulchricornis* a été mis en évidence et a montré une compatibilité prometteuse du champignon dans son rôle d'endophyte avec un autre agent de biocontrôle.

Dans l’ensemble, cette étude suggère que l’endophytisation par *B. bassiana* est un outil prometteur pour réduire l’immunocompétence des Lépidoptères ravageurs. En outre, ces résultats représentent une nouvelle contribution à la compréhension d’interactions dans un complexe multitrophique. L'exploitation de ces connaissances ouvre la voie au développement de nouvelles stratégies de biocontrôle durables au profit de l'agriculture.

Summary

Reducing the use of synthetic pesticides for crop protection is essential to improve the ecological and economic sustainability of agriculture and to minimize their impact on human health. Entomopathogenic fungi (EF) have been proposed as an environmentally friendly alternative to chemical control; however, their effectiveness continues to be limited by their susceptibility to extreme environmental conditions. Nevertheless, EF, thanks to their adaptability, can evolve in the environment through different lifestyles, particularly as plant endophytes. They can thus establish mutualistic symbiotic associations with their plant host, favouring growth and defences against various abiotic and biotic stress agents, including insects. As endophytes, EF could overcome the traditional obstacles of conventional fungal entomopathogens and provide a novel alternative for management of insect pests.

However, the mechanisms associated with this complex network of interactions between EF, plant, insect pest and natural antagonist of insects are still poorly understood. Consequently, the aim of this PhD is to study the functional and molecular basis of multitrophic interactions involving the EF *Beauveria bassiana* colonising tomato plants, and the lepidopteran pest *Spodoptera littoralis*, responsible for significant damage to tomato plants each year.

In the first chapter of this study, feeding bioassays demonstrated the promising endophytic role *B. bassiana* in reducing the immunocompetence of *S. littoralis* larvae, making them more susceptible to other biocontrol agents.

The second chapter provided a better understanding of the mechanisms underlying this interaction. A metabolomic approach showed the effect of *B. bassiana* colonisation on activation of the plant's systemic responses. In addition, metagenomic and metatranscriptomic analysis revealed the contribution of the insect microbiota on the endophytic effect of *B. bassiana* on *S. littoralis* performance.

Finally, in the third chapter, the risk to which beneficial organisms can be exposed using insect feeding on plant conditioned by endophytic colonisation has been determined. Thus, the bottom-up effect of the endophytic colonisation of *B. bassiana* on the endoparasitoid *M. pulchricornis* was studied and showed a promising compatibility of the fungus in its role as endophyte with the other bio control agent.

Overall, this study suggests that endophytisation by *B. bassiana* is a promising tool for reducing the immunocompetence of Lepidopteran pests. In addition, these results represent a new contribution in the understanding of interaction in a multitrophic complex. The exploitation of such knowledge paves the way for the development of a new sustainable biocontrol strategies for the benefits of agriculture.

General Introduction

1. Issues and challenges in agriculture

Feeding the world's entire population is one of the greatest global challenges of the 21st century (Valoppi et al., 2021). Estimates suggest that the global human population will increase by 25% over the next 30 years, reaching 10 billion by 2050. This growth is exponential and, inevitably, to support such a demand, a drastic increase in the food production is required, expecting an increase of 70% by 2050 (Tripathi et al., 2019).

Agriculture is an essential pillar of economic and social development. As well as being a major source of fibre and fuel, it is above all the main source of food supply for human beings, directly or indirectly through animal feed (Abdalla and El-Ramady, 2022). The issues and challenges facing the agricultural sector are therefore very high in the years to come. It has a decisive role to play in meeting the global demand of food increase.
However, agricultural production suffers significant losses due to the abiotic and biotic stress agents. In addition to challenging environmental conditions imposed by climate change, agriculture must contend with herbivores, pathogens, and weeds, all of which significantly reduce production yields (Oerke, 2006). Globally, it is estimated that up to 40% of harvests are lost every year to pests and diseases (FAO, 2022). Of these 40%, insects account for a significant proportion of losses, either through direct damage or indirectly as disease vectors. Such losses contribute to food insecurity (Douglas, 2018). Hence, reducing the impact of insect pests is a key priority.

Pest control is a very ancient practice. The first written records of the use of pesticides date back to 2500 BC, when the Sumerians used sulphur compounds to protect themselves against insects (Perera and Dulmini, 2020). In ancient Egypt, the oldest medical document dating from around 1550 BC mentions hundreds of recipes for insecticides. Around 200 BC, arsenic-based products were used in China to protect seeds against parasites. Century after century, progress continued and, in the 18th and 19th centuries, modern fungicides, herbicides and insecticides were developed. But from the end of the Second World War, agriculture in the world, particularly in the northern hemisphere, intensified enormously to meet growing demand and because of the scientific and technical progress made since the First World War, linked in particular to research into chemical weapons (Bertomeu-Sánchez, 2019). This technological leap in agriculture continued, thanks also to varietal selection, mechanisation and irrigation, and reach a peak between 1960s to the 1990s, named the ‘Green Revolution’. These years were marked by the massive use of inputs, particularly synthetic pesticides. The result was a spectacular increase in agricultural production. Due to their effectiveness and ease of spraying, synthetic pesticides were used on a large scale and with great frequency to protect crops from damage caused by pests (Ngegba et al., 2022).
At the same time, however, a general awareness of the undesirable effects of chemical pesticides and fertilisers on the environment and human health increasingly arose. Although their positive effects on pest control and plant growth were obvious, their indiscriminate use and persistence

in nature caused environmental degradation, leading to widespread pollution, soil erosion, salinisation and significant eutrophication (Savci, 2012; Mahanty et al., 2017). They are also responsible for ozone depletion (Deepa et al., 2011). In addition, they lead to a loss of biodiversity, cause damage to non-target organisms (Hallmann et al., 2017) and generate resistance in pests (Hawkins et al., 2019). Finally, their effects on human health are very harmful, particularly by contaminating foodstuffs, soil and bodies of water, as well as direct exposure of farmers who spray them. According to the World Health Organisation, 200,000 people die every year worldwide because of pesticides (Belmain et al., 2013).
So, despite the short-term benefits of intensive farming practices on crop yields, their abuse has a profoundly negative long-term impact on the environment (Imfeld and Vuilleumier, 2012). But the high production and profitability that these synthetic inputs allow make their use unfortunately still very high today (Figure 1).

The issues and challenges linked to this sector are therefore becoming increasingly important, because the agricultural practices adopted to increase crop productivity have an impact on biodiversity and natural environments.
This is how the term 'sustainability' is now widely used to describe agriculture (Pretty, 2008). Sustainable agriculture is defined as agriculture that: "meets the food needs of a growing human population, while ensuring minimal impact on the environment and the population as well as profitability for producers (Velten et al., 2015)". It takes account of environmental, economic and social issues.

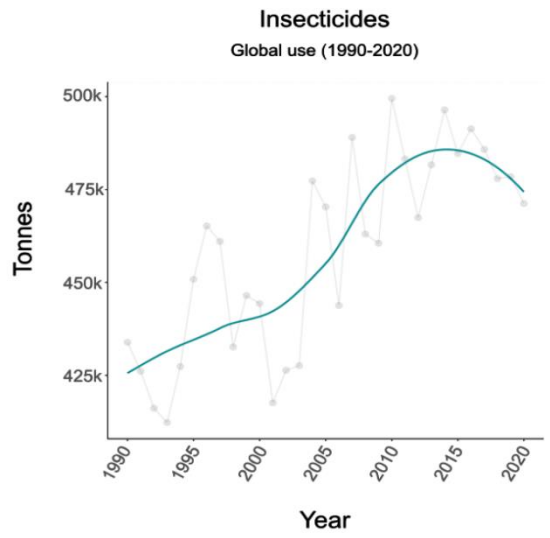


Figure 1: Global use of insecticides since 1990 to 2020 according to FAO. (from Ramirez Serrano 2023, PhD thesis). Data are available in https://www.fao.org/faostat/

Governments in some countries are taking regular action to move towards sustainable agriculture. For example, current legal regulations in the European Union, such as the EU Green Deal, impose a significant reduction of pesticide use in agriculture. Integrated pest management (IPM) programmes are being put in place, involving healthy crop growth with minimal disruption to agro ecosystems, while promoting natural pest control methods (FAO, 2020). If it is not possible to stop using chemical pesticides completely, the aim is to reduce their use to at least a level that is biologically, socially and economically sustainable. However, implementing

such a programme requires the development of new innovative technologies and the introduction of biotechnological tools and strategies alternative to chemical inputs. In this context, practices such as organic crop fertilisation, crop rotations, exploiting the landscape surrounding crops or reducing tillage emerged several decades ago and continue to develop today (Wezel, 2014). Research is focusing increasingly on the development of environmentally friendly methods, with particular emphasis on biological control (Galli et al., 2024).

For more than 400 million years, plants and insects have coexisted and constantly evolved to circumvent plant defences from the insect side, and to reduce herbivory from the plant point of view (Sugio et al., 2015). In addition, both insects and plants are involved in associations with microorganisms that closely interact with both of them, influencing their interaction and the resulting fitness (Frago et al., 2012).
Furthermore, as suggested by Bhattacharyya et al. in 2016, an ideal agricultural system, in addition to producing sufficient resources autonomously and without affecting biodiversity, requires particular attention to nutrient management, energy recycling and preservation of the environment's natural resources, functions in which microorganisms play an essential role in nature. They are therefore fundamental to the proper functioning of ecosystems.

For all these reasons, microorganisms are a formidable source of biotechnological applications for sustainable agriculture. As part of the development of sustainable and effective strategies for crop production, I am going to focus on microorganisms and the promising alternative to chemical controls that they offer.

2. Plants symbiotic microorganisms

Plants form intimate and complex associations, both beneficial and antagonistic, with different communities of microorganisms, such as bacteria, fungi, archaea, viruses and protists (Selosse et al., 2004). All plant-related microenvironments, particularly the rhizosphere, are colonised by microorganisms. Over the last few decades, research has consistently demonstrated the range of beneficial functions that some of these microorganisms, particularly bacteria and fungi, play in the development and health of plants. They are able to promote plant growth and the availability and absorption of nutrients. In addition, although plants have evolved their own defences to resist pathogens and pests, they also depend on these microbial partners to combat the various abiotic and biotic stresses to which they are subjected, either directly or by a priming of their defences (Berg et al., 2009). Obviously, this exchange is not unidirectional, and the host plant also provides metabolic benefits to its microbial associates (Thrall et al., 2007). Understanding and then exploiting these interactions and the functions of the symbionts associated with plants is a major challenge for sustainable agriculture and for reducing the use of chemical inputs.

Among the best-known and most effective types of beneficial microorganisms associated with plants promoting their growth and their resistance to various stressors such as diseases and

phytophagous insects, arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria are the most studied.

Arbuscular mycorrhizal fungi are obligate symbionts that form a mutualistic association with plant roots (Douds and Millner, 1999). These associations are widespread in the environment, including many agricultural crops, and provide numerous benefits to the host plants, such as improved nutrition, increased resistance to soil-borne parasites and pathogens and improved resistance to abiotic stresses (Pozo et al., 2002, Gosling et al., 2006). Their effects on the nutritional value of plants also affect the performance of herbivorous insects (Ramirez-Serrano et al., 2022).

Plant growth-promoting rhizobacteria (PGPR) refers to free-living bacteria in the rhizosphere that improve plant growth directly or indirectly through various mechanisms (Kloepper et al., 1980). Their ability to fix nitrogen, solubilise phosphorus, decrease ethylene concentration or produce phytohormones such as auxins, cytokinins and gibberellins stimulates plant growth (Saranraj, 2014). Their ability to modulate the endogenous level of phytohormones is also crucial in the response of plants to biotic and abiotic stresses (Khan et al., 2020).

Over the last few decades, there has been increasing evidence that endophytic beneficial microorganisms also have important effects on the fitness of their host plant (Yan et al., 2019; Khare et al., 2018). While some microorganisms establish external interactions with plants, endophytic microorganisms colonise and live in tissues of their host plant without causing visible symptoms of infection or disease (Orozco-Mosqueda and Santoyo, 2021). These microorganisms, primarily fungi and bacteria, are hosted by almost all plant species found in nature (Nair and Padmavathy, 2014). They reside and proliferate in all plant tissues such as stems, roots, seeds, fruits and leaves (Chaudhary et al., 2022). In addition, in exchange for protective niches provided by their host, they play an important role in enhancing their host plant fitness by improving nutrient uptake, synthesizing phytohormones and increasing resistance to biotic and abiotic stresses (Figure 2). Given their many benefits to plants, endophytic microorganisms represent a viable alternative to synthetic pesticides, which pose challenges in conventional agriculture (Watts et al., 2023).

The sections below will focus on endophytic microorganisms, with special emphasis on their benefits in promoting plant growth and defence against biotic stress, the latter being the focus of my PhD.

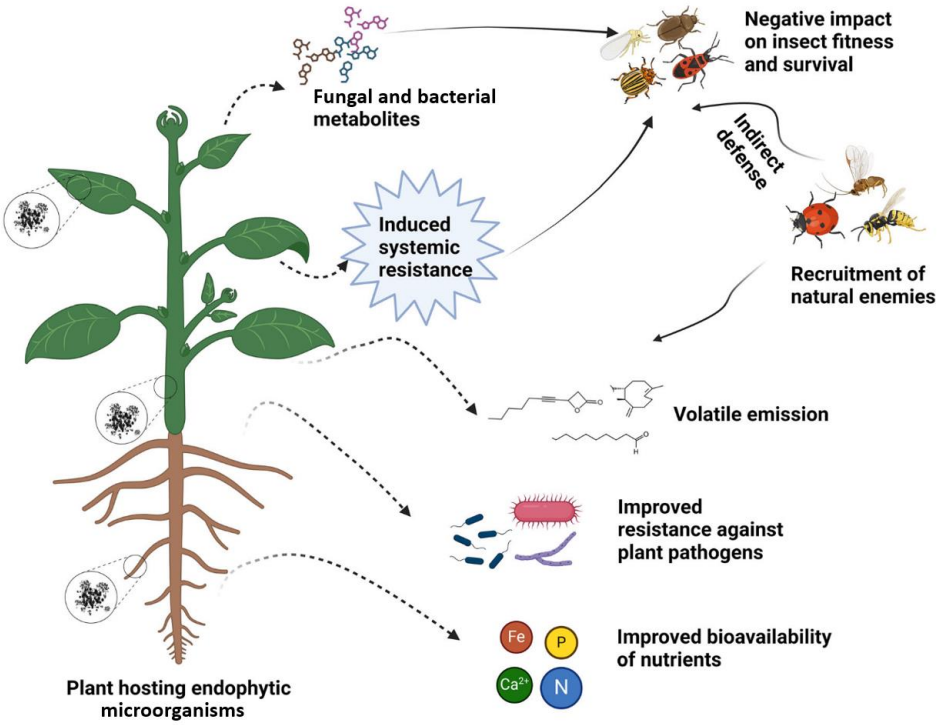


Figure 2: Interaction between plants and endophytic microorganisms. *Endophytes provides benefits to host plants. Fungal and bacterial metabolites and induced systemic resistance enhance the plant's defense mechanisms against pests and pathogens. Volatile compounds emitted by the plant induced by endophytic colonisation attract natural enemies of insect pests. Additionally, microorganisms' colonisation improves the bioavailability of essential nutrients such as iron for the plant (Panwar et al., 2024 adapted).*

2.1. Promotion of plant growth by endophytic microorganisms

An increasing number of studies have demonstrated the ability of several endophytic microorganisms, fungal or bacterial, to promote plant growth after establishment in the host plant (Lopez and Sword, 2015; Jaber and Enkerli, 2017; Sinno et al., 2021). It is now clear that a small change in diversity in plant endophytic communities can have a significant effect on the regulation of host growth (Vandenkoornhuyse et al., 2015). Thus, endophytes constitute promising microbial resources that mimic the effect of fertilisers, by modulating the physiological pathways underlying plant growth (Chaudhary et al., 2022).
I will review some of the mechanisms that may explain this promotion of plant growth by endophytic microorganisms.

First, many endophytes can produce phytohormones involved in plant growth. For instance, the indole Acetic Acid (IAA) production by endophytes is considered an important factor in plant growth regulation. In 2014, Khan et al. reported that the endophytic proteobacterium *Sphingomonas sp*. (endophyte) found in the foliar region of *Tephrosia apollinea* improved the growth of tomato plants via the action of IAA. Other phytohormones involved in plant growth include gibberellins, indole acetamide (IAM), cytokinins and ethylene (De Hita et al., 2020; Hamayun et al., 2017). Beside the production of phytohormones, microorganism colonisation can modulate phytohormones synthesis by the plant.

As an alternative mechanism, some endophytes can regulate the supply of vital plant nutrients. For example, *Gluconacetobacter diazotrophicus*, *Herbasprillum rubrisubalbicans* and *Burkholderia silvantantica* are bacteria that fix nitrogen, an essential macronutrient for plant growth, in the intercellular spaces of sugarcane stems (Lery et al., 2011). Phosphate is a nutrient required by plants to increase their productivity, but it is a limiting factor in the soil. Bacteria such as *Pseudomonas* or *Bacillus* can mobilise phosphorus by mineralisation and solubilisation (Kang et al., 2014). Moreover, inoculating plants with fungal entomopathogens induces the uptake of proteins linked to photosynthesis and energy metabolism, promoting plant growth (Gómez-Vidal et al., 2009). By increasing the supply of nutrients, endophytes enable plants to better combat certain abiotic stresses involving soils deficient in certain minerals.

Endophytes can also produce siderophores, compounds capable of capturing iron and making it available to plants, particularly in deficiency conditions (Rajkumar et al., 2010).

It is important to note that the ability of endophytic microorganisms to promote plant growth depends on the method of inoculation into the plants. For instance, inoculation by foliar spray did not show the same plant growth promotion efficacy for some fungal endophytes compared to inoculation directly through seeds (Jaber and Enkerli, 2017).
Because data focus on a few species and because the underlying mechanisms are still poorly understood, further research is needed to measure the importance of fungal and bacterial endophytes for their application in field conditions in plant growth (Chaudhary et al., 2022).

2.2. The role of endophytic microorganisms in protecting plants from biotic stresses

Cultivated plants are exposed to various biotic and abiotic stress agents during their development, which limit their productivity. While abiotic stress refers to environmental conditions (drought, excess water, extreme temperatures, mineral deficiencies), biotic stress is due to attacks by plant pathogens - such as bacteria, viruses, fungi, nematodes - or herbivorous organisms such as mammals, insects and mites, causing major crop losses (Pandey et al., 2017). As sessile organisms, plants have developed a series of physical and chemical defence barriers, that can be modulated and enhanced by associated microbial symbionts, such as, among others, beneficial endophytes (Chaudhary, 2022).

First, certain pathogens that cause many plant diseases depend on plant resources to develop. However, by already residing in the tissues of their host plant, beneficial endophytes can prevent the development of these pathogens by competing for plant resources from which they also benefit. For example, competition for space and nutrition occurs against fungus *Rhizoctonia solani* and the oomycete *Plasmopara viticola* (Jaber, 2015) in plants colonised by the endophytic fungus *B. bassiana*.

Second, endophytes have also been reported to produce numerous lytic enzymes, such as chitinase, cellulase, proteases, hemicellulases and amylase (Dutta et al., 2014), which are essential for their colonisation in host cells. These lytic enzymes are very interesting in terms

of pathogen control because they enable pathogens cell walls to be degraded (Cao et al., 2009). For example, the fungal cell wall, mainly composed of polysaccharides, can be damaged by the action of these enzymes (Mishra and Arora, 2012).

Third, another mechanism by which endophytes provide protection against plant pests is the production of secondary metabolites, compounds which play a major role in the adaptation of plants to their environment and are often responsible for protection against pests and pathogens. For example, certain endophytic fungi produce alkaloids, which are responsible for reducing herbivore feeding and virus transmission (Bultman et al., 1997). The fungus *B. bassiana* is known to produce certain metabolites such as beauvericin, which has antimicrobial properties (Ávila-Hernández et al., 2020). Endophytes can also stimulate the production of secondary metabolites by plants. For example, Ahmed et al. (2000) demonstrated that the presence of *Trichoderma harzianum* in pepper plants induced a seven-fold higher concentration of the terpenoid phytoalexin capsidiol than in control plants and was therefore considered to be one of the factors responsible for the delay in the development of damage caused by *Phytophthora capsici* Leonian (Oomycota: Peronosporales).

In addition, the presence of endophytes can lead to a change in the quality and quantity of volatile organic compounds (VOCs) released by the plant, as demonstrated by the presence of *Trichoderma longibrachiatum* MK1 in tomato plants (Battaglia et al., 2013). Such a modification can modulate the attractiveness of insects to plants and recruit natural enemies of antagonists (Salazar-Mendoza et al., 2024).

Endophytes induce a modulation of the plant's defences, protecting it from many insect pests and pathogens (Pieterse et al., 2014). They have been shown to elicit induced systemic resistance (ISR) in plants. For example, colonisation of date palms by *Lecanicillium spp*. fungi resulted in the up-regulation of proteins involved in plant defence and stress response (Gómez-Vidal et al., 2009).
In fact, endophytes induce the priming status of plant defences, enabling them to activate their resistance to pathogens, insects or abiotic stresses more quickly and more strongly (Conrath et al., 2006). The induced resistance activated by endophytic colonisation is triggered by phytohormones such as jasmonic acid, ethylene and salicylic acid, which regulate the expression of certain genes and the production of certain compounds such as flavonoids or polyphenols, thereby immunising the plant against future attacks (Leon-Reyes et al., 2009).

Finally, bacterial and fungal endophytes are able to confer additional resistance to their host plant by modifying some of their chemical and physical characteristics. For example, callose is a compound that protects plant tissue from pathogen attack by being deposited between the cell wall and the plasma membrane. Some endophytes have been shown to increase these callose deposits, limiting pathogen infestations (Mishra et al., 2018). Other endophytes, such as the leaf fungus *Colletotrichum*, increase the expression of genes linked to the deposition of cellulose and lignin in *Theobroma cocoa*, protecting it from diseases caused by *Phytophthora spp*. (Mejía et al., 2014).

3. Insect – Symbiont Interactions

It is now widely accepted that not only plants but also most arthropods interact with microorganisms in prolonged and intimate associations known as symbiosis. Insects host a multitude of heritable bacteria whose presence affects their ecology and evolution, either by manipulating their reproduction or by offering them clear advantages in terms of fitness (Brownlie and Johnson, 2009). If insects constitute one of the most diverse and almost ubiquitous groups of living organisms, it is partly thanks to these associations (McFall-Ngai et al., 2013).

While most studies focus on bacterial communities, insects are associated with a wide variety of microorganisms, such as fungi, viruses, protozoa and archaea, which, like bacteria, have a significant influence on the insect life (Gurung et al., 2019).

3.1. Obligatory and optional interactions

Some of the associations between insects and symbionts are described as obligatory (or primary) because they are essential for the reproduction and survival of their host. For example, many insects feed only on substrates that are deficient in certain nutrients, such as plant sap, and their endosymbionts provide them with the missing nutrients that are essential for their survival (Douglas et al.,1998). The association between aphids and the intracellular bacterium *Buchnera aphidicola* is the most famous example (Douglas et al., 1998). Similarly, the TseTsé fly harbours *Wigglesworthia glossinidia*, which is essential for supplying vitamins missing from its diet (Kame-Ngasse et al., 2018).

In addition to these obligate endosymbionts, insects have various associations with facultative (or secondary) endosymbionts, which are not essential to the survival of their host but can confer many advantages under specific ecological conditions. Indeed, as bacteria often possess pathogenicity genes and can produce various bioactive compounds that can function as toxins (Hansen et al., 2012), insects can benefit from new defensive properties provided by their bacterial partners. As well as being transmitted vertically by the mother, these facultative endosymbionts are occasionally transmitted horizontally (Ahmed et al., 2013), acting as mobile elements capable of protecting insect populations from their natural enemies or increasing their ecological niche through the benefits they provide (Oliver et al., 2010). For example, numerous studies have determined the beneficial role of certain facultative endosymbionts in aphids, providing them with adaptation to new host plants (Leonardo and Muiru, 2003; Tsuchida et al., 2004), tolerance to heat stress (Chen et al., 2000; Russell and Moran 2006) and protection against parasitoid wasps (Oliver et al., 2003) or pathogenic viruses (Higashi et al., 2022). Certain endosymbionts also play a role in the formation of the cuticle of their hosts in beetles, improving their performance in dry environments (Vigneron et al., 2014). Hosting these facultative endosymbionts involves maintenance costs for the host, and some even induce costs following the release of toxins. Their impact is therefore established along a parasitism-

mutualism continuum and varies according to environmental conditions (Oliver et al., 2008; Vorburger et al., 2013; Zytynska et al., 2021).
On their side, the symbionts benefit from access to nutritional resources and a stable, protected environment within the host insect (Douglas et al., 2014).

Reproductive manipulators are another category of facultative endosymbionts. Briefly, they are parasites that increase the reproduction of female hosts to the detriment of males using various strategies to increase their spread in the population. This is achieved through reproductive incompatibility between infected and non-infected individuals, elimination of male offspring, feminisation of males or manipulation of the mode of reproduction towards parthenogenesis. The *Wolbachia pipientis* bacterium is widespread in insects and is widely known to manipulate their reproduction through these different strategies (Werren et al., 2008).

3.2. Localisation of microorganisms in their host

Throughout their life cycle, insects establish more or less stable and lasting interactions with microbial populations. Some are defined as resident, due to the high stability of their association with their host. They replicate within their host at a rate greater than the losses linked to their death or excretion. Conversely, others are considered transient, only "passing through" their host (Hammer et al., 2019). Depending on the complexity of the association between microorganisms and their hosts, the function of these microbial populations and their transmission mechanisms, they can be found at different levels of their host, from its surface to the interior of its cells.
Both obligate and facultative symbionts can establish intra- or extracellular lifestyles (Masson and Lemaitre, 2020).

Obligate bacterial symbionts are generally found in derived cells called bacteriocytes within bacteriomes, specialized organs of their host (Sugio et al., 2015; Oliver et al., 2010). These obligate endosymbionts have been associated with their host for millions of years, co-speciating with it through faithful maternal transmission, which explains their specific localisation (Moran et al., 2008).

Some insects also establish external mutualistic interactions with symbionts, usually fungi living outside their insect host. *Macrotermitinae*, for example, form an obligate symbiosis with which *Termitomyces* fungi they have domesticated to obtain nutrients necessary for their survival present in wood that they cannot digest themselves (Rouland-Lefevre et al., 2002).

Optional symbionts may occupy different parts of the host body, such as secondary bacteriocytes or sheath cells at the periphery of primary bacteriocytes (Sugio et al., 2015). Reproductive manipulative bacteria such as *Wolbachia* and *Spiroplasma*, meanwhile, infect insect reproductive organs (Engelstadter and Hurst, 2009).
Microorganisms can also colonise the salivary glands of their insect hosts, modifying the composition of their saliva and modulating the interactions between the insect and the plants

with which they interact, in terms of nutrition and plant-transmitted diseases (Wang et al., 2020).
Once considered a hostile environment for microorganisms, it is now accepted that various microorganisms are sheltered in the hemolymph of their host, either stably or transiently. The costs of housing these microorganisms (Vorburger and Gouskov, 2011) are offset by the benefits these organisms provide to their host. For example, the endosymbiont *Hamiltonella defensa* induces defence mechanisms in the hemolymph of certain aphids through toxin production, protecting them from parasitic wasps (Oliver et al., 2010). This cost-benefit balance is controlled by a fine-tuning of bacteria level through the host immune system (Blow et al., 2019).

