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Unraveling the molecular basis of the microbe-plant-pest network using tomato as a model system

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Tesi di dottorato di:
Dott.ssa Gaia Salvatore Falconieri

Coordinatore del corso
Prof.ssa Roberta Bernini

Tutore
Prof.ssa Roberta Bernini

Prof.ssa Carla Caruso

Co-tutore
Dott.ssa Silvia Proietti

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Abstract

In their natural environment, plants interact with numerous microorganisms, some of which are potentially harmful as pathogens, while others are beneficial as they can promote plant growth and expand the immune response of the plant host. Among them, the fungi *Beauveria bassiana* and *Trichoderma harzianum* can colonize plant tissues in an asymptomatic way, triggering plant metabolic changes which negatively affect both pest insects and plant pathogens. These beneficial fungi have an extremely broad host spectrum, including tomato (*Solanum lycopersicum* L.), a species of great economic importance which is widely cultivated all over the world. In the first part of this work, the research activity focused on understanding and investigating the poorly characterized molecular events that regulate tomato-*B. bassiana* interaction. A time course proteomics analysis showed that *B. bassiana* affects different biological processes linked to primary and secondary metabolism, highlighting a boost of energy and growth-related pathways at a later stage of colonization. At this stage, a plant hormone analysis revealed an interesting up-regulation of gibberellins both in active form and as precursors and also of defense-related hormones such as benzoic acid and jasmonate. Finally, the effect of *B. bassiana* on increasing the protection against *Botrytis cinerea* was tested, revealing that *B. bassiana* was able to mitigate the oxidative stress caused by *B. cinerea*, likely acting on the regulation of the antioxidant defense mechanism. In the second part of the thesis work, the tripartite interaction existing between beneficial microbes-tomato-biotic stress was examined, in order to increase knowledge about the sophisticated way in which beneficial microbes promotes plant health and strengthen defense barriers. In particular, these studies have revealed that *B. bassiana* and *T. harzianum* alone play a role in the activation of different strategies in response to *B. cinerea* and *M. euphorbiae*; in fact, *T. harzianum* significantly contributes to the activation of the phenylpropanoid pathway and to the metabolism of cell wall polysaccharides, while *B. bassiana* appears to be involved in hormone response and endogenous stimulation, in addition to its role in cell communication. These strategies cooperate synergistically in the *B. bassiana-T. harzianum* consortium with a common goal as

strengthening defense responses and supporting plant growth. Further research will be needed to expand knowledge on the molecular mechanisms significantly altered by these two beneficial microbes to obtain a comprehensive snapshot of the ecological impact they cause and in order to stimulate an increasing use of healthy and environmentally sustainable agriculture.

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1 Introduction

The world's population currently is 8 billion people and is likely to increase to around 10 billion in 2050, according to UN projections on population growth. Recent scientific and technological developments achieved by modern agriculture (so-called *green* or *sustainable agriculture*) should be able to meet the resulting increase in food requirements and ensure health through high nutritional quality products in compliance with safety standards. However, the ability to expand agricultural production is greatly limited by the progressive depletion of environmental resources, mainly due to overexploitation, environmental problems, biodiversity erosion, intensive agricultural practice and the effects of climate change, as widely demonstrated by FAO reports, 2017. In addition, the emergence of new pathogens and the re-occurrence of old ones continue to challenge our ability to safeguard the worldwide plants growth and health (Miller *et al*., 2009). Nowadays, in the perspective of an eco-friendly agriculture, the winning approach is to provide for long-term human needs, to preserve nature through the use of renewable resources and to reduce the environmental impact due to the use of synthetic chemicals (Budnik and Baur, 2009). Since the 1970s, the use of chemical compounds in agriculture has caused much damage. The use of ever-increasing quantities of pesticides has resulted in an increased risk of agricultural products contamination, with consequent damage to human health and to environment. Moreover, the repeated use of synthetic products has selected populations of insects, fungi, bacteria and viruses that are resistant to the active ingredients, with the inevitable consequence of having to increase doses to achieve a containment effect. The impact of these chemical compounds on the environment and agri-food products has increasingly stimulated research into alternative methods for fertilization and plant disease control. In this way, plants biotechnology has contributed to the development of new crop varieties with greater resistance to pest, drought and salt stress and with greater nutritional value. Unfortunately, beneficial plant-microbe interactions have often been ignored in crop improvement strategies, even though plant-associated microbes have important functions in plant and in soil ecosystem, increasing plant stress tolerance, providing plant resistance

to disease, expanding nutrient uptake and availability and promoting biodiversity (Dlamini *et al*., 2022; Morrissey *et al*., 2004). However, in recent years several studies have been carried out on the development of integrated or biological disease control strategies (biocontrol strategies). As mentioned before, an interesting contribution may be provided by the use of microorganisms that are antagonistic to pathogens, which are often able to exert not only a bio-pesticidal action but also a bio-fertilizing action towards the plants which they interact, promoting their development and growth.

1.1 Tomato as a model plant

The cultivated tomato, *Solanum lycopersicum L.* belonging to the *Solanaceae* family, is a dicotyledonous herbaceous plant originated from South America where several wild tomato species can still be found today (Spooner *et al*., 2005). After the Discovery of America, its cultivation spread globally representing one of the most important horticultural crops and among the most highly consumed food crops of international acclaim (Knapp *et al.*, 2016). Among the main producers and consumers of tomato is Italy, that, with a production of almost 6 million tonnes in 2022, has always been interested in bringing to market a high-quality product. In fact, tomato is a vegetable of particular importance in many diets, including the traditional Mediterranean diet, due to its notable organoleptic characteristics and health properties, as it is particularly rich in fibre, mineral salts, C and E vitamins, secondary metabolites such as carotenoids (lycopene and β-carotene) and polyphenols, with beneficial action against cardiovascular diseases, tumour growth and aging pathologies (Chaudhary *et al*., 2018). Moreover, within the *Solanaceae* family, it has assumed an important role in genetic and genomic studies, due to some important intrinsic characteristics and applications: in fact, this plant has a modest genome size of 35.000 genes, is very suitable for genetic transformation studies and has a fully sequenced genome. These features make it a valid model organism for the *Solanaceae* family and, in general, for other important plant species. In addition, the cultivated tomato represents an important experimental system for conducting studies on the physiology, the development of the fruit, the genetic improvement, the response to biotic and

abiotic stresses (Tucci *et al.*, 2010). Tomato plant can be subjected to the attack of more than a hundred different pathogens, which target specific parts of the plant and produce characteristic symptoms of the disease. For the defense against these pathogens, in the future it will be increasingly necessary to recur to integrated defense strategies and the use of specifically selected varieties, capable of resisting a wide spectrum of pathogenic species.

1.2 Plant defense mechanisms in response to biotic stresses

In their habitat, plants are constantly faced with abiotic and biotic stresses which can negatively influence their development, growth and reproduction and in extreme cases lead to death. In plants of agricultural interest, they can also determine considerable loss in productivity and in the quality of the products, with consequent depreciation and economic damage. The abiotic stress includes salinity, drought, extremes temperature, floods or lack of nutrients; in contrast with, the action of other living organisms such as herbivorous insects, viruses, bacteria and fungi are included in the biotic stresses. In detail, pathogens act by triggering mechanisms that promote their own proliferation and induce plant changes that affect basic physiological activities and metabolic processes. These physiological changes can lead to reduced photosynthesis capacity and unbalanced growth, resulting in reduced production in both quantitative and qualitative terms. On the basis of their mode of action towards the host, phytopathogens are classified into necrotrophs, which kill cells and decompose plant tissue to feed on them, and biotrophs, which in contrast derive nourishment from living cells. In addition to these, there is another class of plant pathogens, called hemibiotrophs, which exhibit both previous behaviours based on their particular stage of the biological cycle (Pieterse *et al*., 2009) (Figure 1).

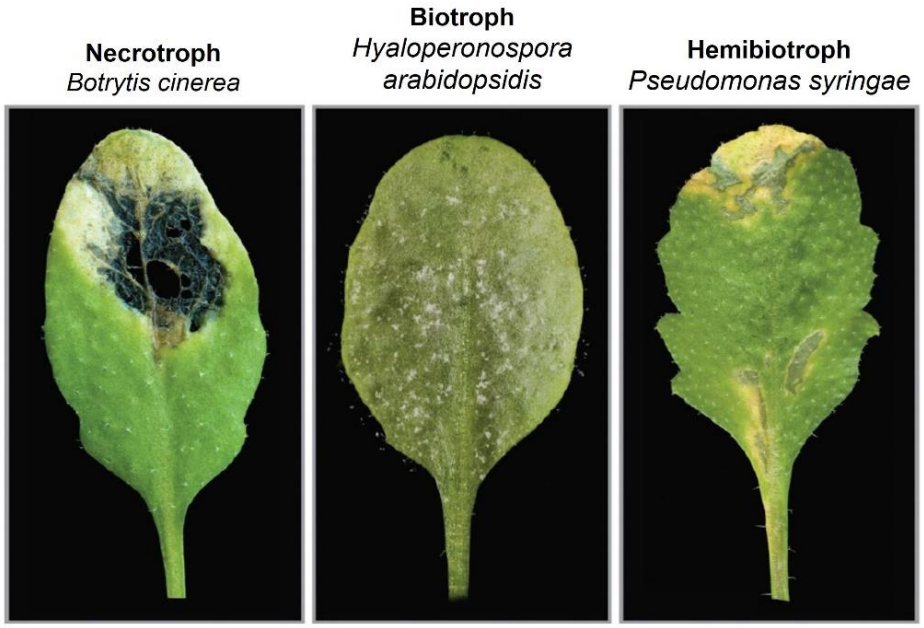

Figure 1. ***Arabidopsis thaliana*** **leaves with symptoms of diseases caused by the necrotrophic fungus** ***Botrytis cinerea,*** **the biotrophic oomycete** ***Hyaloperonospora arabidopsidis*** **and the hemibiotroph** ***Pseudomons syringae.*** From Pieterse *et al.,* 2009.

A typical example of necrotrophic pathogen is the ascomycete fungus *Botrytis cinerea*, belonging to the *Sclerotiniaceae* family of the class Leotiomycetes, which is considered an important model organism with a broad host spectrum for plant pathogenesis studies and for which the entire genome sequence is available (Van Kan, 2006). In tomato, this pathogen can attack all plant epigeal organs, at all stages of vegetative development, being particularly damaging to greenhouse crops. The necrosis is generally manifested by the appearance of soft rot, with loss of consistency of the affected tissues, while the proliferation of the characteristic grey mould, consisting of fungal mycelium, occurs (Williamson *et al.*, 2007). In general, biotic stresses cause various morphological, physiological, biochemical and molecular changes that adversely affect plant growth and cause interference in stress tolerance and adaptation. Therefore, it is important to know the organism-coordinated responses involving mechanisms at the cellular and molecular level.

As plants are sessile in nature, they cannot rush away from a danger; therefore, they have evolved complex defense mechanisms in order to overcome the aforementioned threats. Normally, the development of disease is a rare event, occurring when plant is infected by a virulent pathogen, which either does not activate or suppresses the plant's resistance responses, or

manages to evade the activated defenses, which then fail in their mission. The outcome of the plant-pathogen "battle" (resistance or susceptibility) depends on a balance between the ability and rapidity of pathogen to suppress the plants immune system and the plant rapidity to perceive pathogen and effectively translate this perception into the defense responses activation. In particular, plants have two lines of defense: passive and active defense. The first are structural or chemical barriers that have an exclusionary effect for most pathogens; they are represented by cell wall, waxy epidermal cuticles and bark, used to confer strength and rigidity to plant. Inducible defenses include the production of toxic chemicals and pathogen-degrading enzymes (Freeman and Beattie, 2008). When plant senses stress, a series of processes are activated that in turn activate a cross network of morphological, physiological and biochemical mechanisms. Plants can respond to pathogen invasion by triggering a response to infection first locally and then systemically: at local level, the first line of defense is an intact and impenetrable barrier composed of cell wall and a waxy cuticle. In addition, there are other plant adaptations that include hard shells, thorns (modified branches), and spines (modified leaves). All together they are able to efficiently protect against herbivores. However, external protection can be compromised by mechanical damage, which may provide an entry point for pathogens. If the first line of defense is breached, plant must resort to a different set of defense mechanisms, such as the production of secondary metabolites like toxins and enzymes. Secondary metabolites are compounds that are not directly derived from photosynthesis and are not necessary for respiration or plant growth and development. Many metabolites are toxic and can even be lethal to animals that ingest them. Additionally, plants have a variety of inducible defenses in the presence of pathogens. In fact, they can produce antimicrobial chemicals, antimicrobial proteins, and antimicrobial enzymes that are able to fight the pathogens (Parthasarathy *et al*., 2021). Moreover, plants can close stomata to prevent the pathogen from entering the plant and can activate a hypersensitive response, in which the plant experiences rapid cell death to restrain the invasion. At present, a simple coevolutionary model of plant–pathogen interactions is represented by the zig-zag model: PAMPs (Pathogen-associated molecular patterns) are

recognized by PRRs (Pattern-recognition receptors), activating a so called PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). Successful pathogens evolved effector molecules to break this first line of defense, either by preventing detection of their PAMPs by the host or by suppressing PTI signaling (Bardoel *et al.* 2011; De Jonge *et al.* 2010). Furthermore, plants can acquire a second defense line where resistance proteins (R) recognize these effectors, inducing ETI (Effector-triggered immunity), as shown in figure 2.

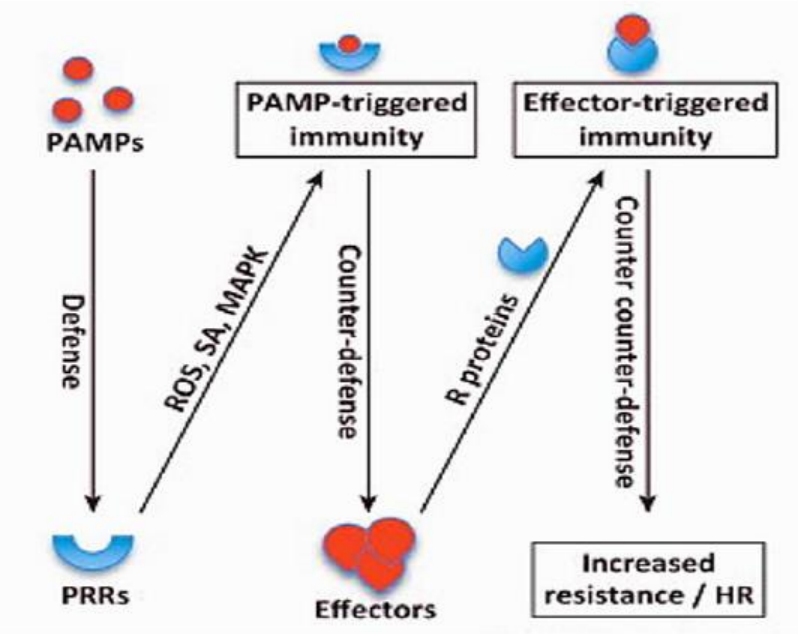

Figure 2. A scheme of plant immune system according with the zig-zag model. From Incarbone and Dunoyer, 2013.

Finally, other plant defense mechanisms toward pathogens include ion fluxes, oxidative burst and induced systemic resistance (Wojtaszek, 1997). Most of these defense responses are mediated by signal molecules such as phytohormones (Pieterse *et al*., 2009; Pieterse *et al*., 2012).

1.3 Phytohormones as central players on plant growth and defense

Higher plants produce many different types of molecules with hormonal activity, also known as phytohormones, which are chemical messengers involved in several cellular processes. They are capable of exerting a strong action on plant metabolic pathways, tissue growth, development regulation and plant defense. Antagonistic and synergistic interactions between different

hormone signal transduction pathways add another layer of regulation (Pieterse *et al*., 2012); this hormone cross talk provides the plant with a powerful capacity to regulate its immune response to the invaders and to utilize its resources in a cost-efficient manner. Salicylic acid (SA) and jasmonic acid (JA) with its derivatives (collectively known as jasmonates) are recognized as major defense hormones and have been well characterized over the years (Browse. 2009; Vlot et al., 2009; Pieterse *et al*., 2009). Auxins (AUX), ethylene (ET), abscisic acid (ABA), gibberellins (GA), cytokinins (CK), brassinosteroids (BR) and strigolactones (SLs), originally recognized as essential regulators of plant growth, development, reproduction and survival, have also been reported to play a role as modulators of the plant's immune signaling network (Pieterse *et al,* 2009) (Fig. 3).

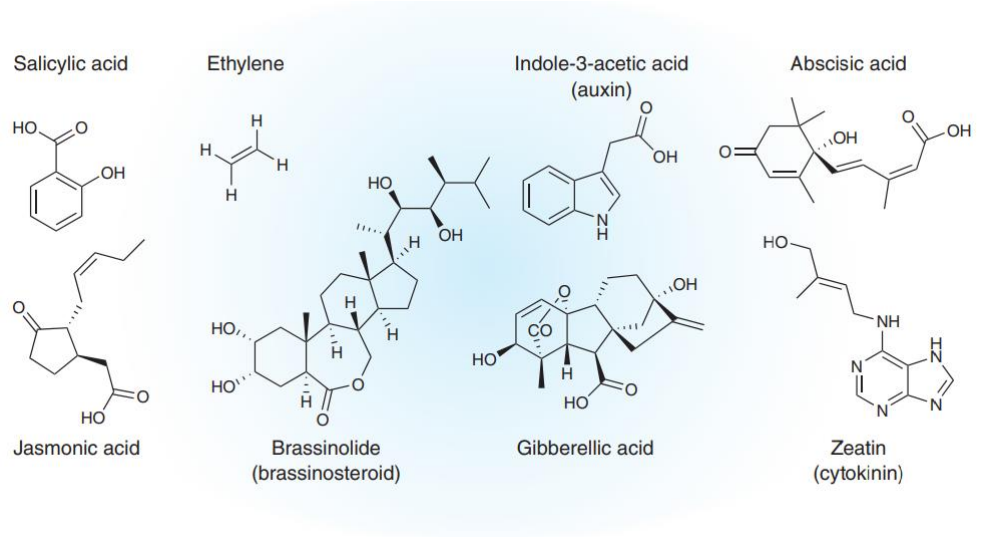

Figure 3. Phytohormones involved in plant immunity signaling network. From Pieterse *et al.,* 2009.

Through the balancing of phytohormones, plants maintain homeostasis and adapt to the environmental changes and this is possible by an efficient and systemic cross talk between various phytohormones. Salicylic acid and jasmonic acid work together in a harmonious manner with abscisic acid, ethylene, brassinosteroids, auxin, cytokinins and gibberellins in mediating plant response to biotic challenges. Elucidating the mechanisms of plant hormone interaction and solving the plant immune network will help understand how plants perceive the orchestrated immune system function.

1.3.1 Plant defense hormones

Among defense phytohormones, salicylic acid, jasmonic acid, ethylene and abscisic acid are the most representative and their importance as primary signals in the regulation of the plant's immune response is well established. Salicylic acid plays an important role in disease resistance signaling, especially against biotrophic pathogens and phloem feeding insects (Glazebrook, 2005; Walling, 2008). SA is a phenolic compound synthesized starting from chorismate through phenylalanine ammonia lyase (PAL) and isochorismate synthase (ICS/SID2) (Garcion and Mètraux, 2006). Signaling pathway activated by SA is largely controlled by the regulatory protein NON-EXPRESSOR OF PR GENES1 (NPR1) which acts as coactivator of defense genes. At basal level, polymeric NPR1 cannot enter the nucleus, due to its ubiquitination and binding to the proteasome, therefore downstream genes cannot be activated (Spoel *et al*., 2009). After a pathogen injury, SA is induced and a redox state change occurs leading to a monomerization of NPR1 through the activity of thioredoxins TRX-H3 and TRX-H5. Thus, monomeric NPR1 can enter the nucleus and, by its binding to members of the TGA subclass of the basic leucine zipper (bZIP) family of transcription factors, it activates the downstream genes (Withers and Dong, 2016). Among these is the PR1 gene, belonging to the pathogenesis related (PR) family. PR genes are a heterogeneous group and several encode proteins with antimicrobial activity (Van Loon *et al*., 2006; Bertini *et al*., 2012). However, once the SA pathway is activated at the site of infection, a similar response is often triggered in distal plant parts to protect undamaged tissues against subsequent pathogen invasion. This broad-spectrum induced resistance is referred to as systemic acquired resistance (SAR) (Vlot *et al.* 2009). On the other hand, when the plant has to defend itself against herbivorous insects or necrotrophic pathogens it activates JA pathway. JA and its structurally related metabolites are lipid-derived compounds: JA biosynthesis starts with the release of α-linolenic acid (α-LA) from membrane lipids (Pieterse *et al*., 2012). At basal level, the SCF complex is free in the cytoplasm and the repressor JAZ is linked to transcription factors, preventing the downstream genes activation. When a stress occurs, JA is tied up by isoleucin to form JA-Ile which binds to the SCF complex and to the repressor JAZ, detaching it

from transcription factors; thus, this complex is linked by ubiquitin for the degradation via the proteasome. So, the downstream genes encoding for Plant Defensin 1.2 (PDF1.2) and Vegetative Storage Proteins (VSPs) can be transcribed. PDF1.2 and VSPs are important proteins: the first belongs to plant defensin family with activity against necrotrophic pathogens, while the second is an anti-insect acid phosphatase. There are two branches of the JA signaling pathway: the MYC branch and the ERF branch that antagonize each other. The first is controlled by MYC transcription factors family that it is responsible of *VSPs* activation. The ERF branch is regulated by members of the Apetala2/Ethylene Response Factor (AP2/ERF) transcription factors family and it activates *PDF1.2* (Pieterse *et al.,* 2012). In general, the ERF branch is associated with a resistance against necrotrophic pathogens, while the MYC branch is involved in insect herbivores defenses (Pieterse *et al.,* 2012). After activation of the JA pathway in the damaged tissue, a similar JA response can be triggered in undamaged plant parts: this resistance helps the plant to protect itself against future pathogen or herbivorous attacks (Howe and Jander, 2008). Moreover, to make defense responses more efficient, different hormone networks interact in a complex interplay of synergistic, antagonistic and additive interactions, a phenomenon known as hormone cross talk that provide a powerful regulatory potential to flexibly tailor the plant's adaptive response to a variety of environmental cues (Aerts *et al.*, 2021). In the context of defense regulation, one of the best investigated cross talk is that between the SA and JA pathways, where antagonism is the prevailing form (Aerts *et al.,* 2021; Pieterse *et al.,* 2012).

Another important hormone involved in plant defense is ethylene, which besides its involvement in plant growth control, also plays a pivotal role in modulating plant immunity exerting both positive and negative effects. In *Arabidopsis*, for example, ethylene potentiates SA-responsive *PR-1* expression (De Vos *et al*. 2006; Lawton *et al*. 1994). Moreover, in combination with JA, it acts synergically on the expression of the ERF branch of JA pathway, while it antagonizes the MYC branch: this leads to a prioritization of the immune signaling network toward JA- and ET-dependent defense signaling associated with resistance to necrotrophs (Anderson *et al*. 2004; Lorenzo, 2004). Also, during SA/JA signal interaction, ET can play a

critical role. Exogenously applied ET can override the requirement of NPR1 for JA suppression by SA, and its signaling makes plant tissues immune to future SA-mediated suppression of the JA pathway (Leon Reyes *et al.*, 2010). Therefore, the final outcome of the SA-JA signal interaction during the complex interactions between plants and their attackers can be shaped by ET produced upon attack (Pieterse *et al.*, 2012). In general, SA, JA and ET mediate a core-signaling pathways of plant defense against biotic agents such as nematodes, insects, and microbial pathogens including virus, bacteria and fungi. The interactions between these hormones enable plants to fine tune energy allocation and minimize fitness costs through a flexible complex signaling network (Yang *et al.*, 2015).

The above-described hormone activate in plants two different adaptive systemic defense responses: the aforementioned systemic acquired resistance (SAR), activated as a response to a biotic stress, mediated by SA and associated with the production of PR proteins, and induced systemic resistance (ISR), mediated by JA/ET, as a result of the interaction of the root system with beneficial rhizosphere soil bacteria, as shown in Figure 4.