Finally, many microorganisms' associates are also hosted in different niches of the insect gut, playing a fundamental role in the development, physiology and immunity of their host (Dillon and Dillon, 2004). The insect gut hosts a wide diversity of microorganisms that depends on their diet (Sugio et al., 2015). This diversity is also strongly shaped by the evolutionary history of the host (Sugio et al., 2015). It is important to be aware that the structure of the gut and its microbial assemblage is of different complexity in different groups of insects (Engel and Moran, 2013). For example, the termite is the insect with the most complex intestinal structure and associated microbiota (Brune and Dietrich, 2015). Conversely, that of Lepidoptera is one of the simplest (Chen et al., 2016). Whether complex or not, it is clear that the intestinal microbiota brings beneficial effects to their host, contributing in particular to their nutrition, physiology, protection against pathogens or the balance of their immunity (Engel and Moran, 2013). In the following paragraph, I will list some of its main functions.

3.3. Gut microbiota: functions and prospects for agriculture

If insects have been able to adapt to a wide variety of ecological niches, some with very nutrient-poor diets, it is thanks to the nutritional symbiosis they operate with their gut microbial associates (Engel and Moran, 2013). Indeed, although direct supplementation of essential nutrients deficient in insect diets is generally provided by intracellular endosymbionts, some gut microorganisms contribute to their supply. In addition, some bacteria that pass through the gut can simply be digested, serving as an additional nutrient supply (Engel and Moran, 2013).
Even more specific nutritional functions may be fulfilled by these microorganisms. For example, in wood-eating insects, host intestinal microbial communities are involved in breaking down cellulose into simpler sugar residues that can be absorbed (Anand et al., 2010). Pollen-feeding insects, on the other hand, harbour microorganisms producing enzymes capable of acting on the pollen wall to release the nutrient-rich content (Ariizumi and Toriyama, 2011).

In addition, insects can feed on plants containing specialised toxic metabolites. This detoxification ability is partly attributed to the insect's gut microbiome and the ability of certain bacteria to neutralise these toxic compounds. For example, the coffee berry borer spends its entire life cycle in alkaloid-rich coffee beans. The bacterium *Pseudomonas fulva* was found to possess a caffeine demethylase gene, giving the insect the ability to degrade caffeine (Ceja-

Navarro et al., 2015). Similarly, insects can resist certain pesticides through the detoxifying action of their intestinal microbiota (Siddiqui et al., 2022).

Moreover, there is a complex interaction between the intestinal microbiota and the modulation of the immune response in insects, which is essential for host resistance to certain pathogens. For example, Upfold et al. (2023) showed that *Galleria mellonella* larvae reared in axenic conditions were more vulnerable to infection by *Bacillus thuringiensis*, and that certain *Enterococcus* species, can stimulate the gut immune response.

In addition to priming insect immunity, gut microbes can increase their host's resistance to parasitic invasions through competition, either nutritional or through antagonistic effects and niche occupation (Engel and Moran, 2013). This confirms that species-rich intestinal communities are more resistant to invasions, due to a higher competitive load for niche colonisation and nutrition. For instance, an inverse relationship between the diversity of the intestinal community and the success of colonisation by the pathogen *Serratia marcescens* was observed in *Schistocerca gregaria* by Dillon et al. (2005).

All these functions demonstrate the importance of the intestinal microbiota in the adaptation of insects to their environment. These microbial associates play an essential role in their nutrition, their resistance to pathogens and pesticides and the modulation of their immune response. It is therefore vital to consider the gut microbiota of insects when developing new pest control measures.

4. Multitrophic Network: exploitation for the benefit of agriculture

Plants are a central nutritional resource within ecosystems, forming part of a complex network of multitrophic interactions (Gruden et al., 2020, Biere et al., 2013; figure 3). Plants interact with phytophagous insects, often to their detriment, with obvious harmful consequences for agricultural production (Bruce et al., 2015). In parallel, plants also interact with microorganisms belonging to different kingdoms such as fungi, bacteria or viruses. Some of these microorganisms are antagonistic, while others are beneficial. Plants establish mutualistic symbioses with the latter, gaining advantages in terms of nutrition and resistance against soil pathogens or phytophagous insects (Ortiz and Sansinenea, 2022). Thus, plants serve as hosts and essential resources for these organisms, enabling interactions that can influence each other, either simultaneously or sequentially, via the plant (Di Lelio et al., 2022; Gruden et al., 2020). Consequently, plants become the focus of competition among these organisms sharing a common host. Although such interactions—especially those involving distant organisms from different kingdoms—are widespread, they remain poorly understood. (Hochberg et al., 1990).

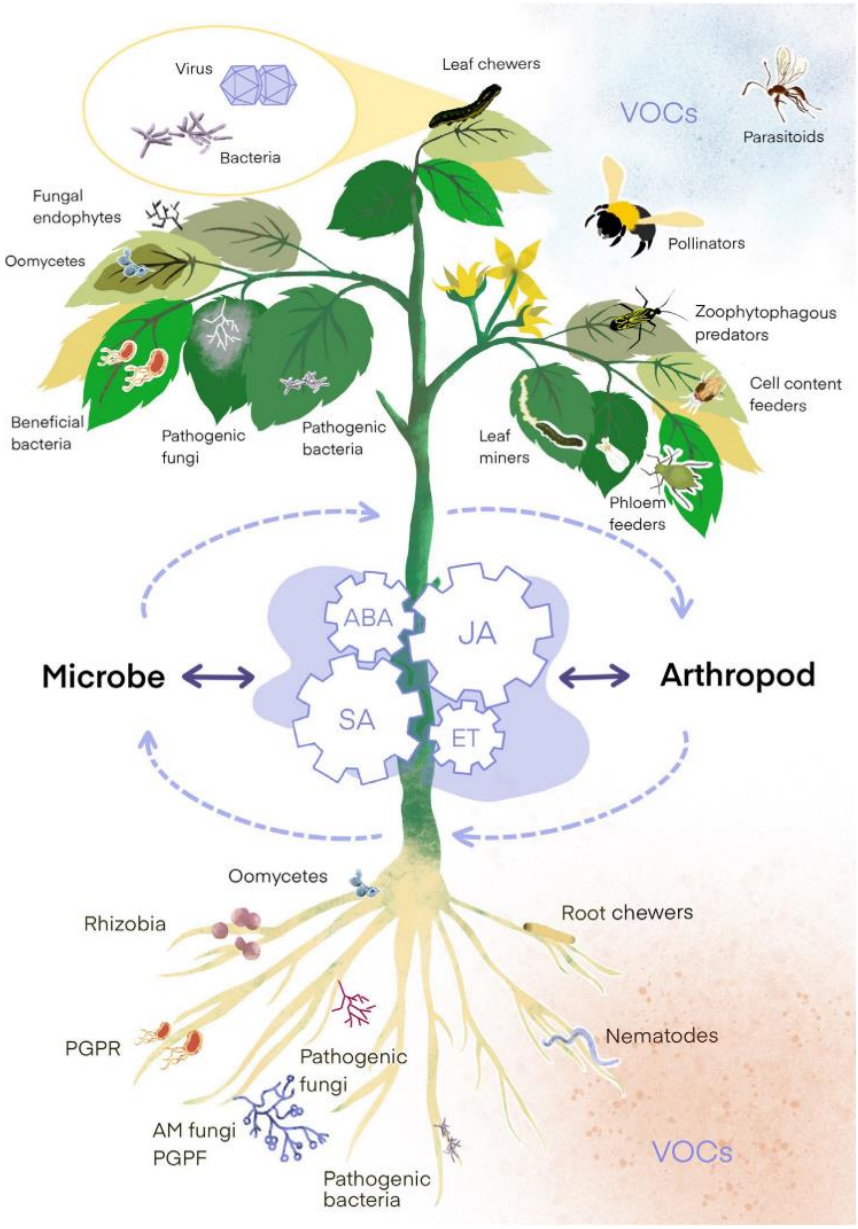


Figure 3: *Illustration of Multiway Interactions between Plants, Microbes, and Arthropods (PMA) and the Main Signalling Pathways Orchestrating the Corresponding Plant Responses (Gruden et al., 2022).*

A further layer of complexity is given by the microorganisms associated with insects interacting with plants. Indeed, the health and development of insects is modulated by their microbiota, which collectively form what is known as the holobiont (Engel and Moran, 2013). Thus, understanding the dynamics of these interactions and associated processes is key to shedding light on how ecosystems function, and to developing effective and sustainable strategies for their protection against their pathogens and pests. In the context of crop protection against insect pests, understanding and exploiting these multitrophic networks is essential for the development of new integrated pest management tools that are both effective and risk-free for non-target organisms and beneficial for the environment.

In this context, beneficial endophytic micro-organisms are of promising interest in agriculture because of the beneficial effects they have on the plant. Recent studies have highlighted the role

of entomopathogenic fungi as endophytes. In addition to their direct ability to infect insects, it has indeed been shown that some entomopathogens can live in mutualistic associations with host plants (Vega et al., 2018). Microorganisms are therefore capable of evolving through different strategies in the environment via different lifestyles. Understanding and exploiting these different strategies should lead to a better understanding of these multitrophic interactions and the development of effective tools for the benefit of agriculture.

Objectives

Entomopathogenic fungi can adopt endophytic lifestyle, developing in a mutualistic association with their host plants (Vega, 2018). Numerous studies have described the beneficial effects associated with such an association such as promoting host plant growth and defences against various stressors including phytophagous insects (Jaber and Ownley, 2018; Sinno et al., 2020). Although the effects of this association on insect performance are increasingly documented, little is known about the mechanisms associated with this complex network of interactions between microorganisms, plant, insect pest and natural antagonist of insects. The physiological changes induced in plants by the colonisation of entomopathogenic fungi are still poorly understood. In particular, the bottom-up effects mediated by the host plant—such as the impact on the structure and composition of midgut bacterial communities in insects feeding on these plants—remain to be elucidated. These changes can, in turn, influence insect performance. Gaining a deeper understanding of these processes, with their ecological, evolutionary and application implications, will pave the way for the development of new, more sustainable biocontrol strategies for the benefits of agriculture.

The general objective of my PhD thesis is to study the impact of endophytic entomopathogenic microorganisms on phytophagous insects and their associated bacterial communities, through changes induced in the physiology of the host plant. Ultimately, the aim is to use this knowledge to develop new, effective and sustainable tools and strategies for controlling insect pests.
This study can be divided into three main points: (i) Determine the impact of endophytic entomopathogenic microorganisms on insect performance, (ii) Understand the mechanisms associated with these interactions, (iii) Assess the effects associated with the endophytic role of these entomopathogenic microorganisms on non-target organisms, in particular biocontrol agents. More specifically, the sub-objectives of the thesis are as follows:

1. To explore the effects of plant inoculation by an entomopathogenic fungus on the performance and immunity of a generalist Lepidopteran chewing pest.
2. To assess the effects of plant inoculation by an entomopathogenic fungus on plant signalling pathways and nutritional value.
3. To determine how the entomopathogenic fungus inoculation affect the abundance, diversity and activity of Lepidopteran pest midgut microbiota.
4. To evaluate the compatibility between the use of an entomopathogenic fungus as an endophyte and natural antagonist of the Lepidopteran chewing pest fed on the colonised plant.

To achieve these objectives, crop model, endophytic entomopathogenic fungus and insect pest used in this work have been selected based on their relevance in agronomy.

Plant

Tomato (*Solanum lycopersicum*) is one of the most important consumed vegetable products in the world. Currently, more than 150 million tons of tomatoes are produced annually worldwide (FAO, 2022). However, it is the target of a multitude herbivorous insects feeding on it and causing huge yield losses every year. It is considered as a model plant for studying plant defence responses to pathogenic micro-organisms and insects, amplifying its relevance to this study.

Insect

Many of the most important crop pests belong to the order of Lepidopteran, displaying the chewing feeding habit with their voracious larvae (Douglas, 2018). They are the source of economic losses and food insecurity for the human population. Thus, many practices are implemented to control them such as prophylactic measures, the use of chemical insecticides, biopesticides and biological control (Khan and Ahmad, 2015). Sustainable strategies are being researched to improve the control of these insects, and to develop IPMs against them. Especially as it is an insect order for whose resistance to pesticide has regularly been mentioned.

Spodoptera littoralis (Boisduval), the Egyptian cotton leafworm, is a major pest of subtropical and tropical agricultural crops, widely found all around Mediterranean Sea. Its larvae have a broad host range, which includes a number of economically important crops, belonging to dozens of different families, including legumes, crucifers, grasses, and deciduous fruit trees. In tomatoes, larvae feed on leaves and drill also into the fruit (Maish, 2019). In addition, the pest can be active for most part of the year, and females are able to lays hundreds of eggs during their life. At a temperature of 25°C, eggs hatch in about 3-4 days, the larval development goes through 6 instars, over about twenty days. The pupal period is spent in the soil and lasts about 11–13 days at 25°C. Biological parameters of this pests, such as longevity and development are affected by the host plants.

Microorganism

Beauveria bassiana plays a key role in the management of many arthropod pests in agriculture, veterinary medicine and forestry. Able to infect a broad spectrum of insects, it is generally applied by spray application of biopesticides formulations. It is produced by obtaining hydrophobic aerial conidia, the main active ingredient in these mycoinsecticides (Mascarin and Jaronski, 2016).

Chapter 1 - Plant-mediated effect of the entomopathogenic fungus *Beauveria bassiana* on the survival, development and immunity of the Lepidopteran pest *Spodoptera littoralis*

1. Introduction

In response to the problem posed by synthetic pesticides for the environment and human health, several integrated pest control strategies have been developed in recent decades to mitigate the impact of herbivorous insect pest, including the use of biological control methods (Hossain et al., 2017). Biological control of phytophagous insects involves the use of their natural enemies such as parasitoids, predators or plants and entomopathogenic microbial agents with the aim of limiting the pest population at levels below economically and ecologically damaging threshold (Eilenberg et al., 2001).

In this context, entomopathogenic fungi (EPF) are widely used as biocontrol agents due to their effectiveness in increasing insect pest mortality (Khan et al., 2012). EPF are ubiquitous organisms contributing to the natural regulation of insect populations by infecting and growing within them (Khan and Ahmad, 2015). They constitute a phylogenetically diverse group, comprising unicellular or multicellular (filamentous) eukaryotic heterotrophic organisms that can reproduce sexually and/or asexually. While they are best known for infecting arthropod carcasses, their primary natural habitat is soil (Meyling, 2007). Given that many insects spend part of their life cycle in the soil, it serves as an ideal environment for fungal infections in insects (Khan and Ahmad, 2015).

Unlike bacteria and viruses, which must be ingested to infect insects, contact between fungal conidia and the host cuticle is sufficient to establish fungal infection. Once in contact, fungi secrete extracellular enzymes capable of degrading insect cuticle's structural components, allowing hyphae to penetrate and proliferate in the host's haemolymph (Srisukchayakul et al., 2005). In addition to these exo-enzymes, EPF produce toxins. The fungus then grows throughout the hemocoele by budding, eventually leading to the emergence of mycelia from the cuticle, the production of conidiophores, and the death of the insect caused to vegetative growth and the action of the toxins (Freimoser et al., 2003). Finally, the fungus pursues saprophytic development on insect cadavers, rapidly producing conidia or other asexual propagules that contribute to its dissemination (Lacey et al., 2015). In general, vectors such as wind and water, or contact between an infected individual and a healthy one, play an important role in horizontal transmission (Zimmerman, 2007a).

The many advantages of EPF - such as their ease of application, the rapid improvement in their formulation, the overexpression of endogenous proteins or toxins that they cause, the vast number of known pathogenic strains and the broad spectrum of target insects (over 700 species in around 90 genera) - make them one of the most widely used microbial control agent (Khachatourians and Sohail, 2008; Wang et al., 2005; Khan et al., 2012). EPF can be used to control a wide range of hosts, including chewing insects such as Lepidoptera (Karthi et al., 2024; Idrees et al., 2021; Resquin-Romero et al., 2016) as well as sap-sucking insects such as aphids (Van Hanh et al., 2007). The most widespread entomopathogenic fungal genera belong

to the Hypocreales order, in the Ascomycetes phylum, including *Fusarium*, *Lecanicillium*, *Isaria* and *Nomurea*. But the two best known are *Metarhizium* and *Beauveria*, in particular *M. anisopliae* and *Beauveria bassiana* (Balsamo) Vuillemin, respectively the green and white muscardine fungi recognisable by their green and white spores (Vincent et al., 2007). Numerous commercial products derived from these two fungi are on the market or under development due to their effectiveness in reducing insect survival (Quesada-Moraga et al., 2006).

EPF are therefore a valuable solution for limiting the action of phytophagous insect pests, but there are still some limits to overcome for improving their efficacy (Vega et al., 2018). They are indeed highly susceptible to UV rays, extreme temperatures, rainfall and low humidity, which reduce their persistence and long-term action (Zimmerman, 2007). In addition, their massive non-targeted spraying can have an impact on certain non-target insect species (Jackson et al., 2010, Bamisile et al., 2021).

Interestingly, these EPF, due to their remarkable adaptive capacities, can adopt a multitude of lifestyles, many of which remain overlooked in the pursuit to develop the ideal strategy for controlling insect pests. For instance, in terms of nutrition, in addition to their role as entomopathogens, these fungi can naturally play a role as saprophytes of the soil, the rhizosphere and the phyllosphere, but also as haemibiotrophs, necrotrophs or mycoparasites. And some of them have adopted more than one way of life (Khan et al., 2012). In nature, EPF are also antagonists of plant diseases or promoters of plant growth through colonisation of the rhizosphere (Jaber and Ownley, 2018). These newly emerged ecological roles offer promising and multiple possibilities for the use of entomopathogenic fungi in integrated pest management strategies (Vega et al., 2009; Lacey et al., 2015; Jaber and Ownley, 2018).

Among these newly discovered roles, a growing number of studies have demonstrated the ability of these fungi to behave like endophytic microorganisms in plants (Azevedo et al., 2000, Vega et al., 2009). These microorganisms colonise the internal tissues of plants without causing any apparent damage. The mutualistic nature of these associations, in accordance with favourable environmental conditions, has been particularly highlighted (Schultz, 2005). One of the first studies to report the benefits of such an entomopathogenic fungus-plant association was conducted by Bing and Lewis in 1991. They demonstrated that the colonisation of maize plants by *B. bassiana* (as an endophyte) resulted in greater mortality among the larvae of the European corn borer, *Ostrinia nubilalis* (Lepidotera: Crambidae). Surprisingly, such a discovery did not immediately lead to other studies, and interest in exploiting this life-history feature of EPF for pest control purposes has only recently gained in popularity. It is now widely established that EPF, by colonising plants as endophytes, contribute greatly to reducing the fitness of a very wide range of phytophagous insects feeding on these host plants (Jaber and Ownley, 2018). For example, this colonisation has been reported to reduce damage by the tomato worm *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) on tomato colonised by *B. bassiana* (Toffa et al., 2021); the banana weevil, *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae) on banana colonised by *B. bassiana* (Akello et al., 2008); but also the black bean aphid, *Aphis fabae* Scopoli (Hemiptera: Aphididae) and the pea leaf miner, *Liriomyza huidobrensis* Blanchard (Diptera: Agromyzidae) with *Hypocrea lixii*, *Gibberella moniliformis*, *Fusarium oxysporum* and *T. asperellum* on beans (Akutse et al., 2013); the Mediterranean corn borer, *Sesamia nonagrioides* Lefebre (Lepidoptera: Noctuidae) with *Metarhizium robertsii* and *Isaria fumosorosea* in sweet sorghum (Mantzoukas et al., 2015); the dark sword grass, *Agrotis*

ipsilon (Lepidoptera : Noctuidae), with *M. robertsii* in *the Zea mays* L. (Ahmad et al., 2020), or *Philaenus spumarius* L. (Hemiptera: Aphrophoridae) in Sowthistle with *M. bruneum* and *B. bassiana* (Yousef-Yousef et al., 2023).

As well as promising insect control agents, these endophytic EPF have also shown antagonistic activity against plant pathogens (Ownley et al., 2008). Moreover, the endophytic establishment of these fungi has been shown to enhance the growth of colonised plants by improving their nutrition (Jaber and Enkerli, 2017). Finally, it has been shown that endophytic colonisation of plants by EPF can activate the release of plant volatile organic compounds, attracting certain natural pest antagonists, further enhancing the effect of the endophyte in pest control (Pec et al., 2024).

The advantages of endophytic EPF are numerous. In addition to the benefits mentioned above, which are ideal for (i) IPM and (ii) agricultural production, the plant-EPF interaction also helps overcome the traditional challenges encountered when using conventional entomopathogens. Firstly, as endophytes, fungi are protected from environmental threats residing inside plants. Secondly, their ability to target only insects feeding on colonised plants minimizes the negative impact on non-target organisms, in contrast to the widespread application used when functioning as conventional entomopathogens. One of the consequences of this is the ability of these endophytes to target certain cryptic pests that are difficult to reach using traditional methods (for example, leaf miners or borers) (Jaronski, 2010). Thirdly, one of the difficulties with conventional entomopathogens is the synchronisation of biocontrol with the target insects. As endophytes, they appear to be able to provide continuous defence for their host plant. Further investigations into the colonisation kinetics of the fungus within the plant have yet to be carried out. However, while spray applications are normally based on the direct action of the sprayed agents, thus not taking successive generations into account, studies have revealed the vertical transmission of certain endophytic EPF (Quesada-Moraga et al., 2014). The use of EPF as endophytes has therefore a very promising potential for the development of multi-purpose strategies as part of IPM strategies.

Tomato is one of the most widely consumed vegetable products in the world. With its abundance of vitamins, minerals, lycopene, flavonoids and carotenoids, its antioxidant, anti-inflammatory and anti-cancer activities, its high nutritional value, and because it is highly appreciated for its taste, it is an essential resource for human beings and their diet (Salehi et al., 2019). With an annual global production of more than 100 million tonnes, tomatoes rank as the second most cultivated vegetable after potatoes, giving it a central place as an agricultural crop (FAO, 2023). However, like many other crops, tomatoes are the target of numerous insect pests, including Lepidoptera, whose larval stage is the most devastating. The damage they inflict on plants by attacking leaves, stems and fruit causes immense yield losses (Simmons et al., 2018). For example, by feeding on the leaves of tomato plants, the plant's photosynthetic capacity and consequently its carbohydrate production is considerably reduced, affecting plant health and fruit production (Maish et al., 2019).

B. bassiana, in addition to its entomopathogenic role is also capable of colonising, both naturally and artificially, different varieties of wild and cultivated, herbaceous and arboreal plants, providing them with effective protection against biotic and abiotic stresses (Singh et al., 2015, Bamisile et al., 2018). Several bioassays on tomato plants have been successfully conducted to obtain endophytic colonisation of *B. bassiana* on tomato plants and evaluate its

potential as a biocontrol agent. In this context, the biocontrol potential of *B. bassiana* as an endophyte against tomato plant pests such as the lepidopteran *Spodoptera littoralis* (Boisduval) (Resquin-Romero et al., 2016), *Helicoverpa armigera* (Qayyim et al., 2015), *Tuta absoluta* (Allegrucci et al., 2017), the aphid *Macrosiphum euphorbiae* (Sinno et al., 2020), the white fly *Bemisia tabaci* (El-Deeb et al., 2012), but also against necrotrophic pathogens such as *Alternaria alternata* and *Botrytis cinerea* has been highlighted (Sinno et al., 2020).
However, the role of *B. bassiana*, and of endophytic EPF in general, in modulating interactions between plants and the environment, and in particular between plants and herbivorous insects, remains little understood. For an endophyte to serve as an effective alternative to synthetic pesticides, its efficacy must be ensured and match that of chemical control. Additionally, the underlying mechanisms involved should be thoroughly understood.
In the present study, we are seeking to gain a better understanding of the impact by the endophytic colonisation of *B. bassiana* of tomato plant on *S. littoralis* (Lepidoptera: Noctuidae), a polyphagous pest species and the underlying mechanisms modulating the complex network of multitrophic interactions.

2. Material and Methods

2.1. *Beauveria bassiana* conidia production

The commercial strain of *B. bassiana*, ATCC 74040, was isolated from the commercial formulation Naturalis® (CBC S.r.l., Grassobbio, Italy) to obtain fungal conidia, following the protocol described by Sinno et al. (2021). Briefly, 100 µL of a 1:100 dilution of Naturalis® in sterile distilled water (ddH_2O) was plated on Potato Dextrose Agar (PDA; Merck, Darmstadt, Germany) in Petri dishes and incubated in a climate-controlled chamber at 25 ± 1°C, 70 ± 5% relative humidity (RH), in complete darkness. After 8 days, 10 mycelial plugs (6 mm^2 each) were gently scraped from the surface of the PDA plates and transferred to a 250-mL Erlenmeyer flask containing 100 mL of sterile Potato Dextrose Broth (PDB; Merck, Darmstadt, Germany). The culture was maintained for 72 h at 25°C under orbital shaking at 120 rpm.
This starter culture was then used to inoculate 500g of autoclaved parboiled rice placed in bags with breathable filter bands for air exchange (SacO2, Flanders, Belgium). The sealed bags were incubated at 25°C under light for 7–10 days until the rice was covered with white conidia. Conidia were harvested by washing the rice with 500 mL of sterile distilled water under aseptic conditions. The initial conidial concentration was determined using serial dilutions and counting in a haemocytometer (Neubauer counting chamber) under a microscope. The conidial concentration was standardized to 1×10^7 conidia/mL (Bb-suspension), which was used for subsequent plant colonisation experiments.