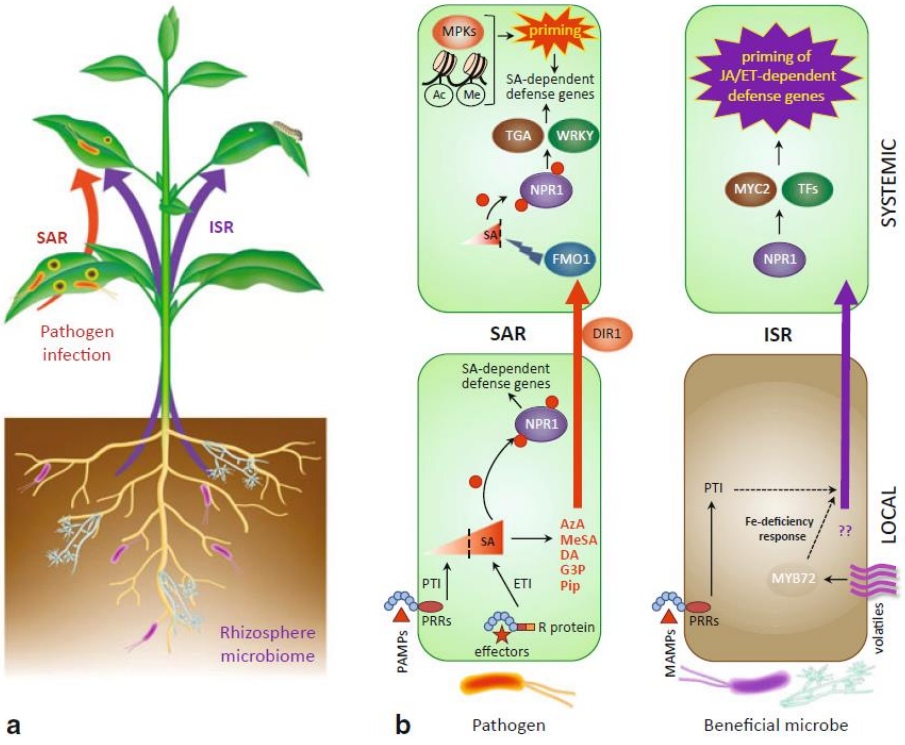

Figure 4. ***a.*** **Schematic representation of biologically induced disease resistance triggered by pathogen infection (SAR; red arrow) and colonization of the roots by beneficial microbes (ISR; purple arrow).** ***b.*** **Schematic representation of molecular components and mechanisms involved in pathogen-induced SAR and rhizobacteria-mediated ISR.** From Pieterse and Van Wees, 2015.

SAR is a kind of immunity due to the activation of dormant protective mechanisms following an initial low-level infectious event, manifested on a subsequent opportunity of encounter with the same or other pathogens. This resistance is systemic, as the defensive capacity does not only increase at the primary site of infection, but also in spatially separated healthy tissues leading to a form of 'immunization', comparable in effect to vaccination. The activation of this response is characterized by the local and systemic accumulation of SA and the induction of specific PR genes with antimicrobial activity, which correlate with a broad-spectrum systemic resistance that is particularly useful in the long term against subsequent infections by biotrophic and hemibiotrophic pathogens (Harman *et al*., 2004a; Pieterse *et al*., 2009). In contrast with, the JA/ET-mediated systemic activation by beneficial microorganisms can provide the plant with broad-spectrum protection against biotic stresses through the mechanism known as ISR,

which does not require PR genes activation (Harman *et al.*, 2004a; Pieterse *et al.*, 1996; Pieterse and van Loon, 1999).

Finally, among defense hormones, abscisic acid (ABA) is an important regulator for plant development and adaptation to abiotic stress (in particular drought and salinity stress) but also modulator of the plant immune signaling network. ABA suppresses SA-dependent defenses and in *Arabidopsis* ABA and SA signaling pathway affect each other at multiple steps, from the level of biosynthesis to intermediate components of the signal transduction pathways (Yasuda *et al.*, 2008). When ABA is expressed in combination with JA, it acts positively on the expression of the MYC branch and negatively on the ERF branch of the JA pathway (Abe *et al.*, 2003; Anderson *et al.*, 2004; Fernandez-Calvo *et al.*, 2011). This results in prioritization of the immune signaling network toward the MYC branch, which is associated with resistance to herbivory, while resistance to necrotrophs is compromised (Anderson *et al.*, 2004). At the same time, JA signaling can exert a positive role for the ABA pathway by inducing genes encoding proteins of the ABA receptor family (Pieterse *et al.*, 2012). Because ABA negatively affects SA signaling while promoting the JA pathway MYC branch, it is likely to affect also the SA-JA cross talk. Additionally, interactions between these three hormones play also a role in the regulation of β-aminobutyric acid (BABA)-related defenses, but the general biological significance and molecular details of this tripartite hormone interaction are not yet clear (Pieterse *et al.*, 2012).

1.3.2 Plant growth hormones

Besides defense hormones, another significant group of phytohormones is represented by growth hormones. They are also called plant growth regulators (PGR) as they are able to influence processes such as root induction, flowering regulation, sexual expression, maturity, plant ageing and modulation of environmental stress. The huge family of the growth hormones includes auxins, cytokinins, gibberellins, brassinosteroids and strigolactones. Auxins were the first category of hormones to be discovered. They are powerful growth hormones produced by plants during their entire growth through two main pathways: the tryptophan (Trp)-independent and Trp-dependent pathways (Woodward and Bartel, 2005; Normanly, 2010). Auxins

are found in shoot and root tips and promote cell division, stem and root growth and can also drastically affect plant orientation by promoting cell division to one side of the plant in response to sunlight and gravity. Moreover, many microbes can produce auxins themselves or manipulate auxin signaling in the host to interfere with normal developmental processes (Kazan and Manners 2009, Robert-Seilaniantz *et al.*, 2011a), as well as some symbiont microorganisms are able to synthesize auxins, thus contributing to the tripartite plant-pathogen-symbiont interaction. Several studies have been demonstrated that this class of hormones can interact with some of the defense hormones. In particular, Chen *et al.*, (2007) showed that auxin signaling can repress SA levels and signaling, so it is not surprising that certain biotrophic pathogens evolved strategies to exploit auxin-mediated suppression of SA to enhance susceptibility of the host. Furthermore, in *Arabidopsis*, the JA signaling suppressor JAZ1 was found expressed in early phases of the auxin response, suggesting that it acts linking auxin and JA responses (Grunewald *et al*., 2009), although the role of the JA-auxin interaction in the regulation of the plant immune signaling network remains to be established.

Cytokinins (CKs) are another important group of growth hormones. The term cytokinins was coined for a series of compounds capable of stimulating cell division in selected cell cultures, but nowadays it is widely recognized that these molecules are able to evoke a large number of other responses. In fact, CKs are involved in diverse processes including stem-cell control, vascular differentiation, chloroplast biogenesis, seed development, growth and branching of root, shoot and inflorescence, leaf senescence, nutrient balance and stress tolerance (Muller and Sheen, 2007). They are adenine derivatives whose initial steps of biosynthesis are catalyzed by adenosine phosphate-isopentenyltransferase (IPT). Numerous organisms other than plants use adenine derivatives as signaling molecules, thus CKs are ideal molecules for interkingdom communication between plants and phytopathogens. Moreover, evidence pointed out that CKs contribute to the SA- and JA- mediated defense responses, acting as hormone modulators of this signaling backbone. Under CKs expression, in *Arabidopsis* the response regulators (ARRs), mediator of CKs signaling pathway, were activated. ARR can be divided into Type-A (negative regulators of CK signaling pathway) and Type-B (positive

regulators of CK signaling pathway). In particular, Type-B ARR can bind to specific SA response transcription factors and positively regulates PR-1 gene expression (Pieterse *et al.*, 2012). Furthermore, a recent study on a type-B response regulator 11 (ARR 11) demonstrated that SA/JA/CK combination has a positive outcome on plant fitness and in resistance against *B. cinerea*, corroborating the role of ARR11 in plant-defense related processes (Proietti *et al.*, 2018; Falconieri *et al.*, 2022).

Gibberellins (GAs) form a large family of tetracyclic diterpenoids originally identified from the necrotrophic fungal pathogen *Gibberella fujikuroi*. GAs are hormones stimulating plant growth and development by regulating DELLA growth-repressing proteins degradation. Among the several hundred GAs, only a few (such as GA1, GA3, GA4 and GA7) are biologically active in higher plants. GA1 and GA4 are the major bioactive GAs with relatively high abundance in various plant species, while GA3 and GA7 are less abundant (Binenbaum *et al.*, 2018). GAs biosynthesis in plants proceeds in steps involving three cellular compartments: plastids, endoplasmic reticulum (ER) and cytoplasm. In particular, it is in the last compartment that the final conversion of GAs into bioactive forms takes place. This hormones class is also involved in cross talk with defense hormones where DELLA proteins are key regulators. In fact, they are negative regulators of GAs and have been found to regulate the immune response modulated by JA and SA in plants. DELLA proteins regulate the balance of SA/JA signaling during plant immunity, supporting JA perception and/or signaling, and limiting SA biosynthesis and signaling (Navarro *et al.*, 2008). These proteins have also been shown to regulate the expression of various genes which are known for producing ROS detoxification enzymes (Achard *et al.*, 2008). Thus, indirectly GAs can be assumed to play a role in the oxidative stress related to salinity and osmosis.

Finally, brassinosteroids and strigolactones are showing increasing relevance in various physiological and morphological processes. Brassinosteroids (BRs) were initially discovered in *Brassica napus* pollen on the basis of their ability to promote growth. BRs have been classified into three main types based on the carbon number of each steroid molecule (i.e. C_{27}, C_{28} e C_{29}) (Fujioka and Yokota, 2003). The 5α-cholestane skeleton is the basic structure

of C_{27} -BR, 5α-ergostane for C_{28} -BR, while 5α-stigmastane is the basic structure of C_{29} -BR. They are a group of polyhydroxylated steroidal phytohormones that are required for the development, growth, and productivity of plants. Moreover, brassinosteroids play a role in regulating the division, elongation, and differentiation of a wide variety of cell types, during the entire plant's life cycle (Manghwar *et al*., 2022). BRs have been found in organs and all parts of the plants, such as leaves, stems, roots, flowers, pollen, anthers, and seeds (Bajguz *et al*., 2011) and are universally distributed in all growing tissues of higher plants. Recent studies have shown how BRs can participate in physiological processes in response to stress by regulating plant growth and improving plant performance by interacting with plant growth regulators or other plant hormones (Trevisan *et al*., 2020; Ahammed *et al.,* 2015). In particular they play an essential role in acclimation to environmental stresses, resistance to pathogens, and cell elongation, resulting in increased crop yield and plant growth (Kim and Russinova, 2020). Furthermore, their role as biostimulants in crops to induce tolerance to abiotic stresses and improve plant efficiency has recently emerged (Trevisan *et al*., 2020).

Strigolactones (SLs) were recently defined as new phytohormones that regulate plant metabolism and, in turn, plant growth and development. They are a group of sesquiterpenes and were initially discovered for their role in stimulating the germination of parasitic plants called Striga and Orobanche, which are detrimental to crop plants. SLs are released by plant roots and act as chemical signals to these parasitic plants, triggering their germination and subsequent attachment to the host plant's roots (Akiyama and Hayashi, 2006). In addition to their role in parasitic plant interactions, SLs have several other functions in plants as shaping the root architecture, inhibiting shoot branching and promoting root branching, in order to help the plant to explore a larger volume of soil for water and nutrients (Mitra *et al.,* 2021). Additionally, SLs promote mycorrhizal symbiosis, assist the plant in nutrient uptake (above all nitrogen and phosphorous) and also appear to play a role in plant responses to abiotic stress, such as drought and nutrient deficiency, by influencing root morphology. Finally, SLs can cross-communicate with other hormones as ABA, CKs, auxins and ET, leading to a synergistic action in response to

environmental changes and in plant growth and development (Wu *et al.*, 2022). Ultimately, similar to the classical defense hormones SA, JA, ET and ABA, also this family of growth hormones is involved in several defense responses, acting as multifaceted regulators of plant immunity.

1.4 Beneficial microbes

In nature plants are protagonists of a complex network in which numerous members make abundant use of their resources. In addition to microbial pathogens and herbivorous insects, plants also nourish a large community of commensal and mutualistic microbes that, for example, provide the plant with enhanced mineral uptake, nitrogen fixation, increased photosynthetic efficiency, growth promotion and protection from pathogens (Pieterse *et al*., 2014). These beneficial effects can be induced by the secretion of microbial small secondary metabolites (SM) that can act as hormone-like plant growth regulators or by the production of SM and proteins that enable microbes to modulate the signaling of plant defense hormones to successfully colonize plant tissues (Manganiello *et al.,* 2018; Stringlis *et al.,* 2018). Many microbial species among plant associated bacteria and fungi can produce hormones on their own. An example is represented by indole-3-acetic acid (IAA) or auxin-mimicking molecules that play a direct role on plant growth and development (Duca *et al.,* 2014). Generally, 80% of rhizospheric bacteria secrete IAA which increase the plant IAA content that in turn exert significant effect on plant growth. IAA released by PGPR (more attractive for the plant compared to endogenous IAA) mainly have a positive effect on the size and weight of the root system, and on increasing the formation of new roots.