2.2. Endophytic Colonisation

Tomato seeds (*Solanum lycopersicum* L., cv. "Dwarf San Marzano") were surface-sterilised by immersion in 70% ethanol for 3 min, followed by washing in 1% sodium hypochlorite for 5

min, and rinsed three times with sterile ddH_2O. The sterilised seeds were germinated on sterile Whatman® filter paper (Sigma-Aldrich, Darmstadt, Germany) in Petri dishes, incubated in a growth chamber at 25 ± 1°C, 70 ± 5% RH, in complete darkness. After root emergence, 1 mL of the Bb-suspension or sterile ddH_2O (for controls) was added to each dish to generate *B. bassiana*-colonised plants (Bb-plants) and control plants (C-plants), respectively.
The dishes were incubated under the same environmental conditions until seedling emergence. After two days, individual Bb-seedlings were transplanted into 96-well polystyrene trays containing a commercial soil mixture (Universal Potting Soil; Floragard, Oldenburg, Germany) and watered with 3 mL of Bb-suspension. Control seedlings received 3 mL of sterile ddH_2O. The treatment was repeated one week later. Plants were grown in a controlled chamber at 25 ± 1°C, 70 ± 5% RH, with a photoperiod of 18:6 h (light:dark). Two weeks after the final treatment, tomato plants were transferred to 10-cm diameter pots filled with the same soil mixture and maintained under the same growth conditions.
After six weeks, when plants reached approximately 15 cm in height with 7–8 fully expanded leaves, the fifth fully expanded leaves were collected from both plants colonised by *B. bassiana* (Bb-plants) and Control plants (C-plants). Samples were immediately frozen in liquid nitrogen and stored at –80°C until DNA extraction using the protocol described by Cubero et al. (1999), with minor modifications. DNA concentration was measured at 260 nm using a Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA), and purity was evaluated based on the 260/280 nm absorbance ratio. DNA samples were stored at –20°C until processed for PCR.
The presence of *B. bassiana* in tomato plants was confirmed via a nested PCR approach, following the protocol by Landa et al. (2013). First-round amplification employed universal primers ITS1F (5′ CTTGGTCATTTAGAGGAAGTAA 3′) (Gardes and Bruns, 1993) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) (White et al., 1990). Second-round amplification used species-specific primers Bb.fw (5′ GAACCTACCTATCGTTGCTTC 3′) and Bb.rv (5′ ATTCGAGGTCAACGTTCAG 3′) (Landa et al., 2013).
PCR products were resolved on a 1.5% (w/v) agarose gel (Euroclone S.p.a., Milan, Italy) containing GelRed (Thermo Scientific, Waltham, MA, USA) in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Bands were visualised under UV light using a UV Gel Doc system (BIORAD), with a DNA size marker (Thermo Fisher Scientific, Waltham, MA) included for size estimation. DNA extracted from the commercial *B. bassiana* strain 74040 grown on PDA was used as a positive control.
Plants were grown under identical environmental conditions (25 ± 1°C, 16:8 h light/dark photoperiod) and irrigated three times weekly.

2.3 Insects rearing

S. littoralis experimental larvae derived from a colony, established in 2009 at the University of Napoli Federico II (Department of Agricultural Sciences, Laboratory of Entomology "E. Tremblay", Portici-NA, IT), regularly refreshed once a year with field-collected insects. The larvae were reared on an artificial diet (41.4 g/l wheat germ, 59.2 g/l brewer’s yeast, 165 g/l corn meal, 5.9 g/l ascorbic acid, 1.53 g/l benzoic acid, 1.8 g/l methyl-4-hydroxybenzoate, and

29.6 g/l agar), as previously described (Di Lelio et al., 2014), at 25 ± 1◦C, 70 ± 5% RH, and photoperiod of 16:8 h light/dark.

2.4 Feeding bioassay

To study the plant-mediated effect of *B. bassiana*, bioassays consisting of feeding *S. littoralis* larvae with Bb-plants and C-plants were conducted.
Groups of 200 newly hatched *S. littorali*s larvae were reared in plastic boxes (30×40×15 cm), bottom lined with 50 mL of 1.5% agar (w/v), on sub-apical leaves of 6 weeks-old Bb-plants (Bb-larvae) or of C-plants (C-larvae). Leaves were randomly selected and excised from tomato plants starting from the second whorl, then offered to larvae. To prevent any undesired effect due to mechanical damage, potentially triggering defense responses, leaves were obtained from plants that were left undisturbed (i.e., not exposed to mechanical damage) for at least three days (Glauser et al., 2008). Leaves were replaced daily, and larvae checked to monitor their development and to select those to be used for the feeding bioassay, which was carried out by comparing two experimental groups made of 16 synchronous newly molted day one 3^{rd} instar larvae, obtained by selecting 2^{nd} instars ready to molt (i.e., showing head capsule slippage) before the onset of the scotophase and collecting those molted in the morning of the following day. In detail, day one 3^{rd} instar larvae were singly weighed, and transferred into multi-well plastic rearing trays (RT32W, Frontier Agricultural Sciences, Pitman, NJ, USA), bottom-lined with 1 mL of 1.5% agar (w/v) and closed by perforated plastic lids (RTCV4, Frontier Agricultural Sciences, Pitman, NJ, USA). The larvae were singly reared on a leaf disk from a C-plant or Bb-plant, giving each larva a portion of a square leaf of a size appropriate to the larval stage (2 cm^2 to 5 cm^2), which was replaced daily. Bioassays were carried out in environmental chamber at 25 ± 1°C, photoperiod 16:8 light/dark. The following parameters were recorded: larval survival (daily), larval weight (every four days), leaf consumption, pupal survival, pupal weight, adult emergence, adult survival, egg number and egg hatching rate per female. Pupal weight was recorded on day three of the pupal stage. To assess the fertility of the emerged adults and the viability of their eggs, each female moth was fed soon after emergence with water/honey solution (50%) and allowed to mate with two males for 24h, then isolated in a box (40 × 30 × 20) to assess the number of eggs laid daily and their rate of hatching. Leaf consumption by day one of 4^{th}, 5^{th} and 6^{th} instars larvae was obtained as difference of the initial and final weight of the leaf disks. The bioassay was replicated 4 times.

2.5. Protein content in Bb-plant leaves and in *S. littoralis* larvae frass

During the bioassays, part of the leaves provided to larvae and the frass produced by larvae were processed to evaluate the protein content. Tomato leaves were collected from 6 weeks old plants, cut in 0.5 cm^2 pieces and placed in 2 mL microcentrifuge tubes. Each sample was formed by approximately 50 mg of leaf pieces obtained from a single plant. Samples were weighed, supplemented with 200 µL 1X PBS and ground using a plastic pestle. After centrifuging at 5000 × g for 5 min at 4°C, protein concentration of the supernatant was quantified using Bradford

method (Bradford, 1976). Then, samples were dried using a speed vacuum at 40°C and weighed again to obtain their dry weight.
The same approach was used to measure protein content of frass collected from experimental larvae in the bioassay trays. Approximately 0.2 mg of frass was collected from each larva. Pools were formed by grouping frass obtained from three larvae.

2.6. Immune Response

To evaluate the immune response of *S. littoralis*, several assays, including haemocyte viability, encapsulation, nodulation, and phagocytosis, were performed on newly molted 6th instar larvae (obtained as described in part 2.4). These methods, adapted from established protocols (Di Lelio et al., 2014; Becchimanzi et al., 2019), provided a comprehensive assessment of the larvae's cellular immune response.

2.6.1. Haemocyte Viability and Phagocytosis Assay

Haemocyte viability was assessed prior to each experiment to ensure sample quality. Larvae were anesthetized on ice for 30 minutes, and haemolymph was collected from a cut thoracic leg into a 1.5 mL Eppendorf tube containing sterile PBS (137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer, pH 7.4) at a 2:1 ratio. For viability testing, an aliquot of haemolymph was mixed with 0.4% (w/v) trypan blue solution (Sigma-Aldrich) in a 1:1 ratio and observed under a light microscope after 5 minutes of staining. Haemocyte samples with viability rates below 98% were discarded.
For the in vitro phagocytosis assay, fluorescein-conjugated *Escherichia coli* (BioParticles, K-12 strain, Invitrogen) were reconstituted following the manufacturer's instructions and suspended in PBS at a concentration of 2×10^6 cells/mL. Haemolymph and bacterial suspensions were mixed in equal volumes (1:1 v/v) and incubated at room temperature in the dark for 10 minutes, with gentle mixing every 2 minutes. Following incubation, 10 µL of the mixture was loaded into a Neubauer chamber, and the total number of haemocytes, as well as phagocytic haemocytes showing fluorescence, were counted using a fluorescence microscope (Axioskop 20; Carl Zeiss Microscopy) with a FITC filter (excitation: 475 nm, emission: 530 nm).

2.6.2. Nodulation Assay

The nodulation response was assessed in vivo, the experimental larvae were anesthetized on ice and surface-sterilised by successive immersion in 70% ethanol and sterile distilled water for 10 seconds each. Larvae were injected intra-hemocoelically with 10 µL of a solution containing 2×10^6 *E. coli* cells in PBS using a Hamilton 1701 RN SYR microsyringe (10 µL, gauge 26s, 55 mm needle length, point style 3). Injected larvae were individually placed in bioassay trays with

fresh tomato leaves as a food source and maintained for 18 hours at 25 ± 5°C, 70 ± 5% RH, under a 16:8 h light/dark photoperiod.
Haemolymph was collected by cutting a thoracic leg and transferring 50 µL of the exuded haemolymph into an Eppendorf tube containing MEAD anticoagulant buffer (1:1 v/v; 98 mM NaOH, 145 mM NaCl, 17 mM EDTA, and 41 mM citric acid, pH 4.5). Samples were gently mixed and 10 µL of the suspension was loaded into a Bürker chamber. Nodules were counted under a light microscope (Axioskop; Carl Zeiss Microscopy) at 400× magnification.

2.6.3. Encapsulation Assay

To evaluate encapsulation in vivo, larvae were sterilised as described above and injected with 5 µL of PBS containing approximately 40 CM Sepharose fast-flow chromatography beads (Pharmacia) using a Hamilton Microliter syringe (702RNR, 25 µL, gauge 22s, 55 mm needle length, point style 3). Injected larvae were individually placed in bioassay trays with tomato leaves and maintained for 24 hours under the same environmental conditions.
Post-incubation, larvae were anesthetized on ice for 30 minutes and dissected in PBS to recover beads. The intestine was gently removed, and any remaining beads were collected by scraping the body cavity.
The beads, detected under the stereomicroscope, were recovered and then placed in 300 µl of PBS, and finally observed under a light microscope (Leica DM IRB Microsystem) to calculate the degree of encapsulation. Five degrees of encapsulation were defined as follows:
0 - no cells adhered to the sphere.
1 - up to 10 adherent cells.
2 - more than 10 adherent cells but with less than one complete layer.
3 - one or more complete layers but no melanisation.
4 - one or more complete layers with melanisation.

An encapsulation index was then calculated using the following formula, presented in Li et al. 2007:

I.I (%) = [Σ (degree of encapsulation x total number of spheres in this degree) / total number of spheres x 4] x 100.

2.7. Evaluation of the susceptibility to commercial biopesticides for larvae feeding on Bb plants

The interaction between the endophytic colonisation of tomato plants by *B. bassiana* (Bb) and the application of commercial biopesticides on the survival and immune response of *S. littoralis* larvae was investigated. Two biopesticides were selected for these studies: Naturalis®, a fungal-based product containing *B. bassiana* ATCC 74040 (10^6 spores/mL, corresponding to a 50% lethal concentration over 9 days), and XenTari® (Valent BioSciences), a bacterial biopesticide containing *Bacillus thuringiensis* subsp. aizawai (containing Cry1Aa, Cry1Ab, Cry1Ca,

Cry1Da, and Cry2Ab toxins, active against lepidoptera). The latter was applied at a sublethal dose of 3 µg/cm², known to delay larval development without inducing mortality.

2.7.1. Effects of Naturalis® Biopesticide

Freshly molted 6th instar larvae (obtained as described in part 2.4) from both control (C) and Bb-colonised (Bb) groups were anesthetized on ice, placed on sterile filter paper, and individually sprayed with Naturalis® solution using a 3 mL manual sprayer. Control treatments were performed with a heat-inactivated solution, prepared by boiling at 100°C for 15 minutes and verified for microbial inactivity on PDA plates. Treated larvae were housed in biological test trays, as previously described, and fed tomato leaves from either C-plants or Bb-plants.
Four treatment groups were established:
- Control larvae treated with an inactivated Naturalis® solution (C).
- Control larvae treated with an active Naturalis® solution (C + Naturalis®).
- Bb-larvae treated with an inactivated Naturalis® solution (Bb).
- Bb-larvae treated with an active Naturalis® solution (Bb + Naturalis®).
Daily mortality was recorded for 15 days. Each treatment was replicated three times.

2.7.2. Effects of XenTari® Biopesticide

The effects of *B. bassiana* colonisation on larval sensitivity to a sublethal dose of XenTari® were assessed by feeding first-day 6th instar larvae (C and Bb groups) with tomato leaves treated with the biopesticide. Preliminary bioassays confirmed the sublethal dose of XenTari®, which caused growth retardation without mortality in control larvae. Tomato plants were sprayed with a XenTari® (3 µg/cm²) or distilled water (control), and the leaves were air-dried before being cut into uniform 4 cm² sections. The experimental larvae were fed treated leaves ad libitum for three days and subsequently with untreated leaves for the remaining duration of the experiment.
Four treatment groups were established:
- Control larvae fed water-treated leaves (C + H2O).
- Control larvae fed XenTari®-treated leaves (C + XenTari®).
- Bb-larvae fed water-treated leaves (Bb + H2O).
- Bb-larvae fed XenTari®-treated leaves (Bb + XenTari®).
Mortality was monitored daily for 8 days, and larval weight was recorded on Day 8. Each treatment group consisted of 16 larvae, and the experiment was replicated twice.

2.8. Statistical analyses

Survival curves of *S. littoralis* larvae and adults were compared by using Kaplan–Meier and Log-rank analysis. Differences between experimental groups (i.e., C-larvae and Bb-larvae) were assessed by unpaired Student's t-test or Mann–Whitney (for pupal weight). Normality of

data was checked with D'Agostino & Pearson normality test, while homoscedasticity was tested with Barlett's test. Statistical analyses were performed using Prism (GraphPad Software Inc. version 6.0b).

3. Results

3.1. Effect of Bb-plants on the development and survival of *S. littoralis*

Feeding *S. littoralis* larvae with leaf disks from Bb tomato plants did not influence larval survival, with 100% survival observed in both Bb-larvae and C-larvae (data not shown). Despite similar leaf consumption rates between the two groups (Figure 1a), from day 22 onwards, Bb-larvae displayed significantly higher weights compared to C-larvae (Figure 1b).

Notably, only 29% of Bb-larvae reached the pupal stage, in contrast to 100% pupation in C-larvae (Figure 1c). Additionally, Bb-pupae exhibited abnormal body shapes (Figure 1e), though the weight of surviving Bb-pupae was not significantly different from that of C-pupae (Figure 1d).

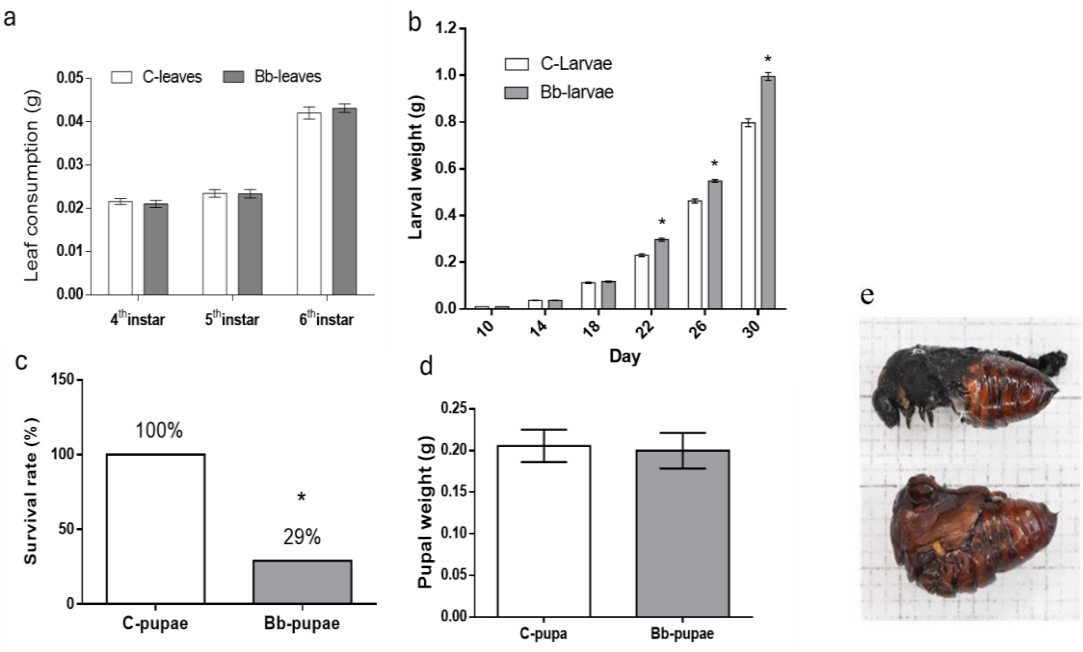


Figure 1: Effects on survival and development of Spodoptera littoralis larvae feeding on leaves from tomato plants colonised by Beauveria bassiana. *(a) Tomato leaf consumption by S. littoralis larvae. No differences in tomato leaf consumption were recorded in C-larvae and Bb-larvae by day one of the 4th (Student's t test: t = 0.544; p-value = 0.588; df = 94; n = 64), 5th (Student's t test: t = 0.05079, p-value = 0.9596; df = 94, n = 64) and 6th instar larvae (Student's t test: t = 0.6512, p-value = 0.5165; df=94; n = 64). (b) Larval weight from third instar to pupation (22th day, Student's t test: t = 46, p-value < 0,001, df = 94; 26th day, Student's t test: t = 203,5, p-value < 0,001, df = 94; 30th day, Student's t test: t = 43,68, p-value < 0,001, df = 94). (c) S. littoralis pupae survival rate (Chi^2 = 66.94, p-value (p-value < 0,001, dF = 1, n = 64). (d) Pupal weight did not differ between C- and Bb-pupae (Mann Whitney test: t = 0.1365, U = 720, n = 64). (e) Bb-pupae showing abnormal body shape. In the graphs the values are means ± SE. In the graph the asterisk indicates a statistical difference to Student's t-test (P < 0.005).*

The emergence rate of adults did not differ significantly between groups, but Bb-adults exhibited significantly reduced longevity (Figure 2a) and fertility (Figure 2b) compared to C-adults.

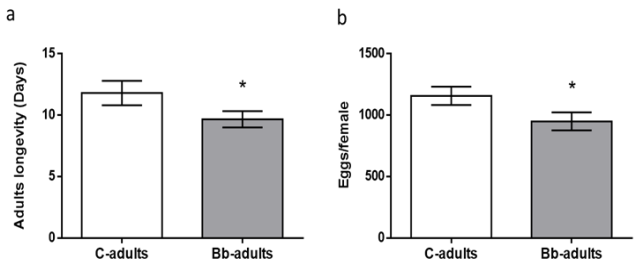


Figure 2: Effects on survival and fecundity of S. littoralis adults from larvae fed on leaves from tomato plants colonised by Beauveria bassiana. *(a) Longevity of the adults emerged from Bb-pupae was lower compared with C-adults (Student's t test: t = 10.40, df = 90, n_c = 64, n_{Bb} = 28); (b) Bb-adults laid a significantly lower number of eggs compared to C-adults (Student's t test: t = 8.743, p-value < 0.0001, df = 38, n_c = 24, n_{Bb} = 16). The values are means ± SE. In the graph, the asterisk indicates a statistical difference to Student's t-test (P < 0.005).*

3.2. Protein and water content in tomato leaves and larval frass

Considering the effect observed on larval weight, we investigated the impact of *B. bassiana* endophytism on the nutritional value of tomato leaves and on the digestion/excretion processes of *S. littoralis* larvae. During the bioassay the total amount of proteins and the percentage of water contained in Bb-plant and C-plant leaves administered to larvae, as well as the amount of proteins and water in the frass of 4th, 5th, and early and late 6th instar *S. littoralis* larvae was evaluated. The protein and water content was higher in Bb-plant leaves compared to C-plant leaves (Figure 3a, b), as well as in the frass collected from Bb-larvae compared to the frass collected from C-larvae (Figure 3c, d).

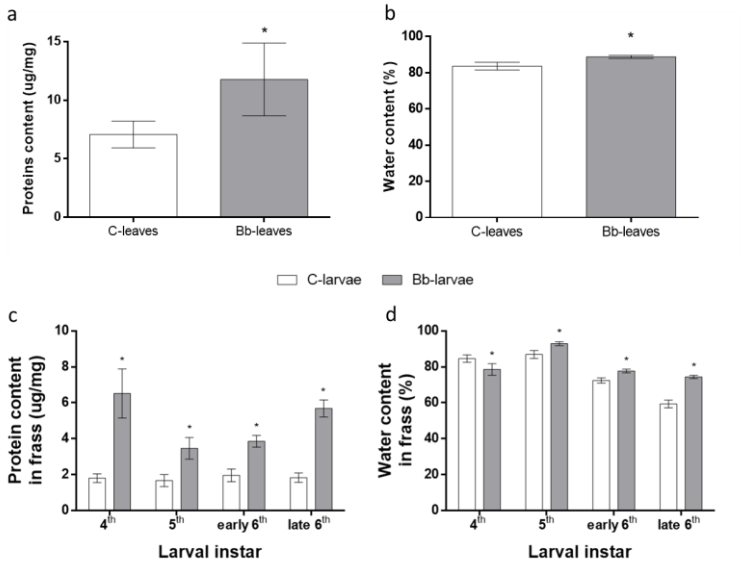


Figure 3: Proteins content and water percentage in plants colonised by Beauveria bassiana. *Proteins content (a) significantly differ between the two experimental groups (Student's t-tests: t = 3.175, dF = 8, p-value = 0.013, n = 5) as water content (b) (Student's t-tests: t = 4.9, dF = 8, p-value = 0, n = 5). Proteins content (c) and water content (d) in frass were statistically different between the considered larval instars. In the graphs the values are mean ± SE, asterisks above histograms indicate significant differences between treatments (p value < 0.05).*

3.3. Effect of Bb-plants on the cellular immune response of *S. littoralis* larvae

To determine whether endophytic colonisation of tomato plants by *B. bassiana* could induce a reduction in the cellular immune capacities of *S. littoralis* larvae feeding on colonised plants, biological tests were carried out to assess phagocytosis, nodulation and encapsulation responses. Surprisingly, the phagocytic activity of haemocytes towards bacteria *in vitro* was significantly higher in Bb-larvae than in C-larvae (Student's t test: $P < 0.0001$) (Figure 4A). In contrast, Bb-larvae showed a significantly reduced capacity to nodulate bacteria *in vivo* compared with C-larvae (Student's t test: $P < 0.0001$) (Figure 4B) as well as a reduced capacity to encapsulate chromatographic beads injected *in vivo* (Student's t test: $P < 0.0001$ (Figure 4C).

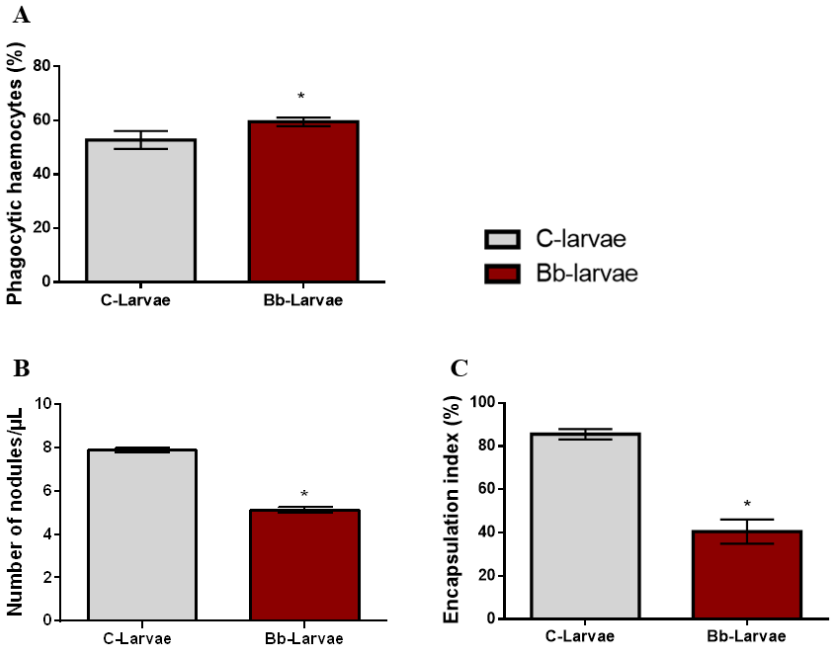


Figure 4: Alteration of Spodoptera littoralis cellular immune response induced by feeding on Bb-plants. *(A) Percentage of phagocytic haemocytes (Student's t-test: p-value < 0.0001, t = 4.619). (B) Total number of nodules per µL of haemolymph (Student's t-test: p-value < 0.0001, t = 13.99). (C) Encapsulation index) (Student's t-test: p-value < 0.0001, t = 40.39). Asterisk above histograms indicate significant differences between treatments (p value < 0.0001).*

3.4. Effect of Bb-plants on the susceptibility of larvae to two commercial biopesticides

We analysed the susceptibility of Bb-larvae compared to C-larvae to two commercial biopesticides: one based on *B. bassiana* (Naturalis®), which acts as a pathogen following contact with the insect integument (i.e. externally), the other based on the entomopathogenic bacterial species *Bacillus thuringiensis* (XenTari®), which causes septicaemia after ingestion.

To investigate if the reduced nodulation and encapsulation response was associated with increased susceptibility to *B. bassiana* (Naturalis®) administered as an entomopathogen, we realized a bioassay by spraying the 5th instar experimental larvae with Naturalis® at CL50 dose at day 9 (10^6spore/ml). Bb-larvae showed a 57% of mortality 7 days after the treatment resulting to be more susceptible to Naturalis® than C-larvae (Figure 5) (Log-rank test: $P < 0.0001$).

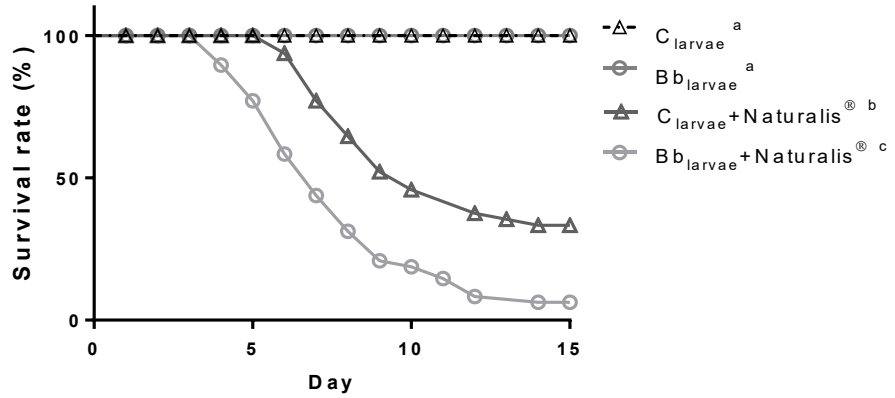


Figure 5: Survival rate of experimental Spodoptera littoralis 6th instar larvae following topic infection with Naturalis®. *Bb-larvae treated with Naturalis showed lower survival rate compared with the other treatments; (Log-rank test: $\chi 2 = 198$; dF=3; p-value < 0.0001, n = 64) reaching a 94% of mortality at day 14. In the graph the different letters indicate a statistical difference to Log-Rank test (P < 0.005).*

The biopesticide XenTari® was applied to sixth instar Bb-larvae at a sublethal dose. Bb-larvae showed a significantly reduced survival rate compared with the other three conditions: untreated C-larvae, untreated Bb-larvae and C-larvae treated with the same dose (Log-rank test: p-value < 0.0001). C-larvae (i.e. fed on leaves of control plants, not colonised by *B. bassiana*) exposed to a sublethal dose of XenTari® showed no significant reduction in survival compared with the two negative controls, not exposed to the biopesticide (Figure 6).

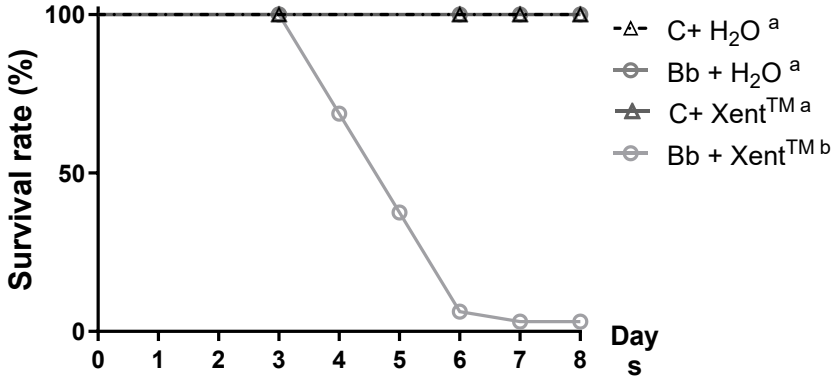


Figure 6: Survival rate of experimental Spodoptera littoralis 6th instar following feeding with XenTari®. *Bb-larvae treated with XenTari showed lower survival rate compared with the other treatments; (Log-rank test: $\chi^2 = 77.97$; df = 3; P < 0.0001). The different letters in the graph indicate a statistical difference in the long-rank test (P < 0.0001).*

4. Discussion

In recent years, a growing number of studies have documented the ability of EPF to develop as endophytes and promote plant growth and defence barriers against phytophagous insects, offering a valuable alternative to synthetic pesticides (Vega, 2018; Jaber and Ownley, 2018). *B. bassiana* is one of the most important EPF that can colonise a wide variety of plant species as

an endophyte, limiting the growth and survival of plant pests and pathogens (Sinno et al., 2020; Mantzoukas et al., 2022). In the present study we contribute to this research topic by studying the effect of tomato plants colonisation by *B. bassiana (*ATCC 74040 strain) on *S. littoralis* performance (Lepidoptera: Noctuidae). To our knowledge, this is the first study to show the immunosuppressive effect of Bb plants on *S. littoralis* larvae.

In the literature, numerous studies have shown that endophytic colonisation by *B. bassiana* could be used as an effective biocontrol tool due to the negative effect it induces on the life cycle of insects feeding on colonised plants, in particular noctuid Lepidoptera and aphids (Sinno et al., 2020; Sinno et al., 2021). In the case of Noctuidae, a reduction in the survival of larvae and other developmental stages has been described several times. For example, Lopez and Sword (2015) observed a reduction in the survival of *Helicoverpa zea* larvae fed on cotton plants colonised by *B. bassiana*. This reduction was neither associated with developmental alterations nor with differences in larval and pupal weights. In 2019, Russo *et al*. showed that the interaction between *B. bassiana* and soybean plants induced a reduction in the survival of *Helicoverpa gelotopopeon* larvae (Lepidoptera: Noctuidae). This reduction was accompanied here by a reduction in leaf consumption as well as negative effects on adult development, fertility and longevity (Russo et al., 2019a). Similarly, reduced consumption of maize plants colonised by *B. bassiana* by *Rachiplusia nu* larvae (Lepidoptera: Noctuidae) was reported by Russo *et al.* (2019b). However, Vianna *et al.* (2018) found no change in the leaves consumption by *H. gelotopoeons* larvae fed on tobacco plants colonised *by B. bassiana*. Larval and adult survival and a reduction in the life cycle were nevertheless observed. In the case of *Phthorimaea operculella*, a potato moth that feeds on plants colonised by *B. bassiana*, a reduction in larval growth, development and adult fertility was also recorded (Zhang et al., 2022). Similarly, *Tuta absoluta* (Klieber and Reineke, 2015) and *Helicoverpa armigera* (Qayum et al., 2015), both fed on tomato plants colonised by *B. bassiana*, showed high mortality at the larval, pupal and adult stages. More recently, Altaf et al. 2023 demonstrated a reduction in fecundity and development time in *S. frugiperda* fed on maize plants colonised by *B. bassiana*. *Helicoverpa armigera* showed also a reduction of larval survival, when feeding on tomato plants colonised by different *B. bassiana* strains with different inoculation methods (Toffa et al., 2021). In 2024, Darsouei et al. observed that endophytic colonisation of sugar beet by *B. bassiana* reduces performance of *S. littoralis*, particularly the weight of larvae, the adult emergence and oviposition by females.

Contrary to the previously cited studies, our results did not show a direct effect of endophytisation by *B. bassiana* on the survival and development of *S. littoralis* larvae with a survival of 100%. However, from the fifth instar, *S. littoralis* larvae fed on colonised plants showed a higher weight than control larvae (Figure 1b). This increase in larval weight was not associated with differential consumption of plant leaves by the larvae (Figure 1a). A lack of alteration in the consumption of plants colonised by *B. bassiana* was observed in other studies, while no differences in weight is usually reported (Leckie et al., 2008; Lopez and Sword, 2015; Vianna et al., 2018). A phenomenon similar to our case was reported in 2003 by Bultman and Bell in *S. frugiperda* larvae fed on fescue plants endophytically colonised by the fungus *Neotyphodium coenophialum*. This colonisation was associated with increased production of proteins in the colonised plants. Based on these results we hypothesized that *B. bassiana* (ATCC 74040) endophytism increases plant nutrient intake in tomato plants, leading to greater weight gain in larvae feeding on them. This could also be linked to greater water retention in the

colonised plants, and that is why we analysed proteins content and water percentage in plants colonised by *B. bassiana* (Figure 3). In plants colonised by the fungus, an increase in water and protein content have indeed been observed (Figure 3a, b), and a positive correlation of water and protein were observed in frass of larvae (Figure 3c, d). Such results could explain the greater weight gain of larvae fed with leaves from tomato plants colonised by *B. bassiana*. From an ecological and evolutionary point of view, it is conceivable that the fungus could benefit from the greater weight of the larvae, making the insect a more suitable host for the entomopathogenic phase of the fungus. In this way, fungi capable of manipulating larval development by making their host plants richer in nutrients could have been selected over time. Modulating plant physiology to obtain larger larvae would be a definite adaptive advantage for the entomopathogenic activity of its own species.

Surprisingly, although the weight of the *S. littoralis* larvae was greater, the chrysalids formed by Bb-larvae had a lower weight and a significantly reduced survival rate compared with C-larvae (figure 1C and D). We can suggest that among increased proteins inside the plants, some act as entomotoxic proteins, thought to be involved in defence mechanisms against insects (Carlini and Grossi-de-Sa, 2002). Furthermore, in view of these results, it would be important to conduct further research and study the mechanisms operating at the time of metamorphosis. In addition, as well as the reduction in pupal weight and survival consistent with the literature, the fecundity of adult *S. littorali*s also showed a reduction (Figure 2) (Russo et al., 2019a; Zhang et al., 2022; Altaf et al., 2023).

Moreover, it is important to note that no fungal growth was detected in the cadavers of pupae from larvae fed on tomato leaves colonised by *B. bassiana*. This suggests that the pupal mortality observed is not due to the entomopathogenic effect of the fungus, and therefore to fungal infection, but is linked to other mechanisms. In many cases, the endophytic colonisation of plants by EPF does not result in direct insect mortality. For example, no fungal growth was observed in *Plutella xylostella* larvae developing on plants colonised by *B. bassiana* (Gautam et al., 2016). Vidal and Jaber (2015) suggested that the effects observed on survival and development in insects exposed to plants endophytically colonised by *B. bassiana* could be linked either to the induction of host plant defence mechanisms or to the direct action of fungal metabolites.

Insects have a powerful immune system, enabling them to defend themselves against foreign bodies and reflecting their evolutionary success (Strand et al., 2008). In the present study, we analysed the endophytic effect of *B. bassiana* on the immune response, particularly the cellular immunity of *S. littoralis*, which is mediated by blood cells (Lavine et al., 2002). Bb-larvae, at day one of the sixth instar, although showing no alteration in haemocyte number or viability, showed a marked reduction in nodulation and encapsulation responses (Figure 4). Conversely, they showed a slight but significant increase in their ability to phagocytose bacteria *in vitro* compared with control larvae. Previous studies have already shown the effect of *B. bassiana*, in its role as an entomopathogen, inducing a reduction in the production of haemocytes and the capacity of insects to phagocytose the spores of the phytophagous fungus (Jinbin and Xiuli et al., 1996). Similar results were demonstrated for larvae of *H. armigera* (Zhong et al., 2017) and *S. frugiperda* (Wang et al. 2022) infected with the EPF *Metharhizium rileyi*, which caused an alteration in their cellular and humoral immune capacity without, however, inducing an alteration in the total number of haemocytes. These results seem to indicate that the presence

of *B. bassiana* in the tissues of tomato plants results in an immuno-depressed phenotype of the larvae fed on the leaves of these treated plants. Although there is an increase in the phagocytosis process, this is slight compared with the decreases in the nodulation and encapsulation processes

The endophytic effect of *B. bassiana* on the cellular immune system of *S. littorali*s may be induced by the plant's systemic defences activated by the presence of the fungus. For example, Mollah *et al*. (2021) showed that consumption of tomato leaves with high levels of endogenous SA caused a significant suppression of cellular immune capacity in *S. exigua*, making the insect vulnerable to attack by entomopathogenic microorganisms. In 2023, Cibills-Stewert *et al.* determined that the endophytic fungus Epichloë, able to produce alkaloids, inducing the activation of host plant defences and increasing the accumulation of silicon by the plant, induced a reduction in the cellular and humoral defences of *H. armigera*. In addition, a reduction in haemocyte numbers was observed in *S. frugiperda* fed on maize plants colonised by different *B. bassiana* strains (Puspita Sari et al., 2023). In future studies, it could be interesting to analyse the metabolic changes in the plant following endophytic colonisation by *B. bassiana* to understand the mechanisms involved in modulating the larval immune system, as well as larval development.

In summary, nodulation and encapsulation are two immunological processes that enable insects to limit fungal infections (Strand, 2008). By reducing the defence mechanisms of the herbivorous insect, EEF would therefore gain a clear advantage by reducing these immune responses, thus facilitating the pathogenesis. On the other hand, phagocytosis is a particularly effective process against bacteria. It is also conceivable that *B. bassiana* may benefit from the increase of phagocytosis, which potentially limits the development of opportunistic bacteria in the larvae parasitized by the fungus. This would enable them to limit competition from organisms capable of infecting, colonising and killing larvae more quickly than them. For instance, it has been observed that the oospore in metabolite of *B. bassiana* acts as an antimicrobial compound to limit microbial competition on *B. bassiana*-killed hosts. It allows the fungus for an optimised use of host nutrients on infected cadavers (Fan et al., 2017).

The reduction in the immune capacity of *S. littoralis* larvae fed on plants colonised by *B. bassiana* results in immunosuppressive individuals. to further explore the benefits of Bb as an endophyte, the susceptibility of the larvae to commercial fungal and bacterial biopesticides widely used in biological control was tested. The results show that the loss of immunocompetence generated by *B. bassiana* is able of promisingly increase the efficacy of these bioformulations. The lethal concentration of Naturalis® significantly amplifies mortality in immunocompromised larvae (Figure 5). These results go hand in hand with a reduction in the activity of the nodulation and encapsulation processes involved in fungal infection (Strand, 2008). In the case of XenTari®, used at a sub-lethal dose, immunocompromised larvae showed a dramatic increase in mortality (Figure 6). While the process of phagocytosis is effective against bacterial attacks, it is not sufficient in the event of major bacterial infection (Becchimanzi et al., 2019), and it is generally the process of nodulation that is used by the immune system in this type of situation. It therefore seems relevant to think that the increase in phagocytosis processes, induced by *B. bassiana*, is not enough to compensate the decrease in nodulation activity in the event of important bacterial infection, as demonstrated here by the use of XenTari®.

In conclusion, this study provides essential results for understanding the mechanisms and consequences underlying the complex multitrophic interactions between plants, insects and microbes. Here, our results suggest that endophytisation of tomato plants by *B. bassiana* is a promising tool to reduce the immunocompetence of Lepidopteran pest, and it can be used in conjunction with other biological control tools to increase their effect. It shows also a direct beneficial effect as a biocontrol agent with the increase mortality observed at the pupal stage. Moreover, it allows for a more targeted approach, affecting the insect feeding on the plant, while avoiding UV rays, low moisture and others abiotic extreme conditions. However, in-depth studies of this tri-trophic interaction between *B. bassiana*, tomato plants and *S. littoralis* are needed in the future to better understand and interpret the phenotypic effects observed. The mode of action of the fungus, its effects on the plant and the plant's response to colonisation are still poorly known. Transcriptomic and metabolomic analyses are needed to gain a better understanding of the physiological changes in the plant induced by the fungus. A study on the insect physiology should also be carried out including the role played by the insect microbiota. The multiple emerging roles played by EPF provide promising biocontrol potential, and such studies are needed to make the most of the endophytic action of these fungi and develop ever more sustainable and effective strategies for controlling plant pests and pathogens for the benefit of agriculture. In addition, understanding the fate and behaviour of microbial control agents is essential to prevent any undesired effect on non-target species.

Chapter 2: Mechanisms underlying the interaction between *Beauveria bassiana* colonising tomato plants and *Spodoptera littoralis* larvae

1. Introduction

Fungal entomopathogens are an effective way of controlling insect pests, which are responsible for considerable agricultural losses every year (Khan et al., 2012). They are therefore an attractive environmentally friendly alternative to chemical pesticides. Given the harmful effects of chemical pesticides on both the environment and human health, their use should be minimized (Imfeld and Vuilleumier, 2012). However, despite over a century of research and applications, fungal entomopathogens still face challenges such as susceptibility to ultraviolet (UV) light, drought and extreme environmental conditions, which hinder their optimal effectiveness in insect pest's control. Interestingly, fungal entomopathogens can adopt a variety of lifestyles, including functioning as endophytic microorganisms within plants (Azevedo et al., 2000; Vega et al., 2009). The ability of these entomopathogenic fungi to act as beneficial endophytic microorganisms has been increasingly investigated in recent years due to the benefits they offer in plant protection (Jaber and Ownley 2018; Vega et al., 2009; see also Chapter 1). Indeed, as endophytes, these fungi live part of their life cycle in the internal tissues of the plant, without causing any visible adverse symptoms to their host (Hyde and Soytong, 2008). Such a mutualistic association has regularly been shown to promote plant growth and affect insects feeding on colonised plants, reducing their performance while avoiding affecting non-target insects (Quesada-Moraga et al., 2022). This endophytic colonization overcomes the weakness of these fungi to the extreme environmental conditions they can face outside the plant (see Chapter 1).

However, the mechanisms underlying these activities are still poorly understood (Vidal and Jaber, 2015). In the literature, a significant number of studies have demonstrated the effect of entomopathogenic endophytes on the performance of insect herbivores (Gange et al., 2019). Nevertheless, in many of these studies, no fungal growth was found on the insect cadavers (Batta et al., 2013; Quesada-Moraga et al., 2009; Akutse et al. 2013). For instance, in 2013, Akutse et al., observed no fungal growth on the cadavers of more than 6,000 larvae of the leafminer *Liriomyza huidobrensis*, even though it was fully affected by the use of beans colonised by the fungus *B. bassiana*. The experiment in Chapter 1 led to the same result, since despite the reduced survival of *Spodoptera littoralis* pupae following the larval nutrition on the leaves of tomato plants colonized by *Beauveria bassiana*, no fungal growth was found on the cadavers of the insects. It would therefore seem that colonisation of the plant does not necessarily lead to direct mortality of the insect through the entomopathogenic effect of the fungus, and that other mechanisms are at work. There is strong evidence to suggest that entomopathogenic fungi use a combination of several mechanisms rather than a single one at a time (Vega et al., 2009; Ownley et al., 2010). In most of the above-mentioned articles, it is suggested that the reduced impact of insect pests is due (i) to the induction of plant defences or anti-nutritional compounds following the colonisation of the plant by the endophyte, with

indirect negative effects on the insect; or (ii) the production of fungal metabolites which have a negative impact on the insect.

It is estimated that arthropods account for 20 to 30% of annual agricultural losses worldwide (Southon et al., 2019). Among these pests, the Lepidoptera order includes some of the most voracious herbivores, causing a considerable reduction in agricultural productivity (Douglas, 2018). To develop new integrated pest management strategies that leverage the colonization of plants by endophytic entomopathogens, it is crucial to understand the mechanisms underlying multitrophic interactions between endophytes, plants, and Lepidoptera. This knowledge is key to effectively controlling these pests while minimizing the risks associated with chemical insecticide use.

First and foremost, it is imperative to understand how the nutritional and defensive plant traits are modulated by the presence of endophytic fungal entomopathogens. In fact, it remains unclear how colonisation alters primary and secondary plant metabolism and modulates general defence responses (Proietti et al., 2023). In particular, amino acids are attracting increasing interest in basic and applied sciences due to their importance in plant development and defence against biotic and abiotic stresses (Trovato et al., 2021). In addition to the roles described above, amino acids are the building blocks of protein synthesis or serve as precursors to numerous primary and secondary metabolites, or to certain phytohormones. For example, tryptophan is converted into indole-3-acetic acid, a plant hormone that regulates plant growth (Patten et al., 2013).

As well as being among the most important growth regulators, phytohormones also play a vital role in modulating plant defence mechanisms against various stressors (Egamberdieva et al., 2017). In tomatoes, the plant growth-promoting activity of *B. bassiana*, in addition to being linked to the production of iron siderophores and phosphate solubilisation, can be explained by the role of phytohormones (Barra-Bucarei et al., 2019). Here again, little is known about the modulation of phytohormone activity following endophytic colonisation. In 2019, Raad et al. demonstrated transcriptional reprogramming of plant defence pathways, particularly those of jasmonic acid (JA) and salicylic acid (SA) following colonisation of *Arabidopsis thaliana* by *B. bassiana*. Understanding how modifications to these nutritional and defensive plant traits by endophytes have an impact on Lepidoptera, and herbivorous insects in general, is also imperative in the context of setting up integrated pest management programmes.

It is essential to recognize that insect gut microorganisms play a fundamental role in their host's nutrition and immunity, which directly influences their performance in the environment (Engel and Moran, 2013). And interestingly, plant defences have been shown to impact the peritrophic membrane and epithelial layer of the insect gut (Konno and Mitsuhashi, 2019). By feeding on plants with different nutrient compositions and chemical defences, the microbial communities of herbivores and all the metabolic activities associated with them are therefore disrupted. For instance, the intestinal profile can be altered, giving way to a proliferation of opportunistic pathogenic bacteria capable of breaking the host's prevention mechanisms and evading immune defences (Vallet-Gely et al., 2008). In other words, plant traits influence the temporal and spatial variation of the gut microbiota and directly influence performances of the insect host (Mason, 2020).

Lepidopteran larvae have relatively simple intestinal morphologies, as well as the bacterial communities they host (Douglas, 2015). Although variable, their gut is generally composed of 5-7 main bacterial taxa belonging mainly to the genera *Enterococcus*, *Klebsiella*, *Enterobacter* and *Pseudomonas*. A few fungal taxa have also been documented (Chen et al., 2020; Mason et al., 2019; Paniagua Voirol et al., 2018). From a functional point of view, the gut microbiota of Lepidoptera is thought to play several roles, including a contribution to plant digestion and nutrient acquisition (Visôtto et al., 2009), resistance to allelochemicals and insecticides (Xia et al., 2018; Siddiqui et al., 2022) and an influence on host immunity (Engel and Moran, 2013). In 2022, Di Lelio et al. demonstrated the functional importance of the host's gut microbiota, particularly its role in nutritional support, by manipulating the gut microbiota of *S. littoralis* larvae. It has also been shown that nutritional and defensive variations in plant traits can affect the gut bacterial communities of Lepidoptera larvae (Hu et al., 2022; Gasmi et al., 2019).

How the insect immune system governs indirect interactions between plants and gut microbes, and how plant physiological traits shape insect microbial communities—particularly those triggered by endophytic entomopathogenic fungi— could open the door to innovative pest control strategies. In the present study, we investigate the endophytic role of *B. bassiana* in modulating the physiological traits of tomato plants, by analysing their amino acids and phytohormones content. In parallel, the impact of plant physiological alterations induced by *B. bassiana* endophytic colonisation on the composition of the gut microbiota and the metatranscriptome of the Lepidopteran pest *S. littoralis* is assessed. At the same time, variations in the haemocelic microbiota are also analysed to provide a more comprehensive functional picture of this system. This multifaceted experimental approach offers unique opportunities for elucidating the underlying functional mechanism governing endophytic fungi, plants and Lepidoptera pests. Based on results from Chapter 1 on plant mediated effect of *B. bassiana* on caterpillar performance, we hypothesized that (i) plant colonisation by *B. bassiana* up-regulates plant defences and nutrition; and that (ii) altered plant traits modify the composition of the gut and haemocelic bacterial community of *S. littoralis.*

2. Material and methods

2.1. *Beauveria bassiana* production and endophytic colonisation

The following experiments are all based on tomato plants (*Solanum lycopersicum* L., cv. ‘Dwarf San Marzano’) colonised by the entomopathogenic fungus *B. bassiana*. Inoculation of the fungus and confirmation of colonisation were carried out as described in the methods in the first chapter (see sections 2.1 and 2.2). This resulted in uncolonised control plants (C-plants), and plants colonised by *B. bassiana* (Bb-plants), all 6 weeks old.

2.2. Insects rearing

S. littoralis experimental larvae derived from a colony, established in 2009 at the University of Napoli Federico II (Department of Agricultural Sciences, Laboratory of Entomology "E. Tremblay", Portici-NA, IT), regularly refreshed once a year with field-collected insects. The larvae were reared on an artificial diet (41.4 g/l wheat germ, 59.2 g/l brewer's yeast, 165 g/l corn meal, 5.9 g/l ascorbic acid, 1.53 g/l benzoic acid, 1.8 g/l methyl-4-hydroxybenzoate, and 29.6 g/l agar), as previously described (Di Lelio et al., 2014), at 25 ± 1◦C, 70 ± 5% RH, and photoperiod of 16:8 h light/dark.

The aphid *Macrosiphum euphorbiae* was collected on tomato crops (Battipaglia, Salerno, Italy) and is permanently reared on tomato plants (*S. lycopersicum*, cultivar Dwarf San Marzano) isolated in rearing cages kept in a greenhouse at 20 ± 2◦C, 70 ± 10% RH, and photoperiod of 16:8 h light/dark.

2.3. Bioassays for amino acid and phytohormone analysis

For the metabolomic analysis, the fully expanded leaf from the first node of 6-week-old C- and Bb-plants was subjected to insect infestation. In experiments involving *S. littoralis*, one larva at the first day of the third instar was placed on each leaf. To prevent the larva from leaving the leaf, it was enclosed in a transparent tulle bag (10 × 15 cm) secured with a drawstring. In experiments with *M. euphorbiae*, tomato plants were placed inside insect-proof cages, and the target leaf was infested with five synchronized 2-day-old nymphs.
Non-infested plants served as controls. The insects were allowed to feed on the leaves for 48 hours in a climate-controlled chamber maintained at 25 ± 5°C, with 70 ± 5% RH and a 16:8 h light/dark photoperiod. After the feeding period, all insects were carefully removed to ensure that only insect-free leaf samples were collected. Non-infested leaves from control plants were also collected.
For each experimental group, a total of 21 plants were infested. Leaves from three plants were pooled to create one biological replicate. The collected leaves were immediately frozen in liquid nitrogen, ground into a fine powder using a mortar and pestle and stored at −80°C for subsequent metabolomic analysis.

2.3.1. Phytohormones analysis

Aliquots of 2 +/- 0.15 mg DW (ca. 10 mg FW) were collected from each sample to perform the analysis of phytohormones according to Prerostova *et al.* (2020). In brief, phytohormones were extracted using cold (-20°C) methanol/water/formic acid (15/4/1 v/v/v). The following isotope-labeled internal standards (10 pmol/samples) were added : $^{2}H_6$-iP, $^{15}N_4$-cZ, $^{2}H_5$-tZR, $^{2}H_6$-iPR, $^{2}H_3$-DZR, $^{2}H_5$-tZ7G, $^{2}H_6$-iP7G, $^{2}H_5$-tZROG, $^{2}H_6$-iPRMP, $^{2}H_6$-ABA, $^{2}H_5$-ABA-GE, $^{2}H_3$-PA, $^{2}H_3$-DPA (NRC-PBI, Canada), $^{2}H_5$-JA, $^{13}C_6$-IAA (Cambridge Isotope Laboratories), ($^{2}H_5$)($^{15}N_1$)-IAA-Asp, $^{2}H_2$-OxIAA (Olchemim, Czech Republic), $^{2}H_4$-SA (Sigma-Aldrich, MO,

USA). A centrifugation (17,000 g, 4°C, 20 min) of extracts has been performed to remove solid debris. Then, they were concentrated using an Alpha RVC vacuum centrifuge (Christ; 40°C, 15 mbar, 1.5 h). Phytohormones were separated using a reverse-phase cation exchange solid-phase extraction (SPE) column (Oasis-MCX, Waters) into an acidic fraction by elution with methanol for auxins, abscisic acid, salicylic acid and jasmonic acid; and into a basic fraction by elution with 0.35 M NH_4OH in 60% methanol for cytokinins. The fractions were dried in a vacuum centrifuge and resuspended in 30 µL acetonitrile (15%) for the acid fraction, and in 5% methanol for the basic fraction. Phytohormones were analyzed using high performance liquid chromatography (HPLC) (Ultimate 3000, Dionex) coupled to 3200 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems). The quantification was performed using the isotope dilution method with multilevel calibration curves ($r^2 > 0.99$). Finally, data were processed with the Analyst 1.5 software package (Applied Biosystems).
Statistical analyses were conducted using the 'R' software version 4.2.2 (R Development Core Team 2022). Figures were obtained using the package ggplot2 (Wickham, 2011). Multifactorial ANOVAs and post hoc tests were conducted to analyse the impact studied factors (endophytisation and herbivory) on free amino acids content. Model validations were performed using Shapiro–Wilk and Levene's tests. To compare the content of some derivates of abscisic acid and auxins between control and colonised plants, Student's t-tests were performed.

2.3.2. Amino acid analysis

Free Amino acids were extracted from samples of 5 +/- 0.15 mg DW with 125µL of extraction buffer H_2O and Methanol (Fisher Scientific OptimaTM, LCMS grade). The samples were then vortexed for 5 minutes, sonicated in a water bath for 1 minute, then centrifuged for 5 minutes (13,000 rpm, 17,900 x g, 4°C, 5 minutes) to remove cellular debris. Finally, the supernatant was collected in vials with inserts. This extraction cycle was performed a second time for each sample, corresponding to a total of 250µL of extract transferred into vials with 250 µL insert (Agilent Technologies) enabling the instrument to take this small volume. Then, to quantify amino acid content, 10µL of each extract was derivatized using the AccQ-Tag Ultra Chemistry Kit and following the manufacturer's protocol (AccQ-Tag Ultra Derivatization Kit Care and Use, 715001331 WatersTM). Norvaline (internal standard) and Amino acid standards were prepared to have a final concentration of 250µM. 1 µL was injected into a ultraperformance liquid chromatography (UPLC, WatersTM Acquity H class) equipped of AccQ-Tag Ultra C18 column (2.1 x 100mm, 1.7µm).
The gradient elution was performed using mobile phase A (Milli-Q water from the laboratory) and mobile phase B (Fisher Scientific OptimaTM, LCMS grade), containing 0.1% of formic acid (LCMS grade Fluka Honeywell) at a flow rate of 0.5 mL/min, column temperature of 50 °C, and gradient conditions as follows 0 to 0.54 min, 95% A and 5% B; 0.54 to 5.74 min, 80% A and 20% B; 5.74 to 7.74 min, 60% A and 40% B; 7.74 to 8.04 min, 40.4% A and 59.6% B; 8.04 to 8.64 min, 10% A and 90% B; 8.05 to 8.64 min, 10% A and 90% B; 8.64 to 8.73 min, 95% A and 5% B; and 8.73 to 12 min, 95% A and 5% B. Then, derivatized amino acids were detected at 260 nm using a photodiode array (PDA) detector (WatersTM Acquity H class).