As shown in figure 5, plant microbiota is predominantly hosted by the root system, which deposits up to 40% of the plant's photosynthetic carbon in the rhizosphere, making this small zone around the roots one of the most energy-rich habitats on Earth (Pieterse *et al*., 2014).

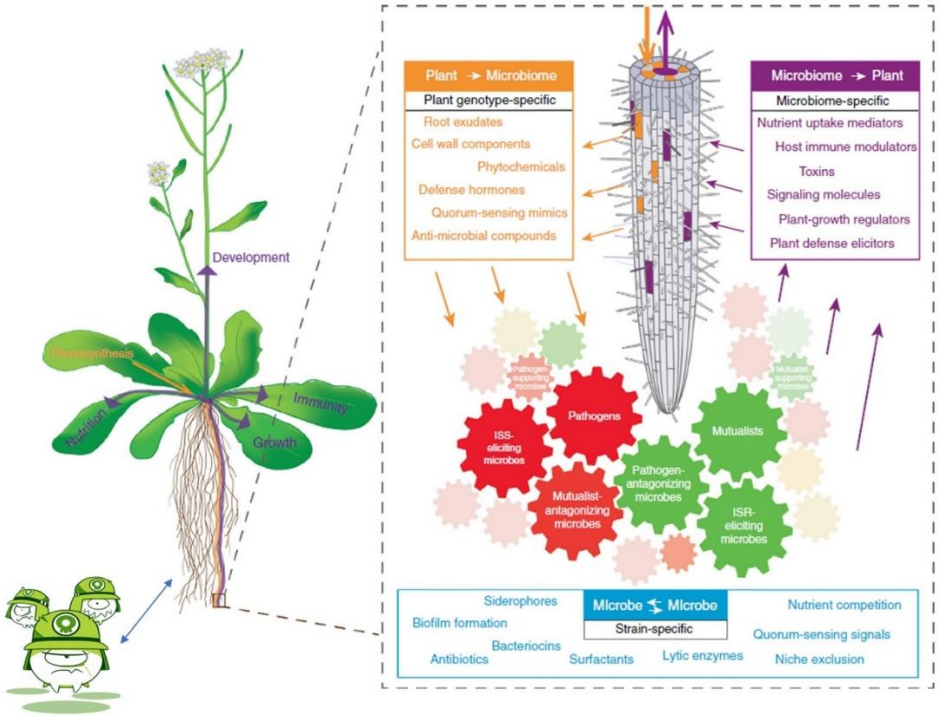

Figure 5. Representation of plant-microbe interaction activity. Plants invest resources in shaping and maintaining the microbial community in and around their roots. In turn, plants benefit from microbiome-encoded functions, resulting in improved growth, development, nutrition and immunity, affecting the activity and structure of the root microbiome, which obtain beneficial compounds for their growth. Modified from Pieterse *et al.*, 2016.

Although plant species tend to assemble relatively distinct microbial communities, these communities can often be largely similar in different geographic environment (Ling *et al*, 2022). In fact, plants exert selective effects on the assortments of microbial pool to acquire specific functional traits necessary for their fitness. Consequently, the microbiome rhizosphere greatly expands the plant's functional repertoire and, despite specific plant nutrient requirements, rhizosphere environments offer broadly similar conditions for microbial life (Ling *et al*, 2022). Microbial activities in the rhizosphere lead to changes in the composition, quality and quantity of root exudates released by plants, which in turn influence the microbial component (Philippot *et al.*, 2013). This phenomenon known as rhizosphere feedback proposes that plants, through rhizodeposition, shape the composition of the microbial community in the rhizosphere which subsequently affects plant growth and productivity (Dessaux *et al.*, 2016). Interactions between plants and microorganisms occur in many different ways and on many different levels. There are plenty of interactions where the plant benefits either through

direct or indirect effects of the associated microbes. In these interactions, plants serve as sheltered habitats for the microorganisms that may colonize different parts of the plant (Schirawski *et al.*, 2018). Many plants release compounds that attract and feed the associated microbes. Associated microbes can secrete compounds that promote plant growth, can make the plant more resistant to abiotic or biotic stresses, or can defend the plant against pathogens (Schirawski *et al.*, 2018). In particular, pathogens act favoring their own proliferation and disturbing the plant physiological and metabolic processes resulting in loss of yield and quality that has always been an important issue for species of agronomic interest. Over time, defense methods have been developed to fight and prevent the spread of the main diseases in plants in order to minimise crop losses. Until about twenty years ago, pest control in agriculture relied almost exclusively on the use of synthetic products, which can be classified according to both the active ingredient and the type of action achieved: an example is represented by contact pesticides that act very quickly by adhering to and penetrating the exoskeleton of insects. On the other hand, systemic pesticides are absorbed by the insect body and can even reach parts of the body not directly treated. The use of these synthetic products creates a long list of problems for the environment and human health, therefore there is a growing demand for innovative and environmentally friendly strategies in agriculture, such as biological control.

1.4.1 Biocontrol agents

Biological control can be defined as "*the use of natural or modified organisms, genes or gene products, to reduce the effects of undesirable organisms, and to favour those beneficial to humans, crops, animals and symbiotic microorganisms*", including in this definition the application of molecular biology techniques to obtain new technologies (US National Academy of Science -NAS, 1987). Over the past two decades, the definition of biological control has been broadened and modified, and today includes the use of any biological entity or derivative that is effective in containing a pathogen or pest. It includes both organisms such as bacteria, fungi, viruses, nematodes, arthropods, vertebrates and protozoa, and any molecule derived

from them. Biological control is proven to be highly successful and economical. The most common and studied beneficial microbial biocontrol agents (BCAs) are bacilli, actinomycetes, pseudomonads, agrobacteria, mycorrhizal fungi and fungi of the genera *Trichoderma* and *Gliocladium* (El-Saadony *et al*., 2022) (Figure 6).

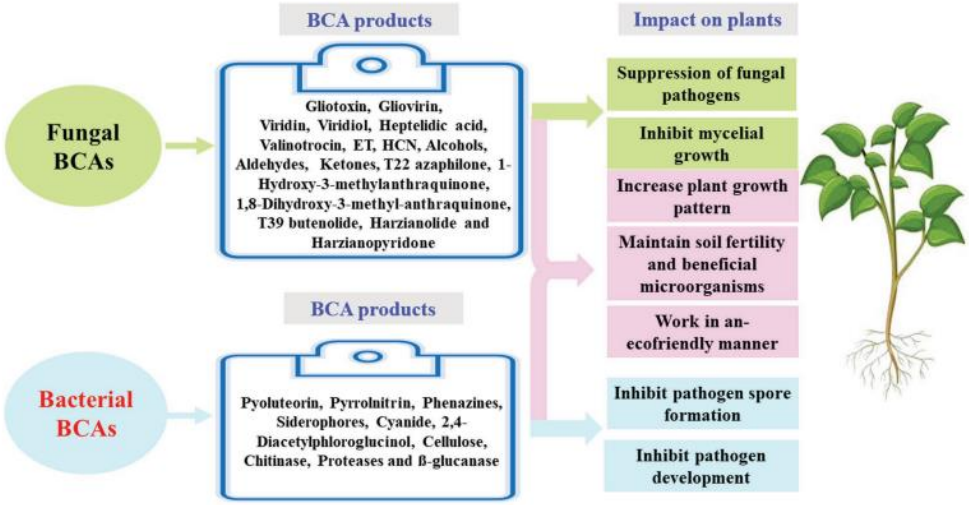

Figure 6. Fungal and bacteria biocontrol agents (BCAs) and their impact on plant. (From El-Saadony *et al.*, 2022).

In general, successful biological control requires not only the study of the potential antagonistic agent, but also the knowledge of the complex ecological interactions that take place between the plant, the pathogen, the natural microflora and the environment. In nature, an antagonist is an organism capable of counteract a target pathogen by interfering with its life cycle (Baker and Cook, 1974), triggering mechanisms that can be classified into direct and indirect. Direct mechanisms are those operating under the antagonist-pathogen interaction and include: parasitism, antibiosis and competition for space and nutrients. Indirect mechanisms include all the morphological and biochemical modifications elicited in the host plant by the antagonist agent and which lead to a greater resistance of plants to abiotic and biotic stresses. Particularly interesting BCAs are the plant-growth-promoting-rhizobacteria (PGPR) which are non-symbiont beneficial bacteria that inhabit the rhizosphere; important members of this group are bacteria belonging to the genera *Pseudomonas, Serratia, Azospirillum* and *Bacillus.* They interact

with the plant by conferring positive characteristics to the host: in addition to increased growth of roots, they are also able to inhibit several plant pathogens by interfering with their ability to infect and invade tissues. In the large family of BCAs, the plant-growth-promoting-fungi (PGPFs) have a prominent role. Fungi of heterogeneous classes and habitats have been shown to function as PGPFs, after the establishment of a mutualistic association. The well-known fungal genera *Aspergillus, Fusarium, Penicillium, Pyriformospora, Phoma* and *Trichoderma* are the most frequently reported PGPFs (Hossain *et al*., 2017). The most commonly reported effects of plant interaction with these fungi are a significant improvement in germination, biomass production, root hair development, photosynthetic efficiency, flowering and yield. It is now widely recognized that PGPF can also control numerous foliar and root pathogens by triggering ISR in host plants. These capabilities are driven by their ability to enhance nutrient uptake and phytohormone production, as well as to reprogramme plant gene expression through differential activation of plant signalling pathways (Hossain *et al*., 2017).

1.5 Beneficial fungi

Beneficial fungi are able to establish a stable association with the host, representing an efficient and biologically relevant plant survival strategy, enabling them to withstand unfavourable conditions. Well-known examples of beneficial fungi are mycorrhizal fungi that can form a symbiosis with approximately 80% of all terrestrial plant species.

Among beneficial fungi are entomopathogenic fungi (EPF) which have the ability to infect insects leading to disease in proper condition, where they directly colonize the insect body by penetrating its cuticles (Bamisile *et al*., 2021). The topic of entomopathogenic fungi as endophytes has been a rapidly-evolving field of research in recent years. In fact, many studies have been performed on EPF-colonized tomato to test their effects on plant performance. A consistent number of species belongs to a group of fungi classified as entomopathogens, including *Akanthomyces spp., Beauveria bassiana, Clonostachys rosea, Cordyceps farinose Lecanicillium spp.,* and *Sarocladium spp*. (Sinno *et al*., 2020). These fungi act as insect control agents, either through a plant-mediated response or by exerting a direct

insecticide effect or by recruiting natural pest antagonists (Coppola *et al*., 2017, Zahran *et al*., 2017, Pineda *et al*., 2017). In particular, concerning the insect pests, biocontrol potential of EPF was apparent on *Aphis gossypii, Bemisia tabaci, Chortoicetes terminifera, Helicoperva armigera, Nesidiocoris tenuis, Spodoptera exigua, S. littoralis, Tuta absoluta,* and *Trialeurodes vaporariorum* (Sinno *et al*., 2020). The overall effects observed on tomato pests included increased mortality, feeding deterrence, reduced growth rate and reproduction, reduced infestation, egg masses colonization, and increased plant defense. Moreover, EPF were reported to counteract the infection of fungal pathogens *Fusarium oxysporum f. sp. lycopersici, Rhizoctonia solani* and *Botrytis cinerea* (Barra-Bucarei *et al*., 2019). In particular, the effects of endophytic colonization on pathogen control were reported as reduced disease symptoms and a disease-suppressive effect. Thus, these fungi have a range of ecological functions suggesting that they may have the potential to be developed as biopesticides with multiple roles for improved production of agricultural systems (Vega *et al.*, 2009; Lacey *et al.*, 2015). Nowadays, there are already different commercial products (also known as bio-formulates) for microbial control of insect pests based on the use of entomopathogenic fungi such as *Beauveria spp., Metarhizium spp., Isaria fumosorosea* and *Lecanicillium spp*. (Lacey *et al*., 2015). Therefore, understanding the mechanisms underlying tomato-beneficial fungus interaction can undoubtedly provide advancement of knowledge in the field for the improvement and quality of crop production.

1.5.1 *Beauveria bassiana*

Beauveria bassiana is the anamorph stage of *Cordyceps bassiana* (Ascomycota, Clavicipitaceae) and it is best known as ubiquitous entomopathogen attacking a broad range of insects, belonging mainly to the order Lepidoptera and Coleoptera (Sung *et al.*, 2007). The spores of this fungus in contact with the insect germinate and penetrate through the cuticle by a combination of mechanical and enzymatic action. The subsequent development of the fungus inside the insect, accompanied by the production of toxins (beauvericin) and the loss of water and nutrients, results in its death in 3-5 days. As shown in figure 7, the infection pathway consists of the

following steps: attachment of the spores to the cuticle due to the hydrophobicity of both the conidia and the cuticular surfaces; germination; penetration through the cuticle by enzymatic and mechanical action; overcoming the host response and immune defense reactions; proliferation within the host by formation of hyphal bodies/blastospores, yeast-like cells; saprophytic outgrowth from the dead host and production of new conidia (Wang *et al*., 2021).

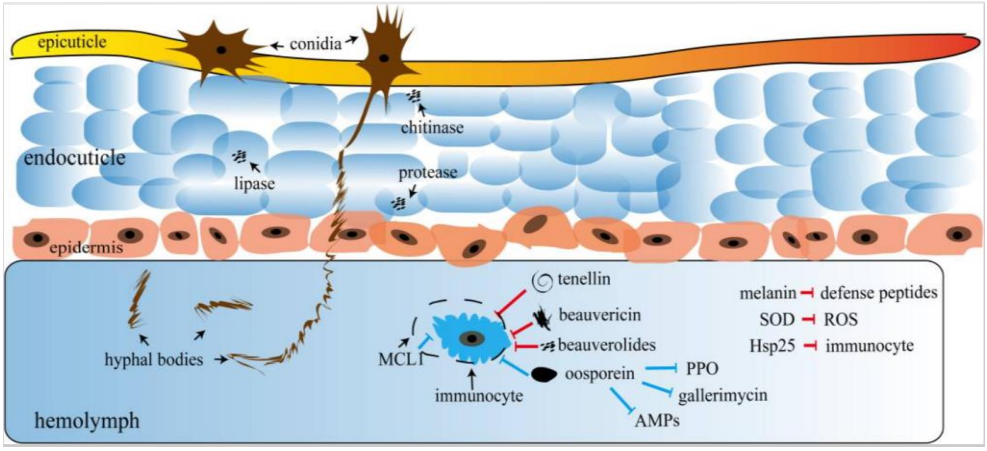

Figure 7. A schematic of *B. bassiana* invasion. Conidia firstly penetrate the epicuticle to reach the endocuticle of the insect and then release plenty of hydrolases (chitinase, protease, and lipase) to rapidly disintegrate the cuticle for more conidia intrusion. Hyphal bodies that pass through the epidermis intrude into the hemolymph of the insects and secrete plenty of insecticidal substances, such as oosporein, beauvericin, beauverolides and tenellin, most of which either suppress the immunocyte or directly destroy the hemolymph. From Wang *et al.*, 2021.

The genus Beauveria is characterised morphologically by its clusters of short-globose to flask-shaped conidiogenous cells. Species identification in *Beauveria* is difficult because of its structural simplicity and the lack of distinctive phenotypic variation. Conidia have been the principal morphological feature used for species identification in Beauveria, although recently molecular approaches have been used to refine the genus (Rehner *et al*., 2011). Conidia can have different shapes: globose, ellipsoidal, cylindrical and different size ranging from 1.7 to 5.5µm (Figure 8). Currently, 15 species are recognised mainly distinguishable by sequence analysis of conserved regions (Rehner *et al*., 2011; Zhang *et al*., 2012).

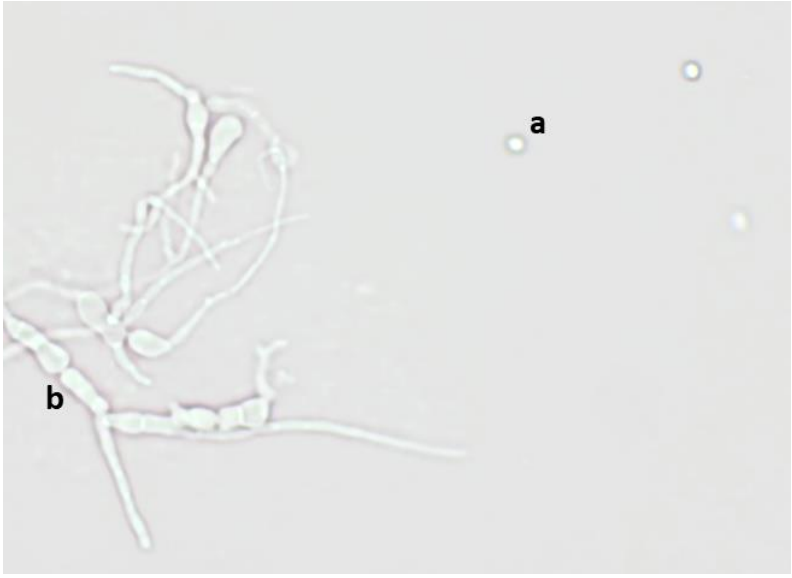

Figure 8. Morphological characteristics of the endophyte *B. bassiana*. (a) Conidia and (b) hyphae. Image acquisition with an optical microscope (Zeiss Primostar 3, 40x).

In 1991, Bing and Lewis demonstrated that *B. bassiana* was able to grow endophytically. Later, *B. bassiana* was reported to negatively affect herbivores and pathogens (Ownley *et al.*, 2008). Colonization of maize (*Zea mays*) by a suspensions of *B. bassiana* spores led to a season-long resistance against caterpillars of *Ostrinia nubilalis*, as result of the *B. bassiana* establishment as an endophyte in the plant (Wagner and Lewis, 2000). The fungus can systemically spread throughout the plant and its presence within xylem vessels has been reported in corn and opium poppy (Wagner and Lewis, 2000). Ownley et al. (2008) reported a protective role of endophytic *B. bassiana* against the plant pathogenic fungi *Rhizoctonia solani* and *Pythium myriotylum*. Studies on *R. solani* in cotton and tomato have established that application of conidia of *B. bassiana* to seed resulted in endophytic colonization and reduction in disease symptoms and plant losses (Ownley *et al.,* 2008). Moreover, a recent study has deep investigated the effect of *B. bassiana* as a biocontrol agent against tomato pathogen and pests (Sinno *et al.,* 2021). In particular, this work demonstrated that *B. bassiana* strain 'Naturalis' was able to promote plant growth, significantly increasing plant height and dry weight, to negatively affect the survival and fertility of the sucking insect pest *Macrosiphum euphorbiae* and to significantly decrease the symptoms caused by the two foliar fungal pathogens *Alternaria*

alternata and *Botrytis cinerea (*Sinno *et al*., 2021*)*. The studies that have demonstrated the efficiency of *B. bassiana* colonization in helping different plant species to cope with pathogens and pests are diverse, however the effect of plant colonization by *B. bassiana* is still poorly understood at the molecular level. Moreover, only a few -omics studies have attempted to investigate *B. bassiana* symbiotic interactions at the host's specific tissue or cellular and subcellular levels, as the investigation at transcriptomic and metabolomic level of the Arabidopsis plant response to *B. bassiana.* These studies showed evidence for transcriptional reprogramming of plant defense pathways, including those related with pathogenesis-related proteins, phytoalexin, jasmonic acid (JA) and salicylic acid (SA) signaling (Raad *et al.,* 2019). Nonetheless, to the best of our knowledge, only one study has investigated the effect of *B. bassiana* colonization on plant proteomes, highlighting the induction of proteins related to photosynthesis, energy metabolism, and defenses (Gomez-Vidal *et al.*, 2009), therefore it would be interesting to investigate these aspects on a large scale.

All these features made *B. bassiana* one of the most widely used biocontrol agent against many major arthropod pests, due to its ability to synthesize a cocktail of proteins, enzymes, organic acids and bioactive secondary metabolites responsible for the entomopathogenic activity and virulence (García-Estrada *et al.*, 2016). The strains of *B. bassiana* currently available in the market are active against all stages (eggs, neanids, pupae and adults) of the aleurodids that attack most horticultural and ornamental crops in greenhouses and open fields (in particular, they are active against *Bemisia tabaci, Bemisia argetifolii, Trialeurodes vaporariorum*). *B. bassiana* bioformulates are guaranteed as harmful for arthropod-specific pathogen and but not infectious, toxic or pathogenic to mammals; moreover, they are not harmful beneficial entomophages. Although the above-mentioned studies have suggested that *B. bassiana* negatively affects herbivores and plant pathogens, the mechanisms underlying this phenomenon still remain elusive. Furthermore, very little is known on how the host plant responds to the presence of *B. bassiana*.

1.5.2 *Trichoderma spp.*

The success of fungi belonging to the genus *Trichoderma* as beneficial microbes depends on its good reproductive capacity, ability to survive in unfavorable conditions, efficient utilisation of nutrients, ability to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi and the effectiveness of plant defense systems (Chen *et al*., 1997; Monte, 2001). To the genus *Trichoderma* belong many species of filamentous saprophytic fungi belonging to the Deuteromycetes group that are naturally present in the soil. *Trichoderma* spp. is a ubiquitous organism, easy to isolate and cultivate, and non-pathogenic to higher plants. It is a strong competitor in its trophic niche and possesses an enzymatic system capable of attacking a wide variety of pathogenic fungi; it is also a mycoparasite capable of producing numerous antibiotics. The antifungal activity of *Trichoderma* spp. is mainly through aggressive competition, mycoparasitism and the production of antibiotic substances. Nutrient competition takes place through the subtraction of substances contained in the exudates present on the surface of plant organs (macro and microelements, sugars, alcohols, pectins, amino acids, organic acids), thus preventing the germination of the pathogen spores. The fungicidal activity takes place when the fungus is attracted to the substances emitted by the hyphae of the host mycelium (parasitisation is specific). Once it reaches the phytoparasite, *Trichoderma* envelops its mycelium with its own hyphae, emitting lytic enzymes (β-1,3-glucanase and chitinase) capable of dissolving the cell wall and allowing penetration into the host mycelium (Figure 9).

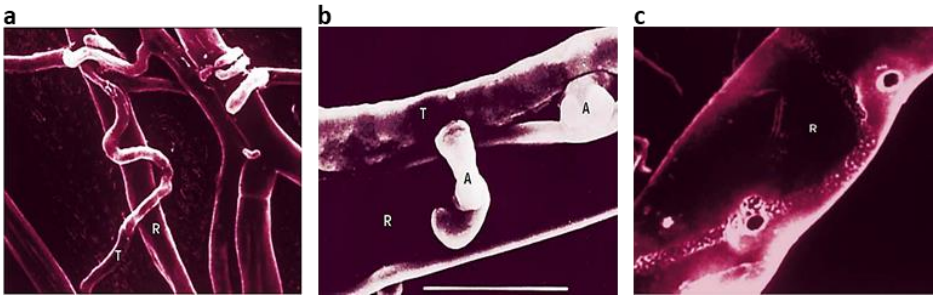

Figure 9. The events leading to mycoparasitism. In the figure, 'R' indicates hyphae of the plant pathogen *Rhizoctonia solani*, 'T' indicates hyphae of Trichoderma spp., and 'A' indicates appressoria-like structures. In a, Trichoderma is in the process of parasitizing a hypha of *R. solani*. In b, a higher magnification of the *Trichoderma–R. solani* interaction, highlighting the appressoria-like structures, is showed and c illustrates an *R. solani* hypha from which Trichoderma has been removed. From Harman *et al*., 2004.

Many *Trichoderma* species are currently used in commercial formulations. It has been observed that some *Trichoderma* species have a synergistic effect with PGPR (Plant Growth Promoting Rhizobacteria) and mycorrhizal fungi (Clavet *et al*., 1993). The species of the genus *Trichoderma* most commonly used for biological control are *T. harzianum* and *T. virens* (Papavizas, 1985). In particular, the chitinolytic enzymes of these two strains have been extensively studied because they are responsible for cell wall degradation in chitin-containing fungi (De la Cruz *et al*., 1992; Di Pietro *et al.* 1993, Lorito *et al.*, 1993b, 1994b). Numerous studies show that *T. harzianum*, alone or in combination with other *Trichoderma* species or chemicals, directly attacks pathogens such as *Rhizoctonia, Fusarium, Pythium, Sclerotinia* and *Botrytis*. Of particular interest is the synergism of *Trichoderma* lytic enzymes with certain fungicide that are inhibitors of sterol synthesis (flusilazole, miconazole), chitin and β-glucans. Cell wall degrading enzymes (CWDEs) favour the penetration of the fungicides into the specific sites of action, which thus reach higher concentrations at the target sites (Wiest *et al*., 2002). For example, MACs (Membrane-Affecting Compounds), among which are the above-mentioned fungicides, induce a degradation of the enzyme complexes chitin and glucan synthase, rendering the fungus unable to repair the damage produced on the wall by the lytic enzymes. The high synergism between the enzymes, fungicides and MACs suggests the application of these compounds to improve plant resistance to disease (Woo *et al*., 1998) and for the production of new fungicide formulations (Lorito *et al.,* 1994), which would allow the quantity of chemical pesticides used in integrated pest management to be reduced, and consequently also the environmental impact.