Amino acid identity was determined with retention times and confirmed, with the mass spectra of Amino Acid Standards. Mass spectra of Amino Acid Standards was performed using a UPLC connected to Synapt G2-Si Q-TOF mass spectrometer (WatersTM) as detector. The MS was equipped with an electrospray (ESI) probe in positive ionization and resolution mode. The MS parameters were set as follows: capillary at 0.5 kV; source temperature at 120 °C; sampling cone at 40 eV; desolvatation temperature at 400 °C and desolvatation gas flow at 600 L/h. Data acquisition and quantification were performed using MassLynx and Quanlynx softwares (WatersTM).
Statistical analyses were conducted using the same approach used for phytohormone analysis (see section 2.3.1).

2.4. Bioassays on *Spodoptera littoralis*

To study the plant-mediated effect of *B. bassiana* on insect microbiota, bioassays consisting of feeding *S. littoralis* larvae with plants colonised by the fungus and uncolonised plants were conducted, as described in the 1st chapter (section 2.4.).
Briefly, newly hatched *S. littorali*s larvae were fed daily with leaves from C-plants or Bb-plants, until day-two 6th instar. Bioassays were carried out in environmental chamber at 25 ± 1°C, photoperiod 16:8 light/dark.

2.5. *S. littoralis* larvae tissues collection

Day-two 6th instar Bb-larvae and C-larvae were cold-anesthetized and surface-sterilized by washing with distilled water, washing with a 4% solution of sodium hypochlorite for 3 min, washing with EtOH 70% in ddH_2O (V/V) for 3 min and finally with ddH_2O. Each of these steps was performed by gently shacking the tubes containing the larvae. Then, excess water was removed using sterilized filter paper and larvae were dissected under a horizontal-flow hood to collect the haemolymph and the midgut under sterile conditions. Briefly a thoracic leg was cut, and 60 µL of the exuding haemolymph from each larva was collected in a 1.5 mL Eppendorf containing 200 µL of TRIzol™ (Life Technologies) and stored at -80°C until RNA extraction, which was performed according to the manufacturer's instructions. The larva was then cut longitudinally, the fat body and tracheal residues were removed, and the explanted midgut was opened lengthwise with micro scissors to separate the midgut epithelium from the peritrophic matrix and its content. The collected midgut was washed with sterile Phosphate buffered saline (PBS) 1x. Each sample was immediately placed in TRIzol™ (Life Technologies) and stored at -80°C until RNA extraction, which was performed according to the manufacturer's instructions. For the haemolymph, the midgut epithelium and the peritrophic matrix content, the RNA extracted from three 6th instar larvae was pooled at equimolar concentration and used as a biological replicate in the High-Throughput Sequencing analyses (midgut epithelium transcriptomics and metatranscriptomics); 7 replicates per group were used for midgut epithelium and microbiota metatranscriptomics.

2.6. Midgut transcriptomics

RNA purity was evaluated by 260 nm/280 nm absorbance ratio with NanoDropTM 1000 spectrophotometer (Thermo Fisher Scientific), while the concentration and the quality were assessed with TapeStation 4200 (Agilent Technologies). RNA samples from midgut and haemolymph were used as templates in library preparation. Each indexed library was prepared from 500 ng of purified RNA with TruSeq Stranded mRNA Sample Prep Kit (Illumina), according to the manufacturer's instructions. Libraries were quantified using the TapeStation 4200 (Agilent Technologies) and Qubit fluorometer (Invitrogen Co.), then equimolar pooled to a final concentration of 2 nM. The pooled samples were subjected to cluster generation and sequencing using an Illumina NextSeq 500 System (Illumina) in a 2×150 paired-end format at a final concentration of 1.8 pmol. A quality check was performed on the 150 nt paired reads using FastQC (Lo et al. 2014) after trimming and adapter removal using trimmomatic (Bolger et al. 2014). Host-derived reads were removed by mapping on the reference genome of *S. litura* (GCA_002706865.1 - ASM270686v1; Cheng et al., 2017) using Bowtie2 (v2.7.5; Langmead et al., 2019). The compositional profile of the composition of the microbial community was generated using Metaphlan and the functional profiling was carried out using HUMAnN 3.0 (Beghini et al., 2021); to allow a direct comparison among samples, abundances were normalized using copies per million (CPM). A principal component analysis leveraging CPM-normalized pathway abundances was performed with R and visualized using ggplot2 (R Core Team, 2020; Wickham et al., 2011). To identify metabolic pathway changes associated with the treatments we leveraged MaAsLin 2 using a linear model and arcsine square root transformation for proportional relative abundance data; pathway abundances were considered to be different when $q < 0.15$ (Mallick et al., 2021).

2.7. Bacterial community characterization

From RNA extracted from the midgut and haemolymph (500 µg total per sample), complementary DNA (cDNA) was synthesized. Libraries were prepared for all samples and extraction blanks using the 341F/785R primer pair (Herlemann et al., 2011), targeting the V3-V4 regions of the bacterial 16S rRNA gene, following the Metagenomic Sequencing Library Preparation (Illumina, US). Sequencing was performed on an Illumina NovaSeq platform (250 bp paired-end mode) at IGATech (Udine, Italy). Raw data were deposited in the NCBI SRA under project ID xx (to be added upon submission). Bioinformatic processing was conducted with QIIME2 version 2022.11 (Bolyen et al., 2019) and R version 3.6.2 (R Core Team, 2019). Initial quality control of the reads was assessed using FastQC (Andrews, 2010), followed by sequence denoising and chimera removal with the DADA2 algorithm (Callahan et al., 2016) to generate Amplicon Sequence Variants (ASVs). Taxonomic classification was performed using a naïve Bayes classifier (Bokulich et al., 2018) trained on the SILVA database v.138 (Quast et al., 2012) (classification confidence > 0.95). The q2-clawback plugin (Kaehler et al., 2019) was used to refine taxonomic weights, improving classification accuracy. The sequences assigned to mitochondria or chloroplasts (0.9% of the reads) and those recovered in the negative control (3% of the reads) were filtered out. Normalized ASVs tables were generated by scaling with

ranked subsampling (Beule & Karlovsky, 2020; Heidrich et al., 2021) at the depth of 203,477 sequences per sample and then used to estimate Bray Curtis and Jaccard dissimilarity matrices. These matrices served as inputs for Permutational Multivariate Analysis of Variance (PERMANOVA) using the Adonis function, to assess the influence of treatment and tissue type on bacterial diversity (Anderson, 2001). The R packages iNEXT (Hsieh et al., 2016) was used to estimate the alpha diversity using the statistical framework of Hill numbers (Roswell et al., 2021) and compare this estimate among different groups of samples with a rarefaction-extrapolation approach (Chao et al., 2014).

3. Results

3.1. Effect of *B. bassiana* endophytism on defence signalling and metabolomic profile of tomato plants

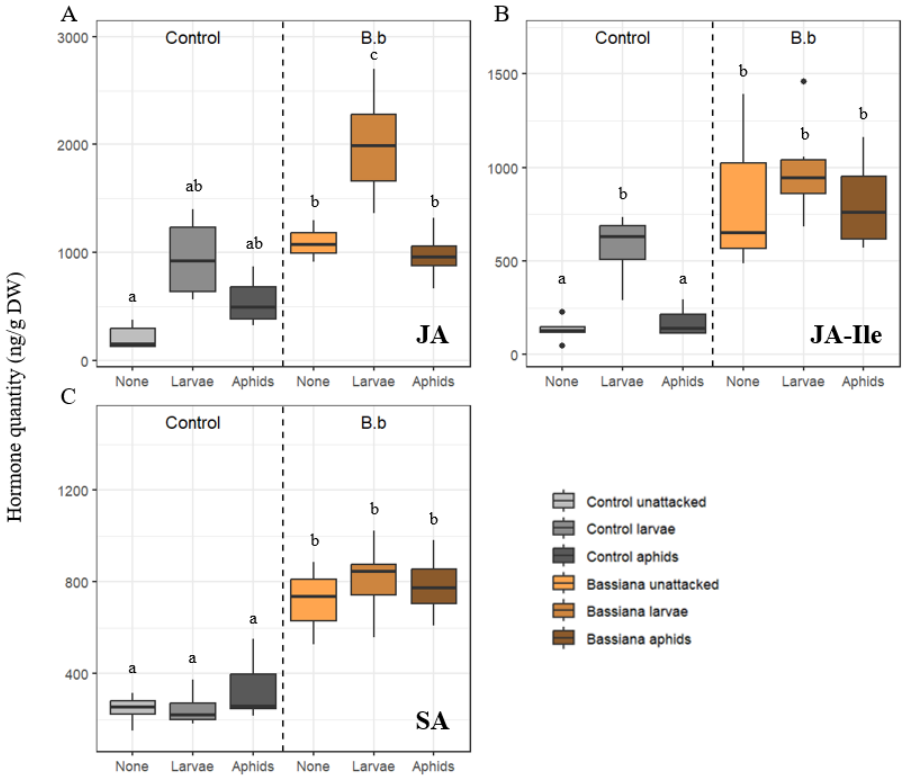


Figure 1: Impact of Beauveria bassiana plant inoculation and herbivory on plant systemic defence hormones content (JA, JA-Ile and SA). *(A) Jasmonic acid. (B) Jasmonic acid-isoleucine. (C) Salicylic acid. Leaves uncolonised by the fungus (grey) and leaves colonised by B. bassiana (orange/marron) subject to no attack (none), Spodoptera littoralis attack (larvae) or Macrosiphum euphorbiae attack (aphids). Boxes represent the interquartile range, inner lines in bold represent the median, whiskers represent maxima and minima within 1.5 times the interquartile range and black dots represent outliers. Treatments not sharing a letter are statistically different based on two-way ANOVA followed by Tukey-Kramer's (HSD) post hoc for unbalanced sample size ($p < 0.05$). JA: Inoculation effect ($p < 0,001$), Herbivory effect ($p < 0,001$), Inoculation x Herbivory effect ($p < 0,05$). JA-Ile: Inoculation effect ($p < 0,001$), Herbivory effect ($p < 0,001$), Inoculation x Herbivory effect ($p < 0,05$). SA: Inoculation effect ($p < 0,001$), Herbivory effect ($p = 0,4291$), Inoculation x Herbivory effect ($p = 0,6222$). The number of replicates for each treatment is the following: Control unattacked = 6, Control larvae = 4, Control aphids =7, Bassiana unattacked = 3, Bassiana larvae = 6, Bassiana aphids = 4.*

Phytohormone and amino acid profile of Bb- and C-plants were assessed (i.e. plants inoculated with *B. bassiana* or with sterile water) to determine whether endophytism of tomato plants by *B. bassiana* induces a reconfiguration of plant defences and metabolomic profile. Moreover, to broaden the scope of our understanding, experimental plants were exposed to two different herbivorous pests (*S. littoralis* larvae, a chewing pest, and *M. euphorbiae,* a sap-sucking pest), allowing to determine if changes in the plant induced by the fungus can be modulated by herbivory. This analysis led to quantification of jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), auxins, cytokinin and some of their precursors, as well as free amino acids (FAAs).

Phytohormones involved in plant systemic defence, such as JA and SA, were induced by *B. bassiana* endophytism (Figure 1). JA titer increased after endophytism and insect feeding, especially chewing insects, while SA titer was upregulated by endophytism alone and not affected by insect feeding. In addition, the jasmonic acid-isoleucine (JA-Ile), active derivate of jasmonic acid, is also induced by *B. bassiana* but not by insect feeding.
Abscisic acid (ABA) involved in physiological processes regulation such as seed maturation, seed dormancy, stress adaptation, water status and stomatal movement, shows an increase in response to endophytism by *B. bassiana*. We observe also a significant increase when attacked by aphids, however, this increase following aphid attack is not found in plants colonized by B. bassiana. In reverse, ABA-GE, the reversibly inactive form of ABA, shows a significant decrease (Figure 2).

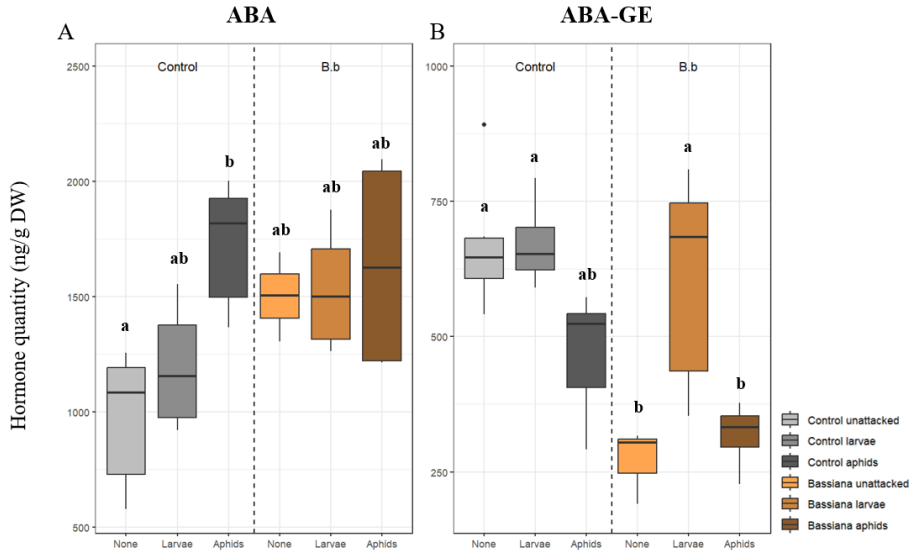


Figure 2: Impact of Beauveria bassiana plant inoculation and herbivory on abscisic acid content (ABA and ABA-GE). *(A) Abscisic acid. (B) Abscisic acid glucosyl ester. Leaves uncolonised by the fungus (grey) and leaves colonised by B. bassiana (orange/marron) subject to no attack (none), Spodoptera littoralis attack (larvae) or Macrosiphum euphorbiae attack (aphids). Boxes represent the interquartile range, inner lines in bold represent the median, whiskers represent maxima and minima within 1.5 times the interquartile range and black dots represent outliers. Treatments not sharing a letter are statistically different based on two-way ANOVA followed by Tukey-Kramer's (HSD) post hoc for unbalanced sample size ($p < 0.05$). ABA: Inoculation effect ($p = 0.1248$), Herbivory effect ($p = 0,338$), Inoculation x Herbivory effect ($p < 0,05$). ABA-GE: Inoculation effect ($p < 0,01$), Herbivory effect ($p < 0,001$), Inoculation x Herbivory effect ($p < 0,05$). The number of replicates for each treatment is the following: Control unattacked = 6, Control larvae = 4, Control aphids =7, Bassiana unattacked = 3, Bassiana larvae = 6, Bassiana aphids = 4.*

As the difference in plant auxin content can only be explained by the inoculation of plants with *B. bassiana*, we decided to show only the comparison of treatments not exposed to herbivory, to make the observation of results clearer.
Our study shows an increase in the title of some auxins and their derivates, such as indole-acetic-acid (IAA) (although not significative), as well as its precursors, I3A and IAM, after endophytism by *B. bassiana* (Figure 3). A slight increase of the *Phenylacetic acid* and its precursor, the *Phenylacetamide,* are also observed, although it is not significant.

The quantity of cytokinins was not influenced by inoculation of tomato plants with *B. bassiana* or by herbivory (data not shown).

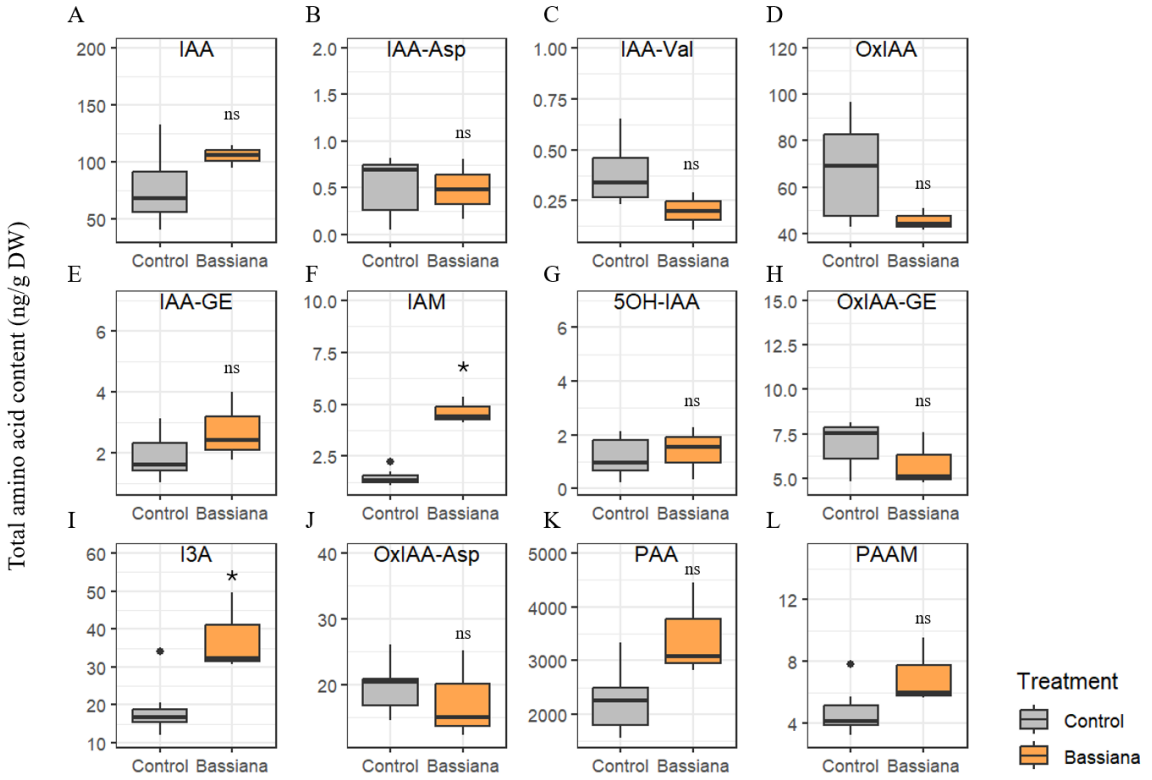


Figure 3: Impact of Beauveria bassiana plant inoculation on auxins content. *(A) Indole-3-acetic acid; (B) Indole-3-acetic acid-aspartic acid; (C) Indole-3-acetic valine acid; (D) 2-oxindole-3-acetic acid; (E) IAA-glucosyl ester; (F) Indole-3-acetamide; (G) 5-hydroxy indole-3-acetic acid; (H) 2-oxindole-3-acetic acid-glucosyl ester; (I) Indole-3-aldehyde; (J) 2-oxindole-3-acetyl-amino acid; (K) Phenylacetic acid; (L) Phenylacetamide. Leaves uncolonised by the fungus (grey) and leaves colonised by B. bassiana (orange). The lower and upper edges of the boxplots represent the 25% and 75% quantiles; the intermediate lines represent the median; the whiskers indicate the smallest and largest non-outliers; black dots represent outliers. Symbols on graphics indicate significant (*p < 0.05) and non-significant differences (ns = p > 0.05) according to whether the tomato plants were inoculated with B. bassiana.*

Finally, a general decrease of free amino acids in leaves in presence of *B. bassiana* was observed (Figure 4). As with auxins, only the effect of the fungus explains the difference between the different treatments, so only these data are shown to make the differences more visible. In detail, we can observe a significant reduction of glycine, glutamic acid and phenylalanine. A reduction of asparagine and alanine is also observed but was not statistically significative (p-value = 0.07259 and p-value = 0.05061 respectively).

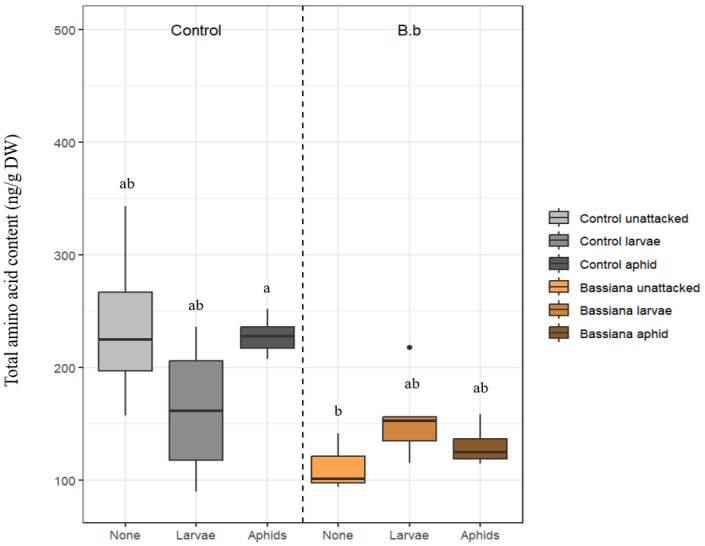


Figure 4: Impact of Beauveria bassiana plant inoculation and herbivory on total free amino acid content. *Leaves uncolonised by the fungus (grey) and leaves colonised by B. bassiana (orange/marron) subject to no attack (none), S. littoralis attack (larvae) or M. euphorbiae attack (aphids). Boxes represent the interquartile range, inner lines in bold represent the median, whiskers represent maxima and minima within 1.5 times the interquartile range and black dots represent outliers. Treatments not sharing a letter are statistically different based on two-way ANOVA followed by Tukey-Kramer's (HSD) post hoc for unbalanced sample size ($p < 0.05$). Inoculation effect ($p < 0,001$); Herbivory effect ($p = 0,2874$); Inoculation x Herbivory effect ($p = 0,003$).*

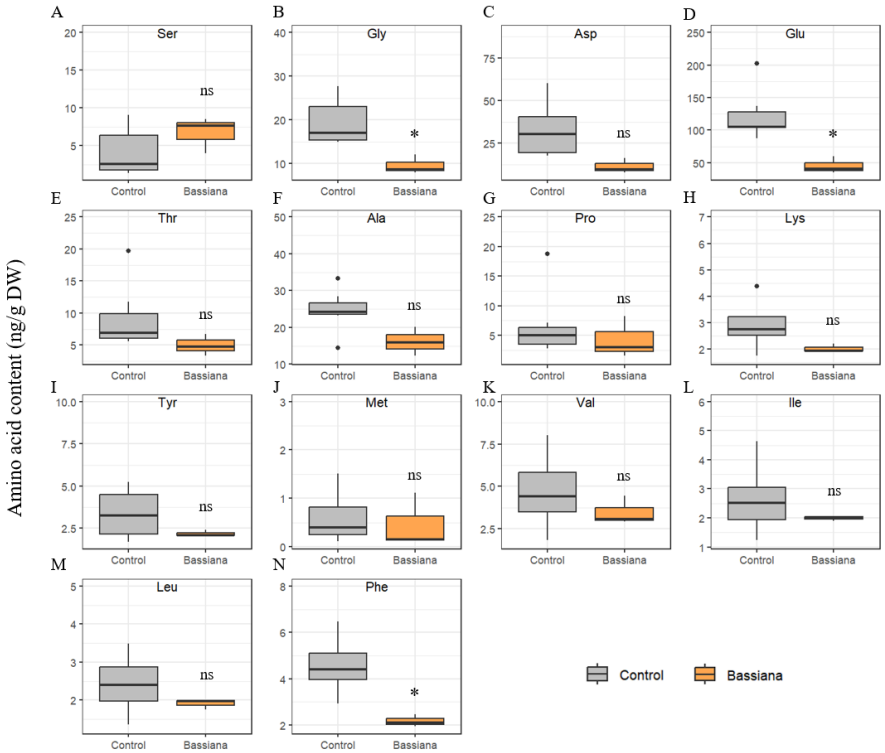


Figure 5: Impact of Beauveria bassiana plant inoculation on amino acids content. *(A) Serine; (B) Glycine; (C)Aspartic acid; (D) Glutamic acid; (E) Threonine; (F) Alanine; (G) Proline; (H) Lysine; (I) Tyrosine; (J) Methionine; (K) Valine; (L) Isoleucine; (M) Leucine; (N) Phenylalanine. Leaves uncolonised by the fungus (grey) and leaves colonised by B. bassiana (orange). The lower and upper edges of the boxplots represent the 25% and 75% quantiles; the intermediate lines represent the median; the whiskers indicate the smallest and largest non-outliers; black dots represent outliers. Symbols on graphics indicate significant (*$p < 0.05$) and non-significant differences (ns = $p > 0.05$) according to whether the tomato plants were inoculated with B. bassiana. All statistical indices are reported in Table S2.*

3.2. Effect of *B. bassiana* endophytism on composition and activity of insect haemocelic and gut microbiota

3.2.1. Bacterial community characterization

A total of 28,702,653 paired-end reads (1,025,095 reads per sample on average) were obtained from sequencing. After denoising and filtering steps, 12,914,368 sequences remained (464,203 reads per sample on average), corresponding to 2,620 unique sequences (ASVs).

Table 1. Relative abundance of dominant bacterial genera (>3% relative abundance) in the samples analyzed. For each sample group, median values of relative abundance per taxon are reported in percentage.

BACTERIAL TAXON	HEAM. C-	HEAM. BB-	PER. C-	PER. BB-
Tyzzerella	49.353	45.167	8.451	8.572
Enterococcus	24.887	42.761	12.401	11.420
Erysipelatoclostridium	14.383	7.881	2.341	3.307
Pseudomonas	0.755	0.701	34.505	19.008
Acinetobacter	0.589	0.671	0.736	5.455
Lactobacillus	0.454	0.437	0.478	16.182
Acetobacter	0.488	0.335	0.215	13.677
Massilia	0.046	0.115	2.810	3.143
Serratia	0.304	0.023	19.863	5.575
Erysipelotrichaceae gen.	3.815	0.011	0.020	1.831
Chryseobacterium	0.120	0.008	12.383	3.066

Abbreviations: Haem. = haemolymph; Per. = peritrophic matrix.

In haemolymph bacterial communities, the dominant genera (group mean relative abundance > 3%) were *Tyzzerella*, *Enterococcus*, and *Erysipelatoclostridium*, present at similar relative abundances both in Bb- and C-larvae (Tab. 1; Fig. 6). In C-larvae, an additional dominant taxon from the family Erysipelotrichaceae was detected. In the peritrophic matrix, bacterial communities were again dominated by *Tyzzerella* and *Enterococcus*, alongside *Pseudomonas*, *Serratia*, *Chryseobacterium*, and *Massilia* (Tab. 1; Fig. 6). *Lactobacillus*, *Acetobacter*, and *Acinetobacter* were dominant only in the BB-larvae peritrophic matrix community, and not in the one of C-larvae (Tab. 1; Fig. 6).