Plant protection by *Trichoderma* can be implemented through classical biocontrol mechanisms of action and through the stimulation of plant disease resistance and defense mechanisms. Several studies show that jasmonic acid and ethylene are involved in the biological control stimulated by *Trichoderma* (Shoresh *et al*, 2005). Furthermore, it has been shown that *Trichoderma* induces overexpression of the plant *Pal1* gene that encodes for the enzyme phenylalanine ammonium lyase. This enzyme catalyses the first reaction of the metabolic pathway that leads to the formation of phenolic compounds, including the phytoalexins (Shoresh *et al*. 2010; Lorito *et al*. 2010).

Inoculation of *Trichoderma* on plant roots produces an under-regulation of PR proteins (Chen *et al*., 2005) and an under-expression of proteins involved in the production of ethylene biosynthesis (Shoresh *et al*., 2005). This local silencing of defense responses in the early stages of plant colonization facilitates symbiotic interactions (Chen *et al.,* 2005). It has been pointed out that *Trichoderma spp.* exert a priming effect in the plant already when it is attacked by a pathogen, leading to higher levels of PR protein than no non-colonized plants. This is also associated with increased levels of phenolic glycosides, highly toxic antifungal compounds (Lorito *et al*. 2010). Noteworthy, priming is an adaptive strategy that improves plant defensive capacity in response to stresses, enhancing activation of induced defense mechanisms (Mauch-Mani *et al.,* 2017). Moreover, Di Lelio *et al.* (2023) investigated how *T. harzianum* can negatively affect the development and survival of the lepidopteran pest *Spodoptera littoralis* feeding on leaves of the treated plants. Through a multifaceted experimental approach, it has been demonstrated that *T. harzianum* influences the dynamics of the interaction between insects and plants by modifying the insect gut microbiome, leading to a gut dysbiosis that results in a negative impact on insect development and survival. The use of omics techniques to study the effects of plant colonization with different species of Trichoderma is much more advanced than that of *B. bassiana* since several transcriptomic, epigenetic, metabolomic and proteomic studies have been carried out highlighting and confirming the beneficial role of these fungi in plant growth promotion and defense strategies (Coppola *et al.,* 2019; De Palma *et al.,* 2019). To the best of our knowledge, how *T. harzianum* interact with other beneficial fungi such as *B. bassiana* at the molecular level in plant is still poorly understood, therefore it would be interesting to fill this knowledge gap to contribute to the discovery of additional action mechanisms underlying *T. harzianum-B.bassiana* interaction. All these data could be particularly interesting for the application of these organisms in biological control, to be used alone or in consortia, since *Trichoderma* species or other beneficial microbes can be selected in order to be compatible with other biological agents. Overall, the use of microorganisms and the exploitation of beneficial plant-microbe interactions

offer promising and environmentally friendly strategies for conventional and organic agriculture worldwide.

2 Goal of the thesis

Tomato (*Solanum lycopersicum L.*) represents the most important non-cereal crop in the world. Together with other species of agricultural interest, it can be subjected to the attack of more than a hundred different pathogens. Beneficial fungi are often associated with physiological and metabolic reprogramming of plants that can promote growth as well as strengthening of defense barriers against pathogens and pests. Among them, the entomopathogenic fungus *B. bassiana* can colonize plant tissues in an asymptomatic way, triggering poorly characterized plant metabolic changes, which negatively affect both pest insects and plant pathogens. Knowledge of these mechanisms is still incomplete, despite their importance for improving agriculture towards eco-compatibility and safeguarding consumer health.

This thesis aims to fill this knowledge gap to contribute to the discovery of additional action mechanisms underlying the interaction between *B. bassiana* and tomato.

Therefore, using tomato as a model system, the research activity initially focused on understanding and investigating the molecular events that regulate tomato - *B. bassiana* interaction. In detail, the set objectives were as follows:

a. To investigate plant metabolic and physiological changes significantly influenced by *B. bassiana*, by a time-course comparative proteomics analysis.
b. To study plant leaf hormones with particular reference to the composition and quantity of phytohormones induced by *B. bassiana*.
c. To investigate the effect of *B. bassiana* on the response and regulation of the antioxidant defense mechanisms and lipid peroxidation in tomato plants infected by *Botrytis cinerea*.

In addition, the thesis focused on the effect of colonizing tomato plants with beneficial fungi used individually and in consortium to investigate the impact of the interaction, particularly in response to biotic stress. This aspect is still underexplored in the literature, and therefore, it holds strategic importance

not only for deepening understanding but also in view to potential practical applications. To this, the experimental work included the investigation of molecular mechanisms involved in the colonization of tomato with the beneficial consortium *B. bassiana*-*T. harzianum*, to understand if these beneficial microbes can together add another layer of regulation in plant growth and defense. In particular, the set objectives were as follows:

a. To reveal new signaling networks underlying plant growth and defense, triggered by *B. bassiana* or/and *T. harzianum*, also in response to biotic stresses caused by the infection with *B. cinerea* or after the attack of the insect *Macrosiphum euphorbiae*, using a comparative proteome analysis approach.
b. To study plant hormonome with particular reference to the quantity and composition of phytohormones produced by the plant colonized by *B. bassiana* or/and *T. harzianum*, also in response to *B. cinerea* and *M. euphorbiae*.

Overall, the study of the complex network of interactions established between the plant and beneficial or pathogenic microorganisms can be useful for the development of new eco-friendly and more sustainable defense strategies.

3 Material and methods

3.1 Material and methods to investigate tomato-*B. bassiana* interaction

Material and methods of the first part of this work, in which we have studied the molecular mechanisms that regulate tomato-*B. bassiana* interaction, are reported in Proietti *et al.* (2023).

3.1.1 *Beauveria bassiana* fungal culture

Beauveria bassiana strain 'Naturalis' was cultured on potato dextrose agar (PDA) maintained at 25 ± 2 °C, and 14:10 h light–dark photoperiod for 20 d. Conidia were harvested by adding 5 ml of sterile distilled water to the sporulating cultures. The surface of the mycelium was gently scraped with a sterile L-spatula to remove spores. The liquid containing mycelia and spores was filtered through synthetic wool to remove the mycelium and collect the spore suspension in a sterile tube. The harvested conidia were shaken to obtain a homogeneous spore suspension. The concentration of the spores in the liquid inoculum was determined by a series of dilutions and counting the number of spores in a hemocytometer (Neubauer hemocytometer chamber) under a microscope. The spore concentration ranged from about 1×10^8 to 10^9 conidia ml^{-1} in approximately 5 ml volume. The spore suspension was stored at 4 °C until use.

3.1.2 Plant growth and colonization by *Beauveria bassiana*

Tomato seeds (*Solanum lycopersicum* cv San Marzano nano, Top Seed, Sarno, Italy) were surface sterilized in 1% NaOCl for 5 min, then rinsed three times with sterile distilled water. Seeds were germinated on sterile filter paper (Sigma-Aldrich, St Louis, MO, USA) soaked with sterile distilled water, in the dark at 24 °C. Germination occurred in 4–5 d. Seedlings were individually transplanted to 110 ml pots containing commercial soil (Type 3 special Brill Soil, Georgsdorf, Germany). After 1 week, the emerged tomato seedlings (two-cotyledon stage) were watered with a variable volume of the *B. bassiana* conidial suspension to obtain a final concentration of 1×10^6 conidia ml^{-1} of

soil volume. Control plants were watered with sterile distilled water. The treatment was repeated after 2 weeks. To confirm endophytic colonization, tissue samples were collected and surface sterilized in 1% NaOCl for 3 min and rinsed three times with sterile distilled water. Each section was dried on sterile paper, then cut into six pieces (about 1 cm^2), placed on 90 mm Petri dishes containing PDA supplied with 1% lactic acid to avoid bacterial contamination, and incubated at 25 °C in the dark. Plant pieces were monitored daily to determine if there was fungal growth emerging from the cut plant (white, cottony, dense hyphal growth). The fungal mycelia were isolated from the substrate close to the plant tissue and transferred to new dishes containing PDA, to obtain pure cultures for identification, which was carried out by examining the morphology of the fungal mycelia under a light microscope (Primostar 3, Zeiss Microscopy, Jena, Germany).

3.1.3 Leaf proteome analysis

3.1.3.1 Plant material

Leaves of adult plants were harvested from two sets of plant samples: (i) root-colonized by *B. bassiana* and (ii) control plants (not colonized), at 5, 7, 12, and 19 d (T1, T2, T3, and T4, respectively) after the second colonization. For each time point, leaf area and stem height were measured with a ruler. Leaf area was calculated on the widest leaf with the following formula: leaf area=leaf length×leaf width. For the following proteome analysis, three biological replicates were used for each time point. In total, 24 samples were obtained.

3.1.3.2 Protein extraction

Sample preparation was performed following Bertini *et al.* (2019b). Briefly, powdered leaves were suspended in a lysis buffer comprising 10% trichloroacetic acid (TCA) in acetone and 10% dithiothreitol (DTT), and then kept at 20 °C for 2 h before centrifugation at 15 000 g for 14 min at 4 °C. Pellets were washed in acetone with 10 mM DTT, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride before being centrifuged again under the same conditions. The pellets were dried in a Speed Vac Concentrator (Savant, Thermo Fisher Scientific, Waltham, MA, USA). Pellets were lysed with 5 ml

of 6 M urea in 100 mM Tris–HCl pH 8.0 in a Branson Digital Sonifier (1min cycle, 1 min run time, 20 s on, 10 s off, 30% amplitude). Samples were centrifuged at 4 °C, 15 min, 15 000 g and the pellet was discarded.

3.1.3.3 In-solution digestion

Samples (20 μg) were reduced with DTT (30 nmol, 37 °C, 60 min) and alkylated in the dark with iodoacetamide (60 nmol, 25 °C, 30 min). The resulting protein extract was first diluted to 2 M urea with 200 mM ammonium bicarbonate for digestion with endoproteinase LysC (1:100 w:w, 37 °C, o/n, Wako, cat. no. 129-02541), and then diluted 2-fold with 200 mM ammonium bicarbonate for trypsin digestion (1:100 w:w, 37 °C, 8 h, Promega cat. no. V5113). After digestion, peptide mix was acidified with formic acid and desalted with a MicroSpin C18 column (The Nest Group, Inc.) prior to LC-MS/MS analysis.

3.1.3.4 Chromatographic and mass spectrometric analysis

Samples were analysed using an Orbitrap Eclipse mass spectrometer (Thermo Fisher Scientific) coupled to an EASY-nLC 1200 (Thermo Fisher Scientific). Peptides were loaded directly onto the analytical column and were separated by reversed-phase chromatography using a 50-cm column with an inner diameter of 75 μm, packed with 2 μm C18 spectrometer particles (Thermo Fisher Scientific). The following buffers were used: 0.1% formic acid in water (buffer A) and 0.1% formic acid in 80% acetonitrile (buffer B). Chromatographic gradients started at 95% buffer A and 5% buffer B with a flow rate of 300 nl min^{-1} for 5 min and gradually increased to 25% buffer B and 75% buffer A in 79 min and then to 40% buffer B and 60% A in 11 min. After each analysis, the column was washed for 10 min with 100% buffer B. The mass spectrometer was operated in positive ionization mode with nanospray voltage set at 2.4 kV and source temperature at 305 °C. An Ultramark 1621 was used for external calibration of the Fourier-transform mass analyser prior the analyses, and an internal calibration was performed using the background polysiloxane ion signal at m/z 445.1200. The acquisition was performed in data dependent acquisition mode and full MS scans with one micro scan at resolution of 120 000 were used over a mass

range of m/z 350–1400 with detection in the Orbitrap mass analyser. In each cycle of data-dependent acquisition analysis, following each survey scan, the most intense ions above a threshold ion count of 10000 were selected for fragmentation. The number of selected precursor ions for fragmentation was determined by the 'Top Speed' acquisition algorithm and a dynamic exclusion of 60 s. Fragment ion spectra were produced by high-energy collision dissociation at normalized collision energy of 28% and were acquired in the ion trap mass analyser. AGC was set to 2E4, and an isolation window of 0.7 m/z and a maximum injection time of 12 ms were used. All data were acquired with Xcalibur software v4.1.31.9. Digested bovine serum albumin (New England Biolabs cat. no. P8108S) was analysed between each sample to avoid sample carryover and to ensure instrument stability, and QCloud (Chiva *et al.,* 2018; Olivella *et al.,* 2021) was used to control instrument longitudinal performance during the project.

3.1.3.5 Data analysis

Acquired spectra were analysed using the Proteome Discoverer software suite (v2.3, Thermo Fisher Scientific) and the Mascot search engine (v2.6, Matrix Science; Perkins *et al.,* 1999). The data were searched against a Uniprot *Solanum lycopersicum* database (as in August 2020, 34 655 entries) plus a list of common contaminants (Beer *et al.,* 2017) and all the corresponding decoy entries. For peptide identification a precursor ion mass tolerance of 7 ppm was used for MS1 level, trypsin was chosen as enzyme, and up to three missed cleavages were allowed. The fragment ion mass tolerance was set to 0.5 Da for MS2 spectra. Oxidation of methionine and N-terminal protein acetylation were used as variable modifications and carbamidomethylation on cysteines was set as a fixed modification. The false discovery rate (FDR) in peptide identification was set to a maximum of 1%. Peptide quantification data were retrieved from the 'Precursor ions quantifier' node from Proteome Discoverer using 2 ppm mass tolerance for the peptide extracted ion current (XIC). The obtained values were used to calculate protein fold-changes and their corresponding adjusted P-values. The raw proteomics data have been deposited into the PRIDE repository (Vizcaíno *et al.,* 2015) and they are available via ProteomeXchange with the identifier PXD029422.

3.1.3.6 Bioinformatics analysis

All downstream data analysis and plotting were generated within the R environment, version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Evaluation of global proteome changes between treatments and across time points was performed by principal component analysis (PCA). To perform PCA, missing label-free quantification values for undetected proteins were imputed to 0. PCA was computed with the package FactoMineR version 2.4 (Lê *et al.*, 2008) on the log2-transformed matrix by applying an offset equal to the minimum value. Differences between PCA clusters were assessed by pairwise Mahlanobis distances with the R package HDMD version 1.2. Distances were calculated between the coordinates of the centroids of sample groups, on the principal components 1 and 2. Hotelling's T2 test was applied to calculate significant differences between centroids distances with the R package Hotelling version 1.0-8. Patterns analysis of expression profiles across time points was performed by a hierarchical clustering analysis with the package pheatmap version 1.0.12 (Kolde, 2019). A joined matrix of the total unfiltered log2 fold change values resulting from the differential expression analysis of *B. bassiana* versus control was built and the fold change missing values of each time point derived from undetected proteins were imputed to 0. Hierarchical clustering was then calculated on rows (proteins IDs) by the complete-linkage clustering method on the Euclidean dissimilarity matrix. Gene ontology (GO) annotation of the *Solanum* proteome was retrieved from the EMBL-EBI Gene Ontology Annotation (GOA) proteome database (https://www.ebi.ac.uk/GOA/proteomes). The downloaded GOA database was then imported in the R environment and a custom database was built with the package mgsa version 1.36.0 (Bauer and Gagneur, 2021). Enrichment analysis was performed with the package clusterProfiler version 3.16.1 (Yu *et al*., 2012) by hypergeometric distribution test. The background universe of the hypergeometric test was represented by a vector of length equal to the total number of detected proteins among all analysed conditions. GOs were considered significantly enriched when passing a P-value cutoff of 0.05 and the Benjamini–Hochberg P-value multiple testing correction. For the GOs, we used clusterProfiler to perform enrichment analysis of over-represented Kyoto Encyclopedia of Genes and

Genomes (KEGG) pathways (Kanehisa *et al.*, 2017) on differentially expressed proteins (DEPs) at each different time point by the command enrichKEGG, which automatically retrieved the annotated pathways of *S. lycopersicum* ('sly') from the KEGG database (https://www.genome.jp/kegg/catalog/org_list.html). KEGG pathways were considered significantly enriched with a P-value <0.05 after Benjamini–Hochberg correction for multiple testing. For each time point, GO and KEGG enrichment analyses were performed on a filtered list of DEPs retaining only annotated proteins.

3.1.3.7 Statistical analysis

The statistical analysis was performed with the R environment, version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). For the PCA plot, the R package Hotelling version 1.0-8 was used to calculate significant differences between centroids distances applying Hotelling's T2 test. Gene Ontology (GO) enrichment analysis was performed with the package clusterProfiler version 3.16.1 (Yu *et al.*, 2012) by hypergeometric distribution test. GOs were considered significantly enriched when passing a P-value cutoff of 0.05 and the Benjamini–Hochberg P-value multiple testing correction.

3.1.4 Western blotting–SUnSET analysis

Western blotting–SUrface SEnsing of translation assay (WB-SUnSET) analysis was performed in leaves of plants root-colonized by *B. bassiana* 12 d after the second colonization (corresponding to the third time point) and in control plants (not colonized), as shown in 'Plant material'. Leaf protoplasts were isolated according to Yoo *et al.* (2007). After incubation at 25 °C with 10 μg ml^{-1} puromycin for 60 min, protoplasts were lysed in 10 mM Tris–HCl buffer pH 7.6 containing 100 mM NaCl, 10 mM $MgCl_2$, 0.1% Triton X-100, and 1 mM EDTA. The lysate was resuspended in Laemmli sample buffer, used for 15% (w/v) SDS-PAGE, and electrophoretically transferred onto nitrocellulose membrane for a WB-SUnSET assay. Protein markers were used to assess molecular masses (SMOBIO-PM 2610, SMOBIO Technology, Hsinchu City, Taiwan). Following electroblotting, the membrane was

blocked for 30 min in phosphate-buffered saline (6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 3 mM KCl, 0.15 M NaCl, pH 7.4) containing 0.5% (v/v) Triton X-100 and 3% (w/v) BSA and then incubated overnight at room temperature with a mouse anti-puromycin monoclonal antibody (dilution 1:1000) as a primary antibody (MABE342, EMD Millipore Corp., Temecula, CA, USA). The immunoreactive bands were detected with horseradish peroxidase-conjugated goat-anti-mouse IgG (dilution 1:7000) as a secondary antibody using 4-chloro-1-naphthole as peroxidase substrate (Sigma-Aldrich). The same membrane was stained with Ponceau S (Advansta, Menlo Park, CA, USA) to normalize the protein loading.

3.1.5 Phytohormone and related compound analysis

Analyses of plant compounds were performed in leaves of plants root-colonized by *B. bassiana* 19 d after the second colonization (corresponding to the fourth time point) and in control plants (not colonized), as in 'Plant material', according to the methods reported in Dziurka *et al.* (2016, 2022). About 20 mg of lyophilized and powdered plant material with a mixture of stable isotope-labeled plant hormone as internal standard were extracted three times with an organic solvent consisting of methanol: water: formic acid 15:4:1 (v/v/v). The three extracts were combined and then evaporated under N_2. The dry extracts were resuspended in 3% methanol in 1 M formic acid and then cleaned on hybrid SPE cartridges (BondElut Plexa PCX, Agilent, USA) as previously described (Dziurka *et al.*, 2016). Qualitative and quantitative analyses of plant hormones and related compounds were performed on a HPLC-MS/MS system with UHPLC apparatus (Agilent Infinity 1260, Agilent, Germany) coupled to a triple quadruple mass spectrometer MS/MS (6410 Triple Quad LC/MS, Agilent, USA) equipped with electrospray ionization. Separation of plant hormones and related compounds was achieved on an Ascentis Express RP-Amide analytical column (2.7 μm, 2.1 mm×150 mm; Supelco, Bellefonte, PA, USA) at 60 °C, at linear gradient of water versus acetonitrile both with 0.01% formic acid. Gradient elution at flow rate of 0.5 ml min^{-1} was used. As internal standards the following were used: [$^{15}N_4$]dihydrozeatin (DHZ-N15), [$^{15}N_4$]kinetin (K-N15), [$^{2}H_5$]trans-zeatin riboside (t-ZR-D), [$^{2}H_5$]indole-3-acetic acid (IAA-D),

[$^2H_4$]salicylic acid (SA-D), [$^2H_2$]gibberellin A1 (GA1-D), [$^2H_2$] gibberellin A6 (GA6-D), [$^2H_2$]gibberellin A5 (GA5-D), [$^2H_2$]gibberellin A4 (GA4-D), [$^2H_6$]cis,trans-abscisic (ABA-D), [$^2H_5$]benzoic acid (BeA-D), [$^2H_5$] jasmonic acid (JA-D5), and [$^2H_5$]dinor-12-oxo-phytodenoic acid (dinor-12-oxo-PDA-D). All standards were from OlChemim (Olomouc, Czech Republic), except JA-D which was supplied by CND Isotopes (Quebec, Canada), and dinor-12-oxo-PDA-D, which was supplied by Cayman Chemical Corp. (Ann Arbor, MI, USA), and were at the highest available purity, and all solvents were of LC-MS grade from Honeywell (Poznan, Poland). Multiple reactions monitoring transitions were used for the identification and quantification of all compounds of interest. Analyte quantification was based on calibration curves obtained with a pure standard of all compounds taking account of the recovery rates of internal standards used; details are given in Cioć *et al.* (2022). Physicochemical determinations were performed in three biological replicates.

3.1.6 Pathogen bioassay

Tomato plants were grown and colonized by *B. bassiana* as described in 'Plant growth and colonization by *Beauveria bassiana*' paragraph (3.1.2). The infection with *Botrytis cinerea* was carried out as follows. *Botrytis cinerea* strain B05.10 (van Kan *et al.*, 1997) was grown for 2 weeks on half-strength PDA (Difco Laboratories, Leeuwarden, the Netherlands) plates containing penicillin (100 μg ml^{-1}) and streptomycin (200 μg ml^{-1}) at room temperature. *Botrytis cinerea* spores were subsequently collected, filtered through glass wool, and resuspended in half-strength potato dextrose broth (PDB; Difco Laboratories, Leeuwarden, the Netherlands) to a final density of 1×10^5 spores ml^{-1} and left 3 h under incubation. Four sets of plant samples were set up: (i) control; (ii) *B. bassiana* colonized plants, treated with pure PDB droplets; (iii) *B. cinerea* infected plants and (iv) *B. bassiana* colonized + *B. cinerea* infected plants, treated with droplets containing *B. cinerea* spores in PDB. Treatments (ii) and (iv) were carried out 19 d after the second colonization by *B. bassiana*. Treatments (ii), (iii), and (iv) were performed applying 5 μl droplets of the suspension to four leaves of each plant. All plants were placed under a lid increasing relative humidity to 100% to stimulate the

infection. Three days after *B. cinerea* inoculation, lids were removed, and the symptoms were scored in five disease severity classes ranging from no symptoms (class I), non-spreading lesion (class II), spreading lesion (class III–class IV), up to severe spreading lesions with tissue maceration (class V) (Van Wees *et al.*, 2013).

3.1.7 Reactive oxygen species detection

Reactive oxygen species (ROS) were detected according to methods available in previous studies (Proietti *et al.*, 2019; Falconieri *et al.*, 2022), on the leaves of plant samples (i), (ii), (iii), and (iv) described above ('Pathogen bioassay'). Briefly, ROS production was detected by using 2′,7′-dichlorofluorescein diacetate (DCFH2-DA; Sigma-Aldrich), which is oxidized to highly fluorescent dichlorofluorescein (DCF) when ROS are present. ROS were detected on 2 mm leaf sections cut around the spotted PDB/B. cinerea spores. A half leaf section was incubated at room temperature in 20 mM DCFH2-DA in 10 mM Tris–HCl solution (pH 7.4) for 45 min in the dark. As a negative technical control, the other half was incubated in 10 mM Tris–HCl (pH 7.4) only, under the same conditions. After the staining, samples were washed three times in 10 mM Tris–HCl (pH 7.4) for 10 min to remove the fluorophore excess and ultimately mounted on glass slides. Fluorescence was then observed under an LSM 710 confocal microscope (Zeiss Microscopy) with a Plan Neofluar 20/1.30 objective. Two laser excitations lines were used (488 nm for probe detection and 561 nm for chlorophyll autofluorescence). Quantification of green fluorescence detected by using 2′,7′-DCFH2-DA in all four tested conditions was performed with ImageJ, version 1.53s (NIH, Bethesda, MD, USA) as in Falconieri *et al.* (2022) and reported as the integrated density mean from three biological replicates.