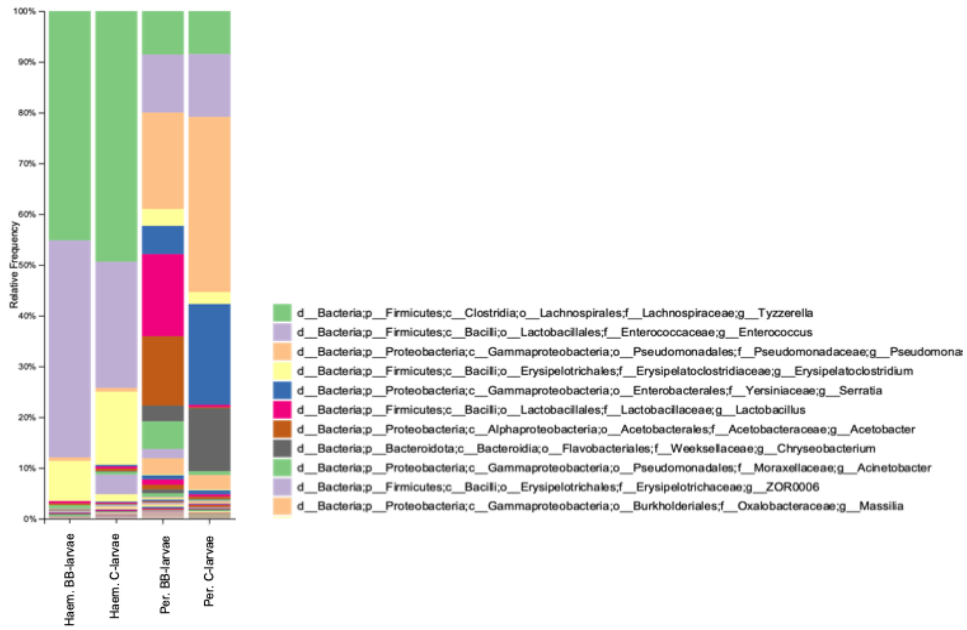


Figure 6: Taxonomic composition of the bacterial communities of haemolymph and peritrophic matrix of C- and Bb-larvae. *Taxonomic identifications are shown at genus level. Abbreviations: Haem. = haemolymph; Per. = peritrophic matrix.*

The bacterial community's alpha diversity was higher in the peritrophic matrix of BB-larvae compared to C-larvae (Fig. 7). In contrast, the diversity of haemolymph-associated bacterial communities was moderately but significantly lower in Bb-larvae than in C-larvae, for all values of q (Fig. 7). PERMANOVA analysis on the Bray-Curtis dissimilarity matrix indicated a significant effect of treatment on microbiota structure (ADONIS $R^2 = 0.124$, $P < 0.001$), although the tissue type was the primary determinant (ADONIS $R^2 = 0.606$, $P < 0.001$; Tab. 2). Similarly, PERMANOVA on the Jaccard dissimilarity matrix revealed a significant influence of both treatment and tissue type, though in this case, the effect of tissue type was closer in magnitude to that of treatment (treatment: ADONIS $R^2 = 0.103$, $P < 0.001$; tissue type: ADONIS $R^2 = 0.153$, $P < 0.001$; Tab. 3). Collectively, these results indicate that differences in microbiota structure between tissues are primarily driven by variations in the most abundant taxa, while treatments induce structural changes that similarly impact both taxon richness and abundance.

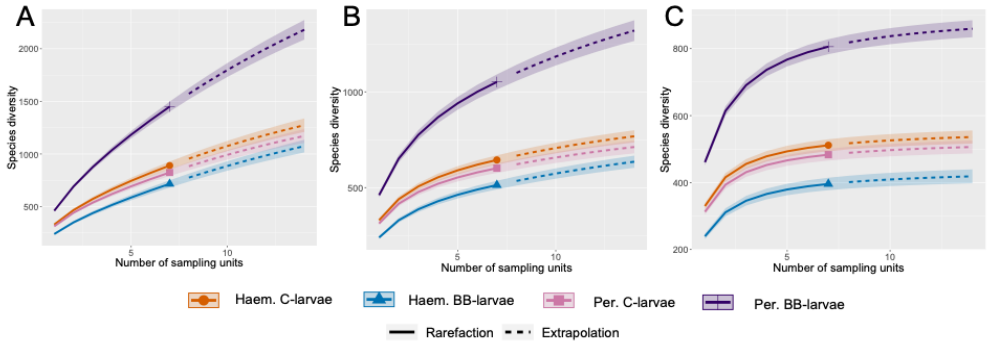


Figure 7: Alpha diversity of the bacterial community of Bb- and C-larvae. *Coverage-based interpolation and extrapolation curves depict alpha diversity, estimated using Hill numbers for different values of q: (A) q = 0, (B) q = 1, and (C) q = 2. The x-axis represents the number of samples, while the y-axis shows the alpha diversity estimates. Colours indicate the analysed groups, and line types denote the methodological approach used. Abbreviations: Haem. = haemolymph; Per. = peritrophic matrix.*

Table 2. Results of PERMANOVA analysis on the Bray-Curtis dissimilarity matrix

	DF	Sums Of Sqs	Mean Sqs	F stat.	R^2	P-value
Treatment	1	0.616	0.616	21.140	0.124	> 0.001
Tissue	1	2.997	2.997	102.930	0.606	> 0.001
Treatment:Tissue	1	0.637	0.637	21.860	0.129	> 0.001
Residuals	24	0.699	0.029		0.141	
Total	27	4.948			1	

Abbreviations: DF = degrees of freedom; Sqs = squares; stat. = statistic; Haem. = haemolymph; Per. = peritrophic matrix

Table 3. Results of PERMANOVA analysis on the Jaccard dissimilarity matrix

	DF	Sums Of Sqs	Mean Sqs	F stat.	R^2	P-value
Treatment	1	0.712	0.712	3.812	0.103	> 0.001
Tissue	1	1.055	1.055	5.651	0.153	> 0.001
Treatment:Tissue	1	0.667	0.667	3.574	0.096	> 0.001
Residuals	24	4.483	0.187		0.648	
Total	27	6.918			1	

Abbreviations: DF = degrees of freedom; Sqs = squares; stat. = statistic; Haem. = haemolymph; Per. = peritrophic matrix.

3.2.2. Transcriptional changes in midgut microbiota

The metatranscriptomic experiment, after removing host reads and trimming, leveraged a total of 157,936,274 reads for C-larvae and 220,678,726 reads for BB-larvae, with average read counts per sample of 22,562,324 and 44,135,745, respectively. The midgut microbial community of Bb-larvae shows a significant increase in both the total number of expressed metabolic pathways and the diversity of transcriptionally active microbial species (Fig. 8A and Fig. 8B, respectively; Wilcox test $p < 0.0001$ for both comparisons). Across samples, *Enterococcus casseliflavus* emerged as the most transcriptionally active species. Notably, the abundance of *E. casseliflavus*, which plays a critical role in the nutritional support of *S. littoralis*, was significantly reduced in Bb-larvae compared to C-larvae (t-test $p = 0.042$; Figure 8C). Furthermore, multivariate analyses separate C-larvae and Bb-larvae based on either abundance or completeness values. Comparative transcriptional analysis based on pathway coverage - calculated from the presence and abundance of genes associated with a specific pathway - reflects the functional completeness of pathways within the microbial community. This analysis highlights that the pathways involved in the biosynthesis of amino acids (e.g., L-

arginine, L-lysine, and L-ornithine), as well as fatty acids, coenzyme A, aromatic amino acids, and branched-chain amino acids are more complete in Bb-larvae compared to C-larvae (Figure 9). Taxonomically, the two genera most associated with these changing pathways are *Enterococcus* and *Pseudomonas*, with the latter contributing substantially to increased pathway completeness (Figure 10). Using Maaslin2, an expanded repertoire of pathways with diverse functional roles was identified as changing abundance, including several that also exhibited changes in their coverage. These pathways include those for the biosynthesis of many amino acids, including L-ornithine, L-phenylalanine, L-citrulline, L-aspartate, L-asparagine, and the super pathway of aromatic amino acid biosynthesis.

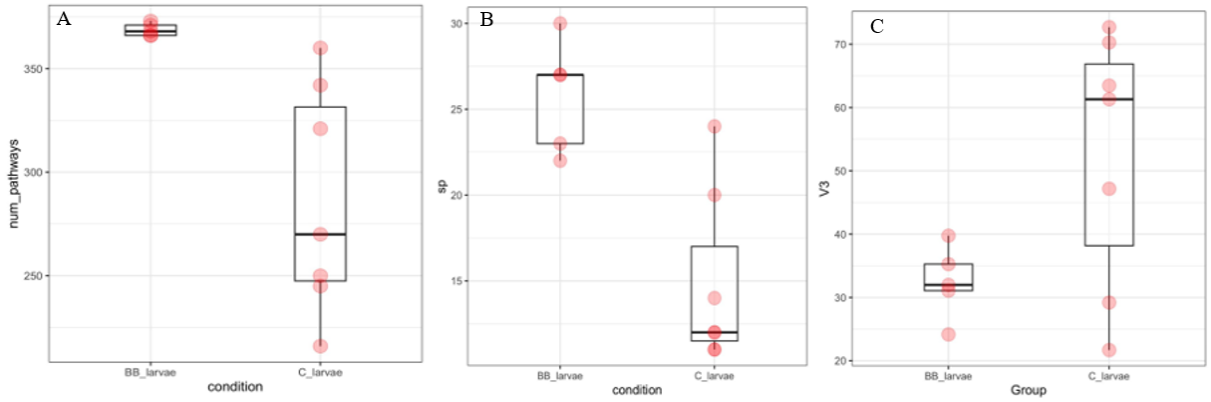


Figure 8: Functional composition of the bacterial communities in the C-larvae and BB-larvae peritrophic matrix. *The left panel shows the number of expressed pathways in each sample (A), while the middle panel displays the number of species (sp) detected as transcriptionally active (B) and the right panel the abundance of Enterococcus casseliflavus (C). Comparisons are statistically significant (p<0.0001).*

3.2.3. Transcriptional changes in midgut tissue

Transcriptional profiling of midgut cells in BB larvae generated 400,606,804 reads, mapping to 13,280 genes, with an average of 60% of reads assigned to annotated genes per sample. The differential expression of BB-larvae compared to C-larvae revealed significant differential expression of 297 genes, with 160 upregulated and 157 downregulated. Gene Ontology (GO) enrichment analysis of upregulated genes (Fig. 11A) identified key biological processes related to cellular ion homeostasis (GO:0006873), organic acid transport (GO:0015849), sodium ion transport (GO:0006814), and broader chemical homeostasis (GO:0048878). These findings suggest enhanced nutrient uptake mechanisms in BB larvae. Notably, enriched terms also included muscle cell differentiation (GO:0042692) and response to steroid hormones (GO:0048545), alongside evidence of immune response activation. Downregulated genes (Fig. 11B) were significantly enriched for GO terms associated with defence response to bacterium (GO:0042742) and cellular response to xenobiotic stimulus (GO:0071466). From a metabolic perspective, enrichment was observed for broader catabolic processes (GO:0009056), including proteolysis (GO:0006508) and purine nucleobase metabolism (GO:0006144).

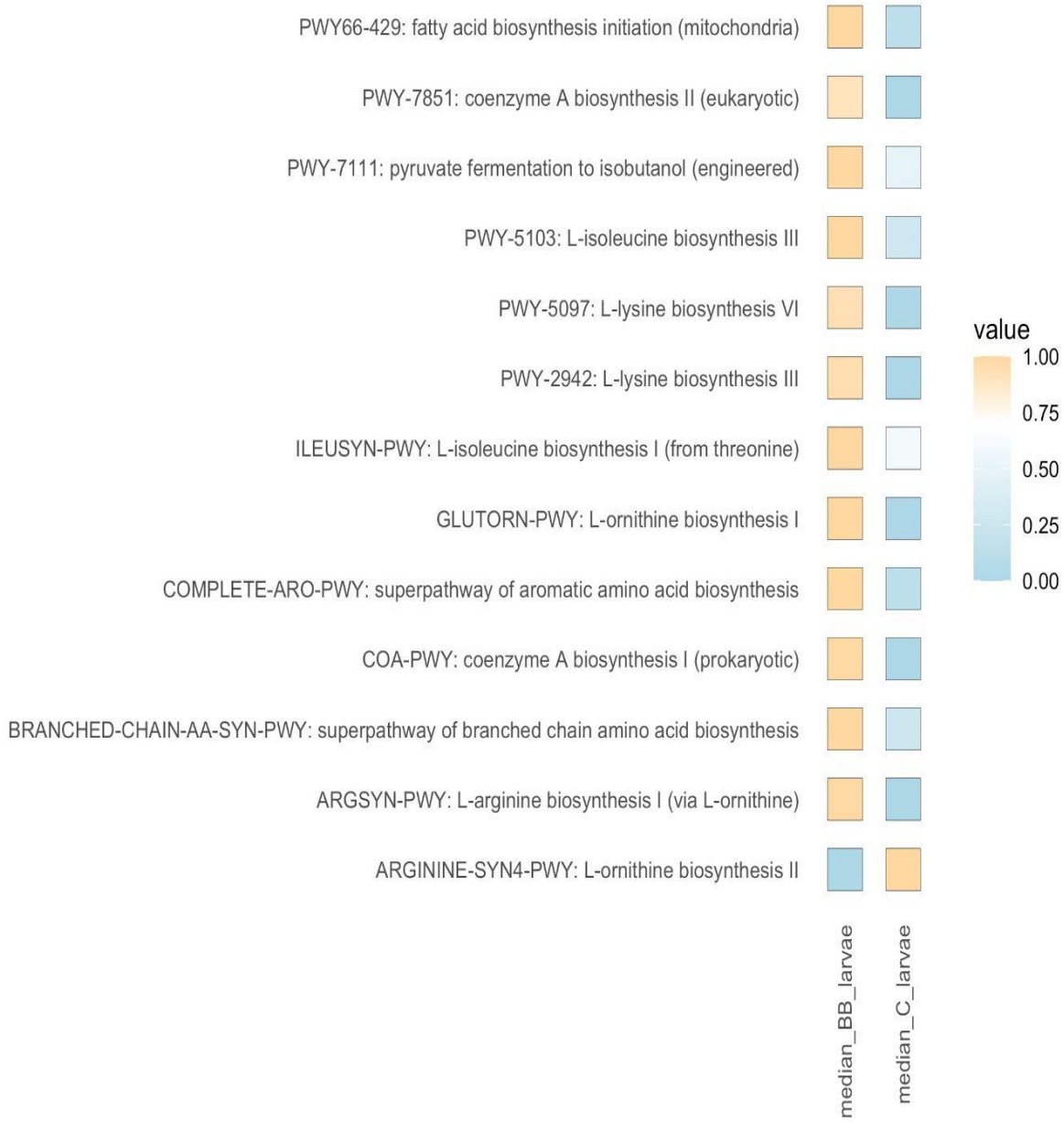


Figure 9: Metatranscriptomic pathways whose coverage is significantly associated with Bb-larvae. *The titles on the right of each pathway represent pathway completeness, ranging from 0 to 1. A pathway with a coverage of 1 is considered confidently detected, as this implies that all of its member reactions were also confidently detected. Conversely, a pathway with a coverage of 0 is considered less confidently detected.*



Figure 10: Pie charts representing the relative contribution of bacterial taxa to each specific pathway whose coverage is significantly associated with Bb-larvae.

id	term	p	sig/exp
GO:0098662	inorganic cation transmembrane transport	0.00011	3.9
GO:0015849	organic acid transport	0.00013	6.1
GO:0046942	carboxylic acid transport	0.00013	6.1
GO:0006820	anion transport	0.00015	4.1
GO:0098656	anion transmembrane transport	0.00018	4.4
GO:0098655	cation transmembrane transport	0.00033	3.4
GO:0015672	monovalent inorganic cation transport	0.00042	3.9
GO:0015711	organic anion transport	0.00066	4.1
GO:0035725	sodium ion transmembrane transport	0.00078	6.8
GO:0006812	cation transport	0.00079	3.1
GO:0045214	sarcomere organization	0.00107	8.7
GO:0051146	striated muscle cell differentiation	0.00110	5.1
GO:0006814	sodium ion transport	0.00139	6
GO:0048878	chemical homeostasis	0.00139	3.7
GO:0042692	muscle cell differentiation	0.00185	4.6
GO:0000768	syncytium formation by plasma membrane f...	0.00224	7.1
GO:0006949	syncytium formation	0.00224	7.1
GO:0007520	myoblast fusion	0.00224	7.1
GO:0140253	cell-cell fusion	0.00224	7.1
GO:0007525	somatic muscle development	0.00229	5.4
GO:0030239	myofibril assembly	0.00243	7
GO:0014902	myotube differentiation	0.00263	6.9
GO:0042391	regulation of membrane potential	0.00272	5.2
GO:0043401	steroid hormone mediated signaling pathw...	0.00310	10
GO:0071805	potassium ion transmembrane transport	0.00356	6.3
		0.01	0
		0	0

id	term	p	sig/exp
GO:0072523	purine-containing compound catabolic pro...	0.00021	13.3
GO:0006144	purine nucleobase metabolic process	0.00024	12.9
GO:0009112	nucleobase metabolic process	0.00126	8.3
GO:0050830	defense response to Gram-positive bacter...	0.00165	12.5
GO:0006805	xenobiotic metabolic process	0.00187	12
GO:0071466	cellular response to xenobiotic stimulus	0.00187	12
GO:0009056	catabolic process	0.00225	2.1
GO:0042742	defense response to bacterium	0.00270	5.2
GO:0042278	purine nucleoside metabolic process	0.00294	10.3
GO:0046128	purine ribonucleoside metabolic process	0.00294	10.3
GO:1901575	organic substance catabolic process	0.00304	2.2
GO:0006508	proteolysis	0.00340	2.3
GO:0009617	response to bacterium	0.00403	4.7
GO:0050829	defense response to Gram-negative bacter...	0.00557	8.3
GO:1901658	glycosyl compound catabolic process	0.00659	16.7
GO:0051923	sulfation	0.00774	14.3
GO:0010469	regulation of signaling receptor activit...	0.00810	7.1
GO:0009119	ribonucleoside metabolic process	0.00868	7
GO:0010951	negative regulation of endopeptidase act...	0.00897	13.3
		0.01	0
		0	0

Figure 11: Enriched Gene Ontology (GO) biological processes *for upregulated (orange, left) and downregulated (blue, right) genes are visualized, with the significance of each term (P-value) represented by a color gradient. The "sig/exp" columns indicate the ratio of observed to expected GO annotations for the analyzed gene sets, providing insights into term overrepresentation. Bar lengths are proportional to these ratio values. A detailed list of all enriched terms can be found in the supplementary materials.*

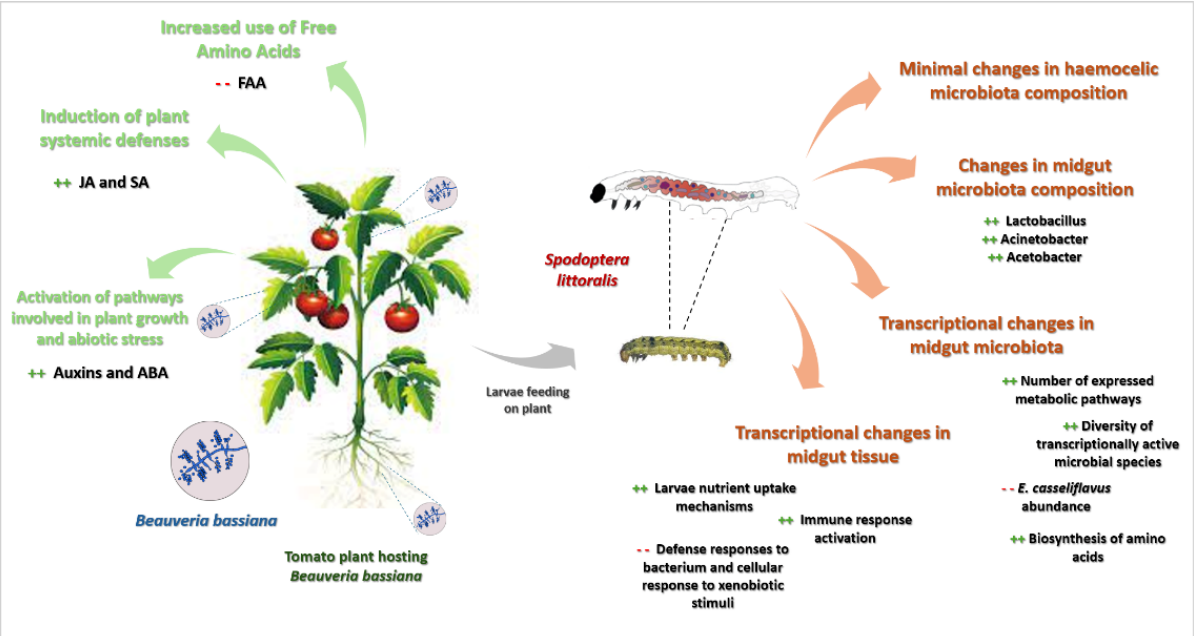


Figure 12: Graphical abstract of results from 2nd chapter. *FAA = Free Amino Acid; JA = Jasmonic acid; SA = Salicylic acid; ABA = Abscisic acid*

4. Discussion

Interactions among plants, insects, and endophytic entomopathogenic fungi have attracted significant interest in biological control. Recent studies link these interactions to the physiological reprogramming of plants, enhancing growth and strengthening defence mechanisms. This study investigates the interaction between tomatoes and *B. bassiana*, focusing on molecular changes in tomato leaves induced by fungal colonization, an area rarely explored (Wang et al., 2023; Proietti et al., 2023). Notably, we provide the first insights into how these changes are influenced by herbivory from two insect species representing distinct nutritional guilds.

Phytohormone analysis in the leaves of *Bb*-plants revealed upregulation of key hormones involved in systemic plant defence — JA and SA. Previous studies have highlighted the role of endophytic fungi in modulating JA levels in host plant (Han et al., 2020; Martinez - Medina et al., 2017; Chen et al., 2022). For instance, Chen et al. (2022) demonstrated that *Piriformospora indica* colonisation enhances herbivore resistance in rice through JA-signalling. Similarly, our findings align with research in tobacco, where *B. bassiana* triggered both SA and JA defence pathways, with SA being more strongly upregulated (Qin et al., 2020). JA-mediated signalling is well-known for regulating antiherbivore defences, particularly against chewing insects, while SA is often induced in response to sucking insect attack (Vos et al., 2013; Huot et al., 2014; Singh et al., 2018). In this study, we observed a marked activation of the JA pathway in response to *S. littoralis* larvae. Interestingly, this response amplified the baseline JA increase induced by *B. bassiana* colonization, further enhancing the plant's resistance to chewing insect attacks.

The elevated SA levels observed in *Bb*-plants may suggest that the endophytic fungus either triggers Systemic Acquired Resistance (SAR) or is perceived by the plant as a potential pathogen. Indeed, SA-dependent signaling plays a pivotal role in plant-microbe interactions, particularly during early stages of a symbiosis, where non-pathogenic microbes are often sensitive to SA-mediated defences (Pangesti et al., 2013; Rivas-Franco et al., 2020). We hypothesise that *B. bassiana* might initially be recognized as a biotrophic pathogen, prompting a rapid activation of the SA pathway. Proietti et al. (2023) similarly reported an upregulation of defence hormones in *B. bassiana*-colonized tomato plants, with an increase in JA and a downregulation of SA. This aligns with findings that, during established symbiosis, SA levels tend to normalize while JA levels remain elevated (Bastias et al., 2021). Interestingly, in our study, despite the fungus having colonized plants for several weeks, SA levels remained elevated, raising questions about the plant's continued recognition of the fungus. Future studies measuring SA content at different time points post-inoculation could provide deeper insights into how this defence pathway is dynamically regulated. Notably, the observed SA increase in this study occurred independently of herbivory, further emphasizing the need to explore its role in plant-fungal interactions over time.

Usually, it exists a trade-off between SA and JA (Erb et al., 2012). When one increases the second is suppressed. In our case we observe an induction of both pathways. This is interesting as it can prime the plant for different types of attackers. Basically, *B. bassiana* clearly primes plant defences against chewing insects with a double strategy: (i) increasing basal level of constitutive defences and (ii) priming the plant for a higher induced defence to chewing herbivores. While SA is primed but doesn't show a specific induction by herbivores even sap-sucking aphids. Hence, the SA pathway is primed but more as a constitutive defense with an increase of basal level. Globally, our results show that *B. bassiana* seems to trigger two different defence mechanisms leading to two different defence strategies for the plant.

Beyond enhancing systemic defences against biotic stresses, *B. bassiana* may also increase plant response to abiotic stresses through the upregulation of ABA. ABA is a key phytohormone that regulates vital physiological processes, including water balance and stomatal reaction in response to environmental changes (Sharp et al., 2000). Under stress conditions, plants often accumulate ABA to induce stomatal closure, minimizing water loss (Lim et al., 2015). This aligns with our earlier findings, which revealed increased water content in leaves of Bb-plants (see Chapter 1). Additionally, ABA can be temporarily inactivated through conjugation to form ABA-GE, which is stored in vacuoles or apoplasts (Spence et al., 2015). When dehydration or salt stress occurs, ABA-GE can be rapidly deconjugated by β-glucosidases releasing active ABA to trigger defence response. The contrasting levels of ABA and ABA-GE observed in our study further support the hypothesis that *B. bassiana* induces ABA upregulation, potentially enhancing the plant's ability to cope with environmental stressors.
ABA-deficient mutants of tomato and *Arabidopsis thaliana* have been shown to exhibit increased susceptibility to insect infestation, with a transcription profiling highlighting ABA's role in regulating herbivore-induced gene expression (Ton et al., 2009). This may explain the elevated ABA levels observed in control plants attacked by *M. euphorbiae*. Several studies have already linked ABA to plant defence against sap-sucking insects. For instance, its role in

countering green bug phloem feeding in sorghum has been well-documented (Morkunas et al., 2011). In our study, aphid attack appears to trigger significant ABA accumulation. However, fungus inoculation, alone or in combination with herbivory, does not appear to trigger significant ABA accumulation, suggesting a more complex interplay between ABA signalling, herbivory and fungal colonization.
Overall, our findings demonstrate a substantial enhancement of tomato plant defences following *B. bassiana* inoculation, which likely accounts for the observed detrimental effects on insect pests (Youssef-Youssef et al., 2023; Sinno et al., 2020; see also Chapter 1).

The increased levels of IAA and its precursors, IAM and I3A, in leaves following *B. bassiana* inoculation suggest a dual role in promoting both plant growth and defence, as previously reported (Jaber and Vidal, 2015). Interestingly, IAA production is not exclusive to plants; it is also synthesized by various microorganisms, including *B. bassiana* itself (Liao et al., 2017). However, these observations should be interpreted with caution. Sinno et al. (2021) highlighted strain-specific effect of *B. bassiana* on plant growth promotion. The ATCC 74740 strain used in this study has not been linked to significant growth enhancement in tomato plant, which may be explained by the modest increase in IAA observed here.

The overall reduction in free amino acids observed in leaves of Bb-plants aligns with the previously reported increase in protein content and synthesis (Proietti et al., 2023). This reduction likely reflects the utilization of free-amino acids for protein synthesis or the production of secondary metabolites (Trovato et al., 2021). For example, aromatic amino acids like phenylalanine serve as precursors for a wide range of secondary metabolites (Oliva et al., 2021), which may explain their decreased levels in Bb-plants.

In conclusion, *B. bassiana* colonization appears to prime plant defences (Conrath et al., 2006), enhancing their readiness for potential herbivore attacks. Moreover, these fungus-induced defences can be further amplified by herbivory, particularly through the jasmonic acid pathway in response to chewing insects like Lepidoptera. A more comprehensive metabolomic and transcriptomic analysis would offer deeper insights into how *B. bassiana* simultaneously promotes plant growth and strengthens defence mechanisms.

Using a 16S rRNA gene metabarcoding approach, we uncovered key differences in the microbiota of *S. littoralis* larvae fed on Bb-plants versus C-plants. This analysis revealed variations in bacterial composition, diversity and potential functional roles within the gut microbiota. Investigating Lepidoptera gut symbionts is particularly relevant due to their status as agricultural pests and the limited understanding of their microbiota's functions that limit their possible manipulation as an innovative tool for a sustainable agriculture (Voirol et al., 2018).

Consistent with previous studies on Lepidoptera (Chen et al., 2016), *S. littoralis* gut microbiota was poorly diversified at the genus level, with a few bacterial genera—such as *Tyzzerella*, *Enterococcus*, *Pseudomonas*, *Serratia*, *Chryseobacterium*, and *Massilia*—dominating the relative abundance of reads. The prominence of *Enterococcus* in the caterpillar community aligns with results from other plant-feeding caterpillars, including *S. littoralis* (Chen et al.,

2016; Ramírez-Serrano et al., 2022). The stability of this core microbiota across both experimental treatments suggests these bacteria may play crucial roles in the fitness and development of *S. littoralis*. This also implies potential vertical transmission, as the microbiota was conserved between treatments (Querejeta et al., 2022), supporting the idea of a stable, beneficial association between *S. littoralis* and its gut core microbiota.

Additionally, the Bb-larvae exhibited shifts in their bacterial communities, marked by increased diversity and the emergence of new dominant taxa including *Lactobacillus, Acetobacter* and *Acinetobacter*. These changes in bacterial composition between two treatments suggest environmental acquisition. Since leaf-associated bacteria are commonly found in insect gut microbiota (Hammer et al., 2017; Hansen and Enders, 2022), the altered defensive and nutritional properties of *B. bassiana*-treated plants may directly influence the microbiota of insects feeding on them (Hu et al., 2022).
The increase in systemic defence observed in Bb-plants suggests that secondary plant metabolites may influence the larval microbiota (War et al., 2020).
Additionally, our results show elevated expression of amino acid producing pathways in larvae from Bb-plants, particularly in *Pseudomonas*, which may be a response to the reduced amino acid content in the plants. This could indicate a microbial adaptation to limited free amino acids, with nitrogen-producing bacteria outcompeting other resources. These bacteria likely explain the increased amino acid expression in the larval gut. Furthermore, the enhanced nutrient uptake mechanisms observed in Bb larvae midgut tissues further support this idea.
Notably, the abundance of *E. casseliflavus*, which plays a critical role in the nutritional support of *S. littoralis*, was significantly lower in Bb-larvae compared to C-larvae. Its role in controlling the expansion of opportunistic bacteria may help to explain the increased diversity observed in the microbiota of Bb larvae (Shao et al., 2017).

While the impact of *B. bassiana* inoculation on plant nutritional and defensive changes is well-documented, the contribution of the resulting microbiota to insect performance remains less understood. In our previous study, larvae fed on Bb-plants exhibited increased size but reduced immunocompetence, alongside higher pupal mortality. Here, we demonstrate that the metabolic changes in tomato plants induced by *B. bassiana* can alter the bacterial composition in larvae, influencing their development, survival, and immunity. For instance, *Acetobacter* species, more abundant in *B. bassiana*-infected larvae, are known to support improved insect development (Newell PD et al., 2014). Similarly, *Acinetobacter* bacteria contribute to nitrogen availability, supporting the biosynthesis of proteins, nucleotides, and other essential biomolecules for insect growth (Bar-Shmuel et al., 2020; Ayayee et al., 2014). We hypothesize that these bacteria play a role in the increased size of *B. bassiana*-infected larvae.

The immune response activation observed in the larvae's epithelial tissue can be attributed to the increased diversity in their gut microbiota, likely under the control of the insect's immune system (Engel and Moran, 2013). Additionally, the higher abundance of *Lactobacillus* in the larval microbiota, known for providing protection against pathogens and supporting digestion and immune functions, may contribute to the immune response increase (Broderick et al., 2012).

Interestingly, there was minimal change in the composition of the haemocelic microbiota between the two experimental treatments, despite its close regulation by the immune system (Blow et al., 2019). This suggests a certain stability in the haemocoel, despite the significant reduction in immunocompetence observed in *B. bassiana* larvae.

At this stage, drawing definitive functional conclusions is challenging. The immunosuppressive roles of certain bacteria still need to be clarified to fully understand the cause-and-effect relationships between gut microbiota changes and larval performance. Further functional studies, such as microbiota reinoculation or specific antibiotic treatments, are necessary to explore the complete microbial functionality and identify potential pathogens in the insect microbiota

Overall, our results show that *B. bassiana*, via plants, can influence the gut microbiota profile of *S. littoralis*, as well as its performance. These results help to improve our understanding of the mechanisms involved in plant protection conferred by endophytic entomopathogenic fungi, highlighting their potential for the targeted development of new biocontrol products against pests.
Insect-plant interactions are part of a much wider and more complex network of interactions, finely modulated by microorganisms. Direct and indirect plant responses to microbial interactions can largely modulate plant-insect relationships through connections between organisms' microbiota. Taken together, these results show that interactions between living organisms can only be fully understood when they are analysed at the multitrophic level.

Chapter 3 - Compatibility between the endophytic entomopathogenic fungus *Beauveria bassiana* and the parasitoid *Meteorus pulchricornis* for the control of *Spodoptera littoralis* on tomato

1. Introduction

With nearly a million described species, the insects (class Insecta) represent one of the most diverse and ecologically significant groups on Earth (Yang and Gratton, 2014). Despite their critical contribution to ecosystem processes, their role as herbivores often brings them into conflicts with agricultural production, causing direct damage through feeding and indirectly by disease transmitting (Douglas, 2018).

To mitigate these impacts, biological control agents - including predators, parasitoids, plants and microorganisms - have been extensively studied as sustainable alternatives to synthetic pesticides in integrated pest management (IPM) strategies (Ons et al., 2020). While most research has focused on the effect of a single biological control agents, fewer studies have explored the synergistic potential of combining multiple agents (Anuar et al., 2023). However, relying on individual biological control agents is not always effective in reducing pest populations below economic thresholds, particularly in open-field conditions where ecological interactions are more complex. IPM addresses this challenge by integrating diverse control methods to optimize pests and pathogens suppression while minimizing reliance on chemical pesticides. Combining biocontrol agents with each other, or combining them with various cultural practices, can helps to minimise the weaknesses of each and allows for a more relevant solution under natural field conditions (Xu et al., 2011). In addition, by deploying agents with multiple modes of action, it reduces the likelihood of pests developing resistance (Tabashnik, 1989; Khan et al., 2012). Economically, it can lower input costs by reducing the need for each biocontrol agent. Demographically, targeting multiple pest life-history stages improves overall population management and minimizes pest resurgence. Thus, combining biocontrol agents within an IPM framework delivers a more sustainable and robust solution for pest control in modern agriculture.

Multitrophic interactions in biological control are inherently complex, making the combination of multiple biocontrol agents a strategy that requires careful evaluation. Ensuring compatibility between agents is critical, as their interactions - both direct and indirect - can yield positive or negative outcomes (Khan and Ahmad, 2015). While synergistic combinations can enhance pest suppression, incompatibilities may inhibit the effectiveness of one or both agents, undermining control efforts (Xu et al., 2011).

These interactions can be broadly classified into **three categories** (Caesar and Cech, 2019; Anuar et al., 2023; Koller et al., 2023):

1. **Positive Interactions** – When combined, the agents deliver **greater pest control** than their individual effects, either through **synergistic** (greater-than-additive) or **additive** impacts. Even interactions that are **sub-additive**, where the combined effect is less than additive but still exceeds individual contributions, are considered **compatible** and advantageous.
2. **Neutral Interactions** – The combination of agents produces **no significant improvement** over individual performance. While not synergistic, these combinations remain **compatible** and can be safely deployed in tandem.
3. **Negative Interactions** – Antagonistic effects between agents reduce overall efficacy, rendering the combination **incompatible** for integrated pest management.

The **effectiveness** of combined biocontrol agents is influenced not only by species-specific dynamics but also by **application timing** and **dosage rates**, factors that must be carefully optimized to maximize impact (Roy and Pell, 2000; Miranda-Fuentes et al., 2021). As such, developing **multi-agent strategies** requires a nuanced understanding of these variables to harness their full potential in **sustainable pest management**.

Entomopathogenic fungi (EPF) represent a key group of biocontrol agent, widely used in agriculture to combat herbivorous insects and plant pathogens. However, as their name suggests, these fungi attack insects, and some species such as *Beauveria bassiana* are highly pathogens, capable of infecting a broad range of insect hosts (Uma Devi et al., 2008). Given their broad-spectrum activity, the compatibility of EPF with other biocontrol agents and their potential risks on non-target organisms are rigorously evaluated (Wraight and Ramos, 2005; Midthassel et al., 2016). Another key group of biocontrol agents are, primarily the orders Hymenoptera and Diptera, while rarer one are Coleoptera, Lepidoptera and Neuroptera. Combining EPF with parasitoids holds significant promise for enhancing insect control programmes. However, the interactions between these two biological control agents, particularly any adverse effects that EPF might exert on parasitoids, must be carefully investigated before their combined use can be recommended.

An increasing number of research efforts aim to exploit the different lifestyles that entomopathogenic fungi can adopt, which extend beyond their primary route of infection through the insect cuticle. Remarkably, EPF can also colonize plants systemically or transiently (Quesada-Moraga et al., 2022). This endophytic association has demonstrated positive effects on plant growth, nutritional status and immunity against herbivorous insects (Gange et al., 2019), as well as protection against other biotic (Posada-Vergara et al., 2022) and abiotic stresses (García-Espinoza et al., 2023). While the endophytic function of EPF shows promising potential for pest control and sustainable agriculture, its impact on natural enemies of pests such as parasitoids remains largely unexplored. Assessing such interactions is crucial before integrating endophytic EPF into IPM strategies.

Compatibility studies between EPF and parasitoids have mostly focused on the indirect harmful effects of fungal biocontrol agents in their conventional use. These interactions are inherently complex, particularly when involving higher trophic levels such as predator and parasitoids (i.e. natural pest antagonists). Findings have been mixed, with some studies reporting compatibility under specific conditions (Mesquita and Lacey, 2001; Rashki et al., 2009), while others highlight reduced performance, increased mortality and resources competition (Oreste et al., 2016; Miranda-Fuentes et al., 2021). In a promising approach, researchers explored synergistic strategies that combine the strengths of both biocontrol agents. For example, Zeng et al., 2024, addressed the challenge of controlling the wood borer *Semanotus bifasciatus* (Coleoptera: Cerambycidae), where parasitism rate by *Sclerodermus guani* (Hymenoptera: Bethylidae) were insufficient and the pest remained inaccessible to EPF *B. bassiana*. By equipping parasitoid wasps with fungal spores, they enhanced pest control, demonstrating a novel and integrative method for improving biocontrol efficiency. Such innovative approaches highlight the potential of compatibility testing to optimize multi-agent strategies for sustainable pest management.

Interactions involving EPF are inherently complex, particularly as it is now widely recognized that the behaviour and performance of natural enemies are shaped by the metabolites and nutritional profiles of their host plant - factors that are themselves influenced by the presence of endophytic microorganisms (Aqueel MA et al., 2015; Turlings and Benrei, 1998).
Over the past three decades, most studies on the compatibility between endophytes and natural enemies of pests have focused on the obligate association between Clavicipitaceae fungi (Hypocreales) and Poaceae (Saikkonen et al., 1998; Bultman et al., 1997; Harri et al., 2009). These studies revealed a bottom-up influence driven by fungal alkaloid production, which triggered toxic effects throughout the food web, impacting non-target organisms (Barker and Addison,1996; De Sassi et al., 2006). Such findings underscore the ecological intricacies of using endophytic EPF in pest management and highlight the need for comprehensive assessments to ensure their compatibility with natural enemies before being incorporated into integrated pest management (IPM) strategies.

Research on the influence of endophytic colonisation by EPF on natural enemies remains limited, with most studies focusing *B. bassiana* and *Metarhizium spp*. Unlike the well-documented Clavicipitaceae-Poaceae interactions, no consistent patterns have emerged. Generally, EPF colonisation has shown minimal adverse effects on the parasitoid performance, suggesting promising compatibility between these biocontrol agents and their use in an IPM programme (Jaber and Ownley, 2018). Nevertheless, a closer examination reveals subtle negative impacts on certain parasitoid traits. For instance, Akutse et al. (2014) reported that while *B. bassiana* and *Hypocrea lixiia* were compatible with parasitoids *Phaedrotoma scabriventris* (Hymenoptera: Braconidae) and *Diglyphus isaea* (Hymenoptera: Eulophidae), there was a slight decrease in parasitoid offspring survival. Garcia-Espinoza (2024) observed that under greenhouse conditions, although there were no negative effects of the endophytes *B. bassiana* and *M. brunneum* on the rate of parasitism and the longevity of parasitoid *Hyposoter didymator* (Hymenoptera: Icnheumonidae) using *Spodoptera littoralis*, larval and pupal development were slightly prolonged. Miranda-Fuentes et al. (2021) noted that *Hyposoter didymator* (Hymenoptera: Ichneumonidae) parasitoids preferred control melon plants over

those colonised by *B. bassiana*. For predators, Gonzales-Mas *et al.* (2019) reported a slower predation rate by green lacewings (*Chrysoperla carnea* - Neuroptera: Chrysopidae) when using prey fed on colonised plants, but no additional detrimental effects. Jensen *et. al* (2020), found lower parasitoid emergence in black bean aphids reared on plants colonised by *B. bassiana*, though some traits remained unaffected or even improved. These findings underscore the complexity of multitrophic interactions influenced by factors such as plant species, parasitoid type (endoparasitoid or ectoparasitoid), insect feeding behaviour (chewing vs. piercing-sucking), plant variety, endophyte strain, abiotic conditions, and host developmental stage. Such variability highlights the need for species-species evaluation to optimize their use in IPM strategies.

This study aims to investigate interactions between the fungal entomopathogen *B. bassiana* colonising tomato plants, the Lepidopteran pest *S. littoralis* (Lepidoptera: Noctuidae) and its endoparasitoid *Meteorus pulchricornis* (Hymenoptera: Braconidae). The aim is to determine whether the *B. bassiana* endophytic use affects non-target organisms and assess its compatibility with parasitoids for controlling *S. littoralis*, a major threat to agricultural crops in Mediterranean regions (Ahmed et al., 2019). The research will evaluate key fitness traits, including parasitism success (F0) and offspring survival and longevity (F1). Given evidence that chewing insects are often negatively impacted by endophytic fungi (Vega, 2018), it is reasonable to hypothesize that these adverse effects on primary consumers may extend to their parasitoids, as seen in Clavicipitaceae fungi-Poaceae systems. However, findings from studies involving EPF suggest the effects could be more nuanced. Interestingly, the *B. bassiana* strain used in this study has been shown to reduce the immunocompetence of *S. littoralis* larvae while increasing their size (see Chapter 1), potentially benefiting parasitoid offspring development. This research aims to provide critical insights into the compatibility of endophytic EPF and parasitoids, paving the way for sustainable, multitrophic pest management strategies.

2. Material and Methods

2.1. Study organisms

S. littoralis was reared under the same conditions as previously described (See Chapter 1, part 2.1). The parasitoid wasp *Meteorus pulchricornis* was collected from maize fields in Ningbo City, Zhejiang Province, China (121°2’ N, 30°12’E) in September 2020 from an asexual (thelytokous) population and reared using *Spodoptera littoralis* instar under same laboratory conditions than their host. Adult parasitoid wasps were fed in 50 mL falcon with a 30% honey/water (V/V) solution, supplied daily via cotton. For parasitization step, a group of third or fourth instar *S. littoralis* larva were exposed to *M. pulchricornis* females in a 50 mL falcon during the night and collected in a common box the morning. Then, larvae were collected individually some days later, just before the parasitoid larvae emergence. The newly emerged parasitoid larvae are rapidly surrounded by a cocoon they produce in which they transform into pupae and are thereby preserved individually until the emergence of the parasitoid female adults that will be used for a new parasitization cycle.

The isolation and growth of *Beauveria bassiana* (Balsam) Vuillemin strain ATCC 74040 used for this study are the same as previously described (See Chapter 1, part 2.1).

2.2. Endophytic colonisation of plants

The plant colonisation of Dwarf tomato seeds cv San Marzano by *B. bassiana* conidia have been performed in the same way as explained in the Chapter 1 (section 2.2), with successive seed coating and soil watering. Plants were maintained in greenhouse conditions at 25 ± 1°C, 70 ± 5% relative humidity and a photoperiod of 16h light/8h dark. Determination of the occurrence of endophytic colonization was obtained by confirmation on petri dishes and by PCR diagnostic as described by Sinno et al. (2021).

2.3. Assessment of *Meteorus pulchricornis* fitness on *Spodoptera littoralis* larvae fed with tomato leaves from plants colonized by *Beauveria bassiana*

Bioassays were carried out by feeding *S. littoralis* larvae, from late 2nd instar to last day of 4th instar, with endophytically colonized and uncolonized tomato leaves detached from 6-weeks-old plants. As described in the previous study (see Chapter 1, section 2.3), bioassays were carried out using 32-well plastic trays (RT32W, Frontier Agricultural Sciences, USA,) with bottom of each well covered with agar-agar, sealed with perforated clear plastic lids (RTCV4, Frontier Agricultural Sciences, USA), and in the same thermo-hygrometric conditions. On the last day of 4th instar, control larvae and larvae fed on leaves colonized by *B. bassiana* were individually parasitized in small petri dishes by the parasitoid females and denoted as (Parasitized-C-larvae) and (Parasitized-Bb-larvae), respectively. In addition, two groups of negative control were monitored: unparasitized control larvae and unparasitized Bb-larvae. To minimize bias as much as possible, each *S. littoralis* larvae received only one oviposition sting, monitored during controlled parasitization. Each parasitized larva was then individually placed in a well and fed with colonized or uncolonized tomato leaves until the emergence of new parasitoid larvae (called F_1 parasitoids, i.e. the first generation of offspring produced by the parents). New tomato leaves were replaced, and the wells were cleaned daily. Two biological replicates of this bioassay were performed.

From this bioassay the following data were recorded: parasitization success, corresponding here to the number of emerging parasitoid larvae divided by the total number of parasitized larvae; the development time of parasitoids, including time from the oviposition until parasitoid larvae emergence/cocoon formation, and time from cocoon formation until adult emergence. Newly emerged adult parasitoids were maintained in 32-well plastic trays. Their longevity in starvation conditions has been measured. At their death, the size of each parasitoid from the two treatments was determined by measuring the length of the tibia of the hind leg, as a proxy of their body size.

2.4. Statistical analysis

Statistical analyses were conducted using the 'R' software version 4.2.2 (R Development Core Team 2022). Differences in size between experimental groups were compared by Student's t-tests. Validations were performed using Shapiro–Wilk and Fisher's tests. Differences in longevity and time of emergence between experimental groups were performed with a Wilcoxon rank sum test with continuity correction. Survival curves of *S. littoralis* larvae were compared by using Kaplan– Meier and Log-rank analysis. Differences related to the success of parasitism were measured using Pearson's Chi-squared test.

3. Results

To determine the compatibility of tomato plants colonised by *B. bassiana* (ATCC 74040 strain) and the parasitoid *M. pulchricornis*, *S. littoralis* larvae at the end of their 4th larval instar were fed on leaves from these plants and parasitised by the parasitoid.

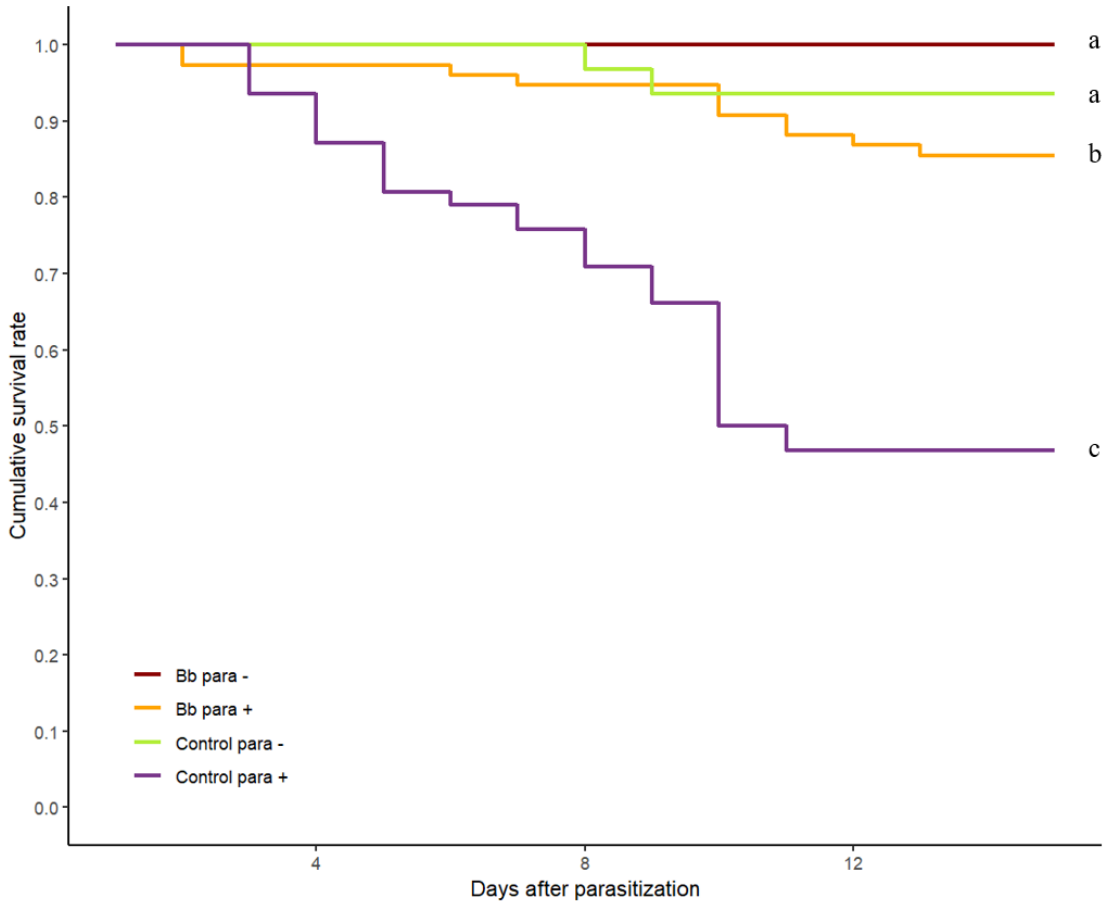


Figure 1: Survival rate of experimental Spodoptera littoralis late 4th instar larvae following parasitization by Meteorus pulchricornis. *Parasitized Bb larvae, unparasitized Control larvae and unparasitized Bb-larvae are in orange, green and red respectively. Parasitized control larvae (purple) showed lower survival rate compared with the other treatments; (Log-rank test: $\chi 2 = 61.2$; dF=3; p-value < 0.001) reaching a 53.2.% of mortality at day 15. In the graph the different letters indicate a statistical difference to Log-Rank test (P < 0.005).*

Initially, even before the first emergence of parasitoid larvae, the survival of these larvae was recorded (Figure 1). The control larvae, parasitized by the parasitoid and fed on leaves from control plants showed a significant marked reduction ($p < 0.001$) of their survival rate in contrast to the larvae parasitized but fed on leaves from plants colonized by *B. bassiana*. To ensure that a difference between these two conditions was not due to the effect of the tomato leaves alone, the survival of 31 non-parasitized control larvae and 31 non-parasitized B.b larvae was also recorded. No significant difference was found between these two negative controls, with a survival rate of 100% for the *B. bassiana* larvae and 93% (two deaths) for the control larvae.

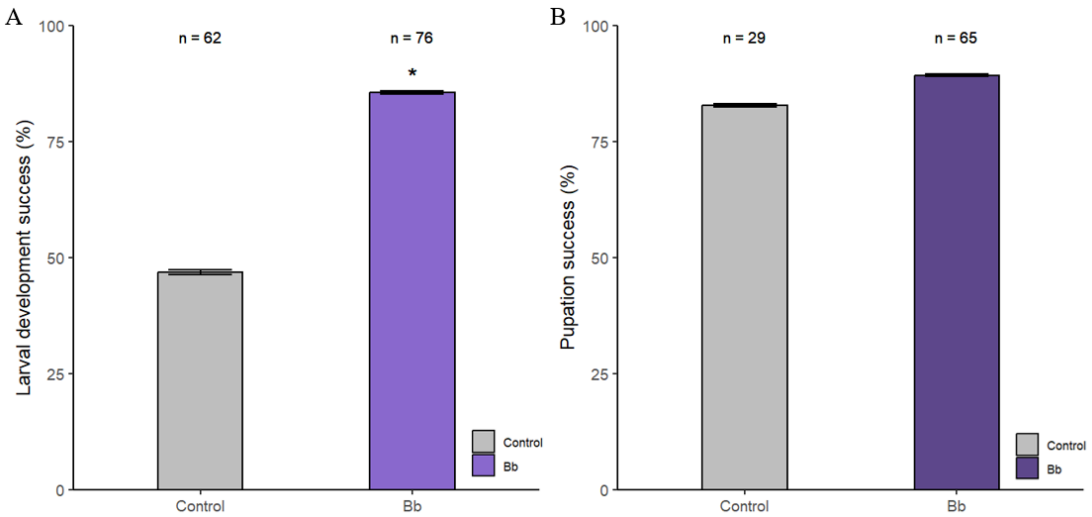


Figure 2: Parasitism success of Meteorus pulchricornis on Spodoptera littoralis late 4th instar larvae fed with leaves from control tomato plants and Beauveria bassiana colonized plants. *(A) Percentage of M. pulchricornis larval emergence/cocoons formed following the oviposition sting of S. littoralis larvae. (B) Percentage of M. pulchricornis adult emergence from cocoons. The results show a marked success of Bb cocoons formation compared to control (Pearson's chi-squared test: X-squared = 21.859, df = 1, p-value = 2.934e-06). In the graph, the letters n represent the number of samples for each treatment and the asterisk indicates a statistical difference to Pearson's Chi-squared test (P < 0.05).*

The success of parasitism by *M. pulchricornis* on host *S. littoralis* larvae fed on tomato plants colonised by *B. bassiana* or on control plants was then evaluated. The results indicate a significantly higher percentage of emergence of parasitoid larvae (confirmed here by cocoon formation) from Bb-larvae (Figure 2A). This is directly linked to the survival of the hosts, as seen above (Figure 1). The high mortality rate observed in control larvae had repercussions on the emergence of parasitoid larvae, which could not live inside dead hosts. Thus, 86% of parasitoid larvae from Bb hosts emerged and formed cocoons, while the emergence rate for controls was significantly lower (X-squared = 21.859, df = 1, p-value = 2.934e-06). In contrast, the pupation success, represented here by the percentage of adults emerging from cocoons showed no significant difference between the two experimental treatments (X-squared = 0.28507, df = 1, p-value = 0.5934) (Figure 2B). In total, of the 76 parasitized Bb caterpillars, 65 (76%) allowed the emergence of new generation adult parasitoids, whereas of the 62 control caterpillars, only 24 (37%) allowed the emergence of adult parasitoids (X-squared = 18.497, df = 1, p-value = 1.702e-05), representing the total parasitism success of *M. pulchricornis*.

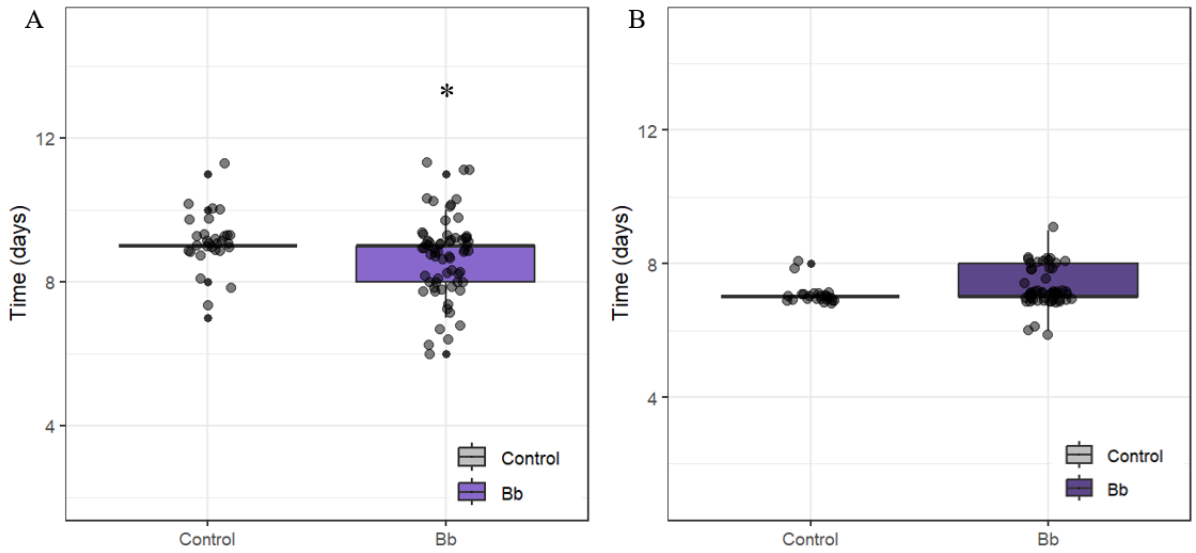


Figure 3: Effect on development of Meteorus pulchricornis derived from Spodoptera littoralis larvae fed on leaves from tomato plants colonized by Beauveria bassiana*. (A) Time of emergence of M. pulchricornis from the oviposition sting to cocoons F1 formation. (B) Time of emergence of M. pulchricornis from cocoons formation to adult emergence. Bb parasitoids showed a reduce time to reach the cocoon formation compared to Controls. (Wilcoxon test: W = 1183.5, p-value = 0.03079, n = 29 for control and 65 for Bb). In the graph the asterisk indicates a statistical difference to Wilcoxon rank sum test with continuity correction (P < 0.05).*

The development time between each developmental stage, i.e. the stage from the sting oviposition until the cocoon formation, and the stage from cocoon formation until adult emergence, was also observed. Parasitoid larvae from Bb-caterpillars emerged and formed their cocoon slightly but significantly earlier than those from control hosts (W = 1183.5, p-value = 0.03079) (Figure 3A). However, the time between cocoon formation and adult emergence was not influenced by the endophytisation of plants by *B. bassiana* (W = 571, p-value = 0.1349) (Figure 3B).

To estimate the size of F_1 parasitoids according to the experimental treatments, the measurement of a hind tibial of *M. pulchricornis* was taken as a proxy of the overall parasitoid size (Allan et al., 2022). The results indicate a significantly higher size for parasitoids derived from *S. littoralis* fed on leaves of tomato plants colonised by *B. bassiana* (t = - 2.8987, p-value = 0.007515; df = 26) (Figure 4).

Finally, the longevity of F_1 adult parasitoids from the two experimental treatments was determined in the absence of food and showed no significant difference (Table 1) (W = 162.5, p-value = 0.2639).

Table 1. Fitness parameters of *Meteorus pulchricornis* parasitizing *Spodoptera littoralis* larvae fed with control plants or *Beauveria bassiana* plants

	Hosts fed with control plants		Hosts fed with *B. bassiana* plants		p-value
	Mean	± Standard error	Mean	± Standard error	
Cocoons formation rate	0.4677	± 0.06	0.8553	± 0.04	<0.001
Adult emergences from cocoons	0.8276	± 0.07	0.8923	± 0.04	0.5934
Total adult emergences	0.387	± 0.06	0.763	± 0.05	< 0.001
Days to cocoons formation	9.103	± 0.1	8.662	± 0.1	0.03079
Days from cocoon formation to adult emergence	7.083	± 0.1	7.246	± 0.1	0.1349
Days to total emergence	16.186	± 0.1	15.908	± 0.1	0.6725
Size (µm)	1528.33	± 22.0	1649.774	± 35.7	< 0.01
Longevity (days)	4.143	± 0.2	4.448	± 0.2	0.264

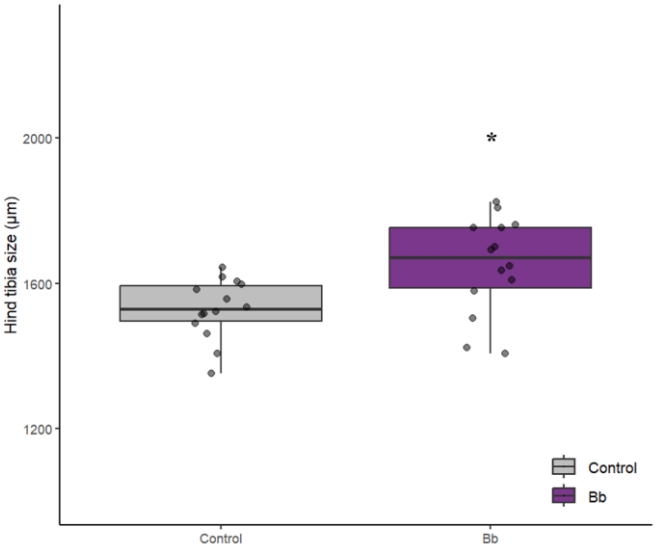


Figure 4: Hind tibia size (in µm) difference between F_1 M. pulchricornis derived from C- and Bb- Spodoptera littoralis larvae. Bb parasitoids show a hind tibia size bigger than Control parasitoids *(Student's t test: t = - 2.8987, p-value = 0.007515; df = 26, n = 14 for each modality). In the graph the asterisk indicates a statistical difference to Student's t-test (P < 0.05).*

4. Discussion

This study investigates the compatibility between the endophytic application of *Beauveria bassiana* and the parasitoid *Meteorus pulchricornis* to assess whether the fungus poses any risks to non-target organisms. The findings provide key insights into the potential for integrating these two biocontrol agents in pest management strategies. While most previous studies on endophytic fungi have focused on grass-endophyte systems (Bultman et al., 2009; Harri et al., 2009; Bixby-Brosi and Potter, 2012), this research stands out as one of the few investigations into the effects of an entomopathogenic fungus on parasitoids. To our knowledge, it is among the first studies to demonstrate a potentially beneficial interaction between a parasitoid and a plant colonized by an entomopathogenic fungus. This contrasts with earlier findings that primarily reported neutral or even negative effects (Miranda Fuentes et al., 2021; Akutse et al., 2014; Van Hee et al., 2024), highlighting the innovative contribution of this study to sustainable pest management research.

This analysis revealed that the percentage of *M. pulchricornis* F1 larvae emerging from parasitized *Spodoptera littoralis* caterpillars fed on leaves from *B. bassiana*-colonized plants and forming cocoons was twice as high compared to the control group (Figure 2). These results suggest that *B. bassiana* enhances parasitoid larval development within the host, likely due to its negative impact on host immunocompetence as previously observed in *S. littoralis* larvae fed on *B. Normally bassiana*-treated leaves (see Chapter 1). Normally, recognition of a foreign body by the insect immune defenses trigger haemocyte rapid proliferation and differentiation, leading to the encapsulation of foreign bodies such as parasitoids (Lavine and Strand, 2002). However, the suppression of encapsulation responses in larvae fed on plants colonised by *B. bassiana*-colonised plants mays explain the higher parasitism success observed in this study.

Interestingly, our experiment also revealed a significant increase in mortality among *Spodoptera littoralis* larvae fed on control plants following parasitism (Figure 1). This increased mortality directly correlates with the reduced parasitism success in the control group, as the death of the host inevitably results in the death of the developing parasitoid larvae within it. Several factors may explain this outcome. First, as indicated by our previous findings, *B. bassiana* colonisation alters the metabolic profile of tomato plants, increasing leaf protein content and modifying the content of free amino acids (cf. Chapter 1). This nutritional enhancement was reflected in larvae feeding on *B. bassiana*-treated plants, potentially providing them with better defenses against parasitism due to an improved protein-to-carbohydrate ratio in their diet (Cotter et al., 2019). *B. bassiana*-induced endophytisation may also optimize nutrients absorption (Gana et al., 2022), further strengthening host resilience to parasitism by *M. pulchricornis*. We can also assume that the secondary metabolite profiles of *B. bassiana*-colonised plants may differ from control plants, potentially altering their toxicity. A more comprehensive metabolomics approach will be required to understand in more detail the metabolic changes in plants induced by *B. bassiana*. A particularly striking observation was the way the control parasitized larvae died. Surprisingly, their bodies very quickly became entirely black, suggesting widespread melanisation throughout the insect's body. This possible melanisation suggests a systemic immune hyper-activation of *S. littoralis* larvae. This excessive immune response may have proven lethal, highlighting a potential trade-off between immunocompetence and survival. In contrast, larvae fed on *B. bassiana*-colonized plants likely experienced reduced immunocompetence, mitigating this lethal melanisation effect after parasitization and lowering mortality rates. While this hypothesis aligns with our findings, it requires further experimental validation to confirm.

Improved survival of pests fed on Bb plants and parasitized by *M. pulchricornis* may seem counterproductive from an effective integrated pest management perspective. However, we observed a reduction in the food intake of parasitized larvae, particularly in the days preceding the emergence of the parasitoid, which would limit the damage done to plants. Moreover, the greater emergence of parasitoid offspring, induced by this improved survival, leads to an increase in the second generation, which has positive consequences for parasitoid populations and the ecosystem services they induce (Noriega et al., 2018). Such an increase is an advantage in biocontrol strategies, particularly inoculative ones where control relies on the progeny of released individuals (Jensen et al., 2020).

In addition to higher parasitic success in parasitoids ovipositing in Bb larvae, some other traits of their offspring showed improvement. The time from oviposition to larval emergence showed a decrease, while the body size of the individuals was found to be larger than that of the controls (Figures 3 and 4). Such information is consistent with the general model that parasitoid development is influenced by ‘host quality’ (Barker et al., 1996). The nutritional modification of plants by fungal infection of *B. bassiana* led to larger caterpillars (see Chapter 1), which could increase the performance of parasitoids. These interesting results are confirmed by the Bixby-Brosi et al. study (2012), which tested the impact of perennial ryegrass colonised by an alkaloid-producing endophytic fungus on two parasitoids, *Copidosoma bakeri*, a slow-developing polyembryonic parasitoid, and the tachinid fly *Linnaemya comta*, a fast-developing

solitary species. In their case, the hosts are negatively affected by the presence of the fungus and this had a negative impact on the performance of the parasitoids, especially those that have developed for a long time in their host. As the parasitoids complete their development on a single host, their development is strongly influenced by their host's diet, which may explain the reduced time taken for the *M. pulchricornis* larvae to emerge and the bigger body size. This result appears to be in contrast with the study by Wilberts et al. (2023) in which, although the quality of *Myzus persicae* aphids was reduced by plants colonised by the entomopathogenic fungus *A. muscarius*, the performance of the parasitoid *Aphidius ervi* was not affected. However, the mummification time of *A. ervi* was longer for aphids feeding on plants inoculated with the fungus than for control plants, which is consistent with our study. Similarly, it was shown that the body size of the egg parasitoid *Trissolcus basalis*, developed in the eggs of the pest *Nezara viridula*, was not altered by the colonisation of sweet pepper by *B. bassiana* and *T. harzianum* (Van Hee et al., 2024). The authors suggest that fungal inoculation did not affect host quality.

Parasitoids emerging from *Spodoptera littoralis* larvae fed on *B. bassiana*-colonized plants exhibit superior fitness, marked by larger size and faster development, making them more valuable for biological control. Larger individuals generally have higher fecundity, mating success, and longevity (Visser, 1994), all of which contribute to greater biological control efficacy (Bennett and Hoffmann, 1998). Additionally, faster development accelerates parasitoid population growth, a critical factor for effective pest management (Roitberg et al., 2001). To confirm these observations, we measured the longevity of F1 (Table 1). Preliminary data suggest no significant difference in longevity between parasitoids from the two experimental treatments when deprived of food. However, a comprehensive measure of fecundity, including daily assessments of parasitised larvae throughout the adult stage, is now necessary and would offer a complete understanding of the indirect effect of *B. bassiana* on the reproductive potential of *M. pulchricornis*.

These preliminary results show that the use of *B. bassiana* (strain ATCC 74040) as an endophyte appears to have promising potential in IPM programmes, given its capacity to reduce the immunocompetence of larvae feeding on colonized plants and triggering plant metabolic changes, enhancing parasitism success rate and parasitoid’s fitness. However, further research is needed to achieve a more comprehensive understanding of these endophyte-plant-pest-parasitoid interactions, particularly in the chemical ecology area. For instance, Miranda-Fuentes et al. (2021), showed that the parasitoid *Hyposoter didymator* was more attracted to *Spodoptera littoralis* control larvae than to those fed on melon plants colonised by the entomopathogenic fungus *M. brunneum*. Hence, it would be interesting to see if and how the profile of plant VOCs is altered by *B. bassiana* endophytism and if it can have an impact on behaviour of both pests and their natural antagonists.

General conclusions

1. The promising role of *Beauveria bassiana* as plant endophyte

Microorganisms are gaining significant attention for their biotechnological potential in enhancing crop productivity and resilience, particularly in the current context of pesticide use reduction (Nadeem et al., 2014). Beneficial endophytic organisms, in particular, play a key role in promoting plant growth and strengthening defenses against environmental stressors, including insect pests (Agrawall and Bhatt, 2023). My thesis underscores the potential of the endophytic entomopathogenic fungus (EEF) *Beauveria bassiana* as a targeted and eco-friendly solution for pest control, advancing sustainable agricultural practices.

This study provides a comprehensive analysis of the impact of endophytisation by the entomopathogenic fungus *B. bassiana* on plant defense against herbivorous insect pests. Focusing on tomato plants and the lepidopteran pest *S. littoralis*, it represents, to our knowledge, the first investigation to simultaneously assess the fungus's effects on plant physiology, insect fitness, immune responses, and microbiota—both gut and hemolymph-associated. By exploring these interactions, this research sheds light on the mechanisms underpinning the potential of *B. bassiana* as a tool for sustainable pest management in agriculture.

One of our key findings highlights *B. bassiana* endophytism as a promising strategy to weaken the immunocompetence of lepidopteran pests, enhancing their vulnerability to commercial biopesticides. Notably, the fungus' impact extends beyond larvae feeding on colonised plant leaves, significantly reducing pupal survival and adult fecundity, thereby disrupting multiple stages of pest development. The multifunctionality of EEFs opens new avenues for innovative tools and strategies in IPM.

Unravelling the mechanisms controlling this multitrophic interaction is however crucial for harnessing *B. bassiana* as endophyte in pest management strategies. The two main mechanisms proposed in the literature to explain EFF-induced reduction in insect performance are the induction of plant defences by the fungus and the release of fungal toxins through the plant. In my thesis, I focused on plant defences activation, revealing that *B. bassiana* triggers a general increase in phytohormones associated with systemic plant defences pathways. Notably, it also enhances hormones linked to plant growth and abiotic stress responses, equipping plants to cope in hostile environments. Furthermore, herbivory was shown to amplify *B. bassiana* primed defenses in certain cases, reinforcing its potential as a dual-action tool for pest protection and plant growth/resilience.

In addition to plant-associated microbes, insect microbiota have also gained significant interest due to their critical role in host health, as demonstrated across numerous insect systems (Frago et al., 2012). Understanding how endophytes influence insect microbial communities—and subsequently disrupt insect function—offers valuable insights for agronomic applications. In my thesis, metagenomic and meta-transcriptomic analyses revealed that *B. bassiana* colonising

tomato plants altered the bacterial composition and diversity in the midgut of *S. littoralis*. These changes also impacted metabolic pathways, providing potential mechanistic explanations for the fungus' effects on insect performance.

Moreover, integrating *B. bassiana* with natural enemies in an IPM framework also shows promise. Assessing these interactions is crucial, especially since EFF-inoculated plants can negatively affect beneficial insects, including parasitoids (Jensen et al., 2020). However, my thesis, demonstrated compatibility between *B. bassiana* and the parasitoid *M. pulchricornis*, evidenced by higher emergence rates, shorter development times and larger parasitoid size in larvae exposed to the endophytic fungus.

Based on our results, I can say that EEFs are promising resources for reducing the use of synthetic chemicals in insect pest control and optimizing crop productivity. *B. bassiana* as an endophyte is a promising tool for sustainable agriculture, capable of overcoming some of the weaknesses/limitations it may show as a traditional entomopathogen.

2. Critical insights and future perspectives

In this section of my thesis, I will now deliver a critical analysis of our results, exploring their broader implications and unveiling new perspectives to guide future research and applications.

"The Holobiont Paradigm: Bridging Gaps in Plant-Insect-Microbe Interactions"

The holobiont concept has gained significant interest in recent years, emphasizing the pivotal role of plant and insect symbionts in shaping plant-insect interactions. Despite this recognition, our study does not address the plant microbiota—but only focuses on a single endophyte. While highly relevant for developing biotechnological tools in sustainable agriculture, my thesis exposes a critical gap in understanding ecological interactions, an oversight that could hinder the practical application of innovative solutions in real-world agricultural systems. Integrated investigations that simultaneously examine microbial communities associated with both plants and herbivorous insects is methodologically challenging and remain scarce, leaving fundamental questions about their complex interactions and ecological dynamics unanswered. Addressing these gaps is essential to avoid potential barriers to the implementation of advanced pest management strategies.

In my thesis, we hypothesised a link between the abundance of specific bacterial taxa and insect pest performance. However, a numerical correlation alone does not necessarily indicate a biological one. Although the current data require further analysis to fully comprehend their implications, to establish the functional effect of certain bacteria, future research should focus on axenic colony studies, comparing their performance with non-axenic colonies. Additionally, experiments involving bacterial (Di Lelio et al., 2022) or antibiotic treatments (Zhang et al., 2022) could shed light on the specific functions of bacterial groups. Manipulating the insect microbiota in this way would offer a more precise interpretation of the observed effects.

"Evaluating the Impact of Beauveria bassiana on Parasitoids and Other Beneficial Organisms: Implications for Sustainable Biocontrol Strategies"

The longevity observed in parasitoids across the two experimental treatments was estimated under food deprivation conditions. However, to gain a more comprehensive understanding, it would be valuable to explore longevity under conditions with a constant food supply. *M. pulchricornis* is a synovigenous parasitoid, with egg maturation occurring throughout its life, making its diet a critical factor (Jervis et al., 2008). The current longevity assessment is based on reserves accumulated during the larval stage; however, evaluating the impact of *B. bassiana* on adult-fed parasitoid longevity would offer crucial insights into how the endophytic fungi really influences lifespan of the parasitoid (Rivero et al., 2001). While, this approach may introduce confounding factors, such as food consumption, sensory capacity, and digestive ability (potentially influenced by fungal-altered insect gut microbiota), it would offer a more physiologically relevant estimate of the fungus's impact on parasitoid fitness. Such insights are critical for optimizing biocontrol strategies.
Beyond parasitoids, the potential effects of EFFs on other beneficial organisms, such as predators and pollinators, must also be thoroughly evaluated to fully harness their potential in sustainable pest management strategies.

"Evaluating the Long-Term Impact of Beauveria bassiana on Tomato Plant Resistance, Development, and Yield in Commercial Greenhouses"

My thesis was carried out under controlled laboratory conditions; however, given the influence of environmental factors on endophyte effects (Di Lelio et al., 2021), it is crucial to assess how fluctuating environmental conditions, such as those in commercial greenhouse settings affect these multitrophic interactions. Additionally, our study relied on feeding bioassays with detached leaves, but experiments involving whole plants would better replicate greenhouse conditions (Minchev et al., 2024).
Furthermore, the long-term effects of fungal colonization on tomato plant resistance warrant further exploration. While plant defenses are known to be costly and can impact plant fitness (Strauss et al., 2002), it would be valuable to investigate potential trade-offs between defense activation and plant development. To fully understand the implications, the impact of fungal endophytes on tomato yields should be studied in commercial greenhouse settings, particularly through to the fruiting stage, a largely unexplored aspect of plant performance.

"Exploring the Tripartite Interactions Between Beauveria bassiana, Tomato Plants, and Herbivores: Costs, Benefits, and Long-Term Implications"

Our interpretations have primarily focused on the effects of *B. bassiana* on plants and the resulting benefits in pest control. However, both phytophagous insects and plant symbionts compete for plant resources, interacting simultaneously or sequentially with the same host, each aiming to benefit from the relationship (Gruden et al., 2020). A deeper investigation into the costs and benefits for each partner of this tripartite interaction would be valuable. On one hand, we observed that *B. bassiana* enhances protein and water content in tomato plants, which may

provide an evolutionary advantage to the plant. However, this also makes the plant more nutritious for insects, which could be detrimental to the plant. Yet, the insect did not consume more plant tissue, and plant defenses were activated, mitigating this potential entomopathogenic cost. Furthermore, plant endophytisation made the insect a more suitable host for the fungus' s entomopathogenic phase—larger in size and with reduced nodulation and encapsulation processes. Additionally, the slight increase in phagocytosis favors the fungus by limiting competition with opportunistic bacteria. Overall, this interaction appears beneficial for both the plant and the fungus. Notably, the survival of larvae appears enhanced after parasitoid oviposition on fungus-colonised plants, in contrast to larvae feeding on non-colonised plants. However, the reduced survival of *S. littoralis* pupae remains a potential long-term disadvantage for the fungus.

"Exploring Inter-Kingdom Ecological Interactions: Distinct Strategies of Endophytic Organisms and Their Implications for Sustainable Agriculture"

Comparing our findings with those of Di Lelio et al. (2022), reveals that different endophytic organisms employ distinct strategies. In their study, endophytisation of tomato plants by the soil fungus *Trichoderma afroharzianum* had a more pronounced negative impact on *S. littoralis*, reducing survival from the larval stage onward, and decreasing larval weight. Such studies highlight how endophytic organisms bridge underground and above-ground environments, influencing energy allocation in nature. They enhance our understanding of the complex ecological networks in which organisms evolve. Understanding the complex inter-kingdom ecological interactions and their underlying mechanisms within the context of real-world crop producing is both a challenging and essential step toward achieving sustainable agriculture.

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Corentin CLAVÉ

Le rôle des microorganismes symbiotiques dans la modulation des interactions plantes-insectes

Vers le développement de nouveaux outils et stratégies pour la lutte contre les ravageurs

Résumé en français

La réduction de l'utilisation des pesticides synthétiques pour la protection des cultures est essentielle pour améliorer la durabilité écologique et économique de l'agriculture et pour minimiser leur impact sur la santé humaine. Les micro-organismes peuvent établir des associations symbiotiques mutualistes avec les plantes, favorisant leur croissance et leurs défenses contre différents agents de stress abiotiques et biotiques, notamment les insectes. L'objectif de ma thèse est de décrypter les bases fonctionnelles et moléculaires des interactions multitrophiques impliquant le champignon endophyte entomopathogène *Beauveria bassiana,* colonisant les plants de tomates, et le lépidoptère ravageur *Spodoptera littoralis*. L'effet de ce champignon entomopathogène sur le développement, la survie et l'immunité de *S. littoralis* a été évalué. En outre, l'impact des barrières de défense des plantes déclenchées par la colonisation fongique sur les insectes nuisibles et leurs antagonistes naturels a été exploré, et les mécanismes sous-jacents de ces interactions ont été analysés au niveau de l'holobionte, en tenant compte du rôle joué par le microbiote associé aux insectes.
L'ensemble de ces analyses a permis de mettre en évidence le lien potentiel entre les changements métaboliques des plantes induit par leur colonisation par *B. bassiana*, le microbiote des insectes et leur performance. Ces informations de base permettront de développer des technologies bioinspirées de lutte contre les ravageurs, basées sur la manipulation des interactions immunitaires entre les différents niveaux trophiques, en utilisant des combinaisons synergiques d'agents de biocontrôle. Une meilleure compréhension de ces processus, avec leurs implications écologiques, évolutives et applicatives, ouvrira la voie au développement de nouvelles stratégies de biocontrôle plus durables, au bénéfice de l'agriculture.

Mots clés : Interactions multitrophiques, Entomologie, Biotechnologies, Défense des plantes, Symbiontes bactériens d'insectes, Contrôle biologique, *Beauveria bassiana*, *Solanum lycopersicum L.*, *Spodoptera littoralis*, *Meteorus pulchricornis*

Summary

Reducing the use of synthetic pesticides for crop protection is essential to improve the ecological and economic sustainability of agriculture and to minimise their impact on human health. Micro-organisms can establish mutualistic symbiotic associations with plants, promoting their growth and their defences against various abiotic and biotic stress agents, including insects. The objective of my Ph.D. is to decipher the functional and molecular bases of multitrophic interactions involving the entomopathogenic endophytic fungus *Beauveria bassiana*, which colonises tomato plants, and the lepidopteran pest *Spodoptera littoralis*. The effect of this entomopathogenic fungus on the development, survival and immunity of *S. littoralis* was evaluated. In addition, the impact of plant defence barriers triggered by fungal colonisation on insect pests and their natural antagonists was explored, and the mechanisms underlying these interactions were analysed at holobiont level, considering the role played by the microbiota associated with the insects.
Taken together, these analyses have highlighted the potential link between the metabolic changes in plants induced by their colonisation by *B. bassiana*, the insects' microbiota and their performance. This basic information will enable the development of bioinspired pest control technologies based on the manipulation of immune interactions between different trophic levels, using synergistic combinations of biocontrol agents. A better understanding of these processes, with their ecological, evolutionary and application implications, will pave the way for the development of new, more sustainable biocontrol strategies for the benefit of agriculture.

Keywords: Multitrophic interactions, Entomology, Biotechnologies, Plant defence, Bacterial insect symbionts, Biological control, *Beauveria bassiana*, *Solanum lycopersicum L.*, *Spodoptera littoralis*, *Meteorus pulchricornis*