3.1.8 Thiobarbituric acid reactive substance measurement

Measurement of thiobarbituric acid reactive substances (TBARS) was conducted on the leaves of plant samples (i), (ii), (iii), and (iv) described in 'Pathogen bioassay'. Following the methodology described in Proietti *et al.* (2021), TBARS level was used as a measure of lipid peroxidation. In brief, 400 mg of frozen leaves was thinly ground using a mortar and pestle while

liquid nitrogen was continuously added. The powder was resuspended in 3 ml of 0.1% TCA and vortexed until homogenized. Following a 10 min centrifugation at 15 000 g, 400 µl of the supernatant (or 0.1% TCA for the blank) was added to either 1 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA (+TBA solution) or 1 ml of 20% TCA (−TBA solution) (dilution factor 1:3.5). The samples were incubated at 80 °C for 30 min before being cooled on ice. After 5 min of centrifugation at 15 000 g, the absorbance was measured at 532 nm, which represents the maximal absorbance of the TBA–TBARS complex, and at 600 nm to allow for non-specific turbidity adjustment. The molar extinction coefficient ($\varepsilon_{\mu M}$) (0.155 μM^{-1} cm^{-1}) of malondialdehyde (MDA), one of the primary products of membrane degradation, was used to determine the TBARS equivalent (nmol ml^{-1}) as follows:

$$[A/\ \varepsilon_{\mu M}\ \text{MDA}] \times \text{dilution factor}$$

where

$$A = [A_{532}\text{nm}\ (+TBA_{sol}) - A_{600}\text{nm}\ (+TBA_{sol})] - [A_{532}\text{nm}\ (-TBA_{sol}) - A_{600}\text{nm}\ (-TBA_{sol})]$$

3.1.9 Reactive oxygen species scavenging enzymes assays

The enzymatic assays were conducted on the leaves of plant samples (i), (ii), (iii), and (iv) described in 'Pathogen bioassay'. One gram of leaf was finely ground with a mortar and pestle in the presence of liquid nitrogen and further processed as in Proietti *et al.* (2021). The powder was resuspended in 5 ml of a cold extraction solution containing 50 mM sodium phosphate buffer (pH 7.5), 1 mM EDTA, 1% (w/v) polyvinylpyrrolidone, 3 mM DTT, and a cocktail of protease inhibitors (Complete ULTRA tablets, Roche, Basel, Switzerland). The homogenate was centrifuged at 9000 g for 15 min at 4 °C (Universal 32R, Hettich, Tuttlingen, Germany), and the supernatant was utilized for enzyme activity tests. According to Proietti *et al.* (2021), superoxide dismutase (SOD) and catalase (CAT) activities were measured. SOD activity was measured using the SOD determination kit (Sigma-Aldrich, Uppsala, Sweden). The SOD Assay Kit is based on the utilization of Dojindo's water-soluble tetrazolium salt (2-(4-iodophenyl)-3-(4-

nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt), which when reduced with a superoxide anion forms a water-soluble formazan dye. The rate of decrease is linearly related to the activity of xanthine oxidase, which is inhibited by SOD. Because absorbance at 440 nm is related to formazan dye concentration, SOD activity may be measured as inhibition activity by measuring the reduction in color development at 440 nm. Thus, inhibition activity corresponds to the quantity of total protein extract required to suppress formazan formation by 50% (IC50). CAT activity was determined by measuring the reduction in absorbance at 240 nm at 25 °C caused by H_2O_2 decomposition (ε_{mM}=0.0436 mM^{-1} cm^{-1}). The reaction mixture (1 ml final volume) comprised 19 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0); the reaction was initiated by adding 50 µg of protein extract. The specific activity of CAT was measured in µmoles of hydrogen peroxide consumed per minute per mg of protein sample. Ascorbate peroxidase (APX) activity was measured according to Bertini *et al.* (2019a). The decrease in absorbance at 290 nm due to ascorbate oxidation was used to calculate APX activity (ε_{mM}=2.8 mM^{-1} cm^{-1}). Reaction buffer contained 50 mM of potassium phosphate, 0.5 mM of ascorbate, 0.1 mM of H_2O_2, and 0.1 mM of EDTA (pH 7.6).

3.1.10 Thiobarbituric acid reactive substance and reactive oxygen species scavenging enzyme data processing

Analyses were performed using three biological replicates and, for any biological replicate, on three technical replicates. Reported in the graphs are the weighted average and the mean standard deviation (SD), following the method reported in Taylor (1997):

$$\text{Weighted average} = \frac{X_1W_1 + X_2W_2 + X_3W_3}{W_1 + W_2 + W_3}$$

Where X_1 is the average of technical replicates of the first biological replicate, $W_1=1/SD^2$, X_2 is the average of technical replicates of the second biological replicate, $W_2=1/SD^2$, X_3 is the average of technical replicates of the third biological replicate, and $W_3=1/SD^2$.

$$\text{Mean standard deviation} = \frac{1}{\sqrt{W_1 + W_2 + W_3}}$$

3.2 Material and methods to investigate tomato-*B. bassiana*+*T. harzianum* consortium-biotic stress interaction

3.2.1 *Beauveria bassiana* fungal culture

B. bassiana fungal culture was obtained according to paragraph 3.1.1.

3.2.2 *Trichoderma harzianum* fungal culture

Trichoderma harzianum strain T22 was cultured on Potato Dextrose Agar (PDA) at 25 ± 2°C, with 14:10 h, L:D photoperiod for about 5 days, until sporulation. Conidia were harvested by adding 5 ml volume of sterile distilled water to the sporulating cultures. The surface of the mycelium was gently scraped with a sterile L-spatula to remove spores. The liquid containing mycelia and spores was filtered through synthetic wool to remove the mycelium and collect the spore suspension in a sterile tube. The harvested conidia were shaken to obtain a homogeneous spore suspension. The concentration of the spores in the liquid inoculum was determined by a series of dilutions and counting the number of spores in a hemocytometer (Neubauer hemocytometer chamber) under a microscope. The spore concentration ranged from about 1×10^8 to 10^9 conidia ml^{-1} in approximately 5 ml volume. The spore suspension was stored at 4°C until use.

3.2.3 Plant growth and colonization with beneficial fungi

Tomato seeds (*Solanum lycopersicum* cv San Marzano nano, Top Seed, Sarno, Italy) were surface sterilized in 1% NaOCl for 5 min, then rinsed three times with sterile distilled water. The sterilized seeds were placed in Petri dishes on Whatman® sterile filter paper (Sigma-Aldrich, Darmstadt, Germany) imbibed with sterile distilled water, and incubated in the dark at 25°C until germination. Seedlings were transplanted individually into 8-cm diameter pots containing 200 mL of sterile commercial soil (Universal Potting Soil, Floragard, Oldenburg, Germany) and maintained in a growth chamber

at the following environmental conditions: 25 ± 2 °C, 70 ± 10% RH, and photoperiod of 14:10 h light:dark. The pots were arranged in a randomized design with 36 plants for each treatment. Collectively, the experimental sample size consisted of 144 plants, treated as follows: 36 plants inoculated with *B. bassiana* strain Naturalis; 36 plants with *T. harzianum* strain T22; 36 plants with a consortium of both the beneficial fungi; and 36 plants served as water control. The experiment was repeated three times. One week after the seedling emergence, the tomato plants were inoculated with the beneficial fungi by soil watering with conidial suspension at different concentrations.

B. bassiana inoculum consisted of 20 mL of conidial suspension for each plant (1×10^7 conidia/mL) to obtain a final concentration of 1×10^6 conidia/mL of soil volume; *T. harzianum* inoculum consisted of 20 mL of the conidial suspension for each plant (1×10^5 conidia/mL) to obtain a final concentration of 1×10^4 conidia/mL of soil volume. Experimental plants were watered with one of the different fungal conidia suspensions or with both in the case of consortium treatment. Control plants were watered with sterile water. The same treatment was repeated after one week.

3.2.4 Plant infection with *Botrytis cinerea*

B. cinerea fungal colonies were maintained in 90mm Petri dishes containing Potato Dextrose Agar medium (PDA, HiMedia, Mumbai, India) in the dark at 25 °C. Conidia were collected in sterile distilled water by scraping the surface of sporulating fungal culture and recovering the spores by centrifugation. Conidia were counted in a hemocytometer (Neubauer counting chamber) under a microscope and the concentration was adjusted to 5×10^6 spores/mL for plant infection.

Three weeks post inoculums with beneficial fungi, 12 plants approximately five weeks old, for each treatment, were placed in a high-humidity chamber (80–90% RH, 20 °C) and infected with *B. cinerea*. Four true leaves for each plant were inoculated with the pathogen laying two 10 µL-drops of conidial suspension on the leaf surface of two opposite primary leaflets. The inoculation points were marked with a permanent marker.

3.2.5 Plant infestation with *Macrosiphum euphorbiae*

The potato aphid *Macrosiphum euphorbiae* was collected in a field from infested tomato plants (Battipaglia, Salerno, Italy) and is permanently reared at the Department of Agricultural Sciences of the University of Naples "Federico II" on tomato cv. Dwarf San Marzano, kept in a growth chamber at the following environmental conditions: 23 ± 2 °C, 70 ± 10% RH, and photoperiod of 14:10 h light:dark. The choice of this insect pest was motivated by its capacity to readily feed on this cultivar of tomato, which led to a successful utilization in previous studies, and by a well-proven protocol of infestation and monitoring already available (Coppola *et al*., 2019; Di Lelio *et al.*, 2021; Sinno *et al.*, 2021).

Approximately 20 days after seedling emergence, four days after the last fungal inoculum, four plants for each treatment were infested with the aphid *M. euphorbiae* as follows: 15 apterous adults were placed on each plant and allowed to give birth; after 6 h, the adults were removed, and only 25 first instar nymphs were left per plant, removing the exceeding ones.

3.2.6 Leaf proteomic analysis

Leaves of adult plants were harvested from twelve sets of plant samples: (i) control plants (not colonized), (ii) *B. bassiana* root-colonized, (iii) *T. harzianum* root-colonized and (iv) *B. bassiana*-*T. harzianum* root-colonized plants, (v) *B. cinerea* infected and (vi) *M. euphorbiae* infested plants, (vii) *B. bassiana* + *B. cinerea*, (viii) *B. bassiana* + *M. euphorbiae*, (ix) *T. harzianum* + *B.cinerea*, (x) *T. harzianum* + *M. euphorbiae*, (xi) *T. harzianum* + *B. bassiana* + *B. cinerea* and (xii) *T. harzianum* + *B. bassiana* + *M. euphorbiae* plants. For the proteome analysis, three biological replicates were used for each sample. In total, 36 samples were obtained.

Leaf proteome analysis was performed as described in "Leaf proteome analysis" (paragraphs from 3.1.3 to 3.1.3.6).

3.2.6.1 Bioinformatics analysis

Label-Free Quantification (LFQ) proteomics data recorded as peak intensity of extracted ion chromatograms (XIC) are reported for each protein identified with at least 2 unique peptides for each sample. Different datasets that

included treatment groups corresponding to each comparison of interest were created, and all analyses were consistently applied to each dataset to ensure uniform comparisons. Downstream analysis, including data normalization, imputation, and differential expression analysis, was performed with the R package DEP (v1.22, Zhang *et al.*, 2018). Specifically, variance stabilization and data normalization were carried out using the "normalize_vsn" function, utilizing the R package vsn (v3.38, Huber *et al.*, 2002). Imputation of missing values was performed using the "impute" function with the "MinProb" method, which employs a left-censored imputation approach by drawing missing values from a Gaussian distribution centered around a minimal value. The choice of the "MinProb" imputation method was based on the multiclass nature of our comparisons and the potential for missing values to be non-random (MNAR). Differential expression analysis was conducted using the "test_diff" function integrated into the DEP package, which utilizes the limma package (v 3.56.2, Phipson *et al.*, 2015). The "all" option for contrast type was employed, allowing for all possible pairwise comparisons between treatments. Datasets were subsequently subset to include only proteins that were found to be differentially expressed with an adjusted p-value <0.05 after applying the Benjamini–Hochberg correction for multiple comparisons. Principal component analysis (PCA) was performed using the "plot_pca" function to visualize data structure, assess sample similarities, and evaluate distances between treatments. To explore expression patterns of differentially expressed proteins, hierarchical clustering heatmaps were generated for each comparison based on the Gower's distance metric analysis applied to the centered $\log_2$ peak intensity values. K-means clustering was then employed to identify clusters of proteins based on their expression profiles. For each identified cluster of proteins, Gene Ontology (GO) enrichment analysis was carried out using the clusterProfiler package version 4.8.3 (Wu *et al.*, 2021) through a hypergeometric distribution test. To establish the background universe for the hypergeometric test, a vector of the same length as the total number of detected proteins across all analyzed conditions was created. Significantly enriched GO terms were identified by applying a P-value cutoff of 0.05, followed by the Benjamini–Hochberg correction for multiple testing. Gene Ontology (GO) annotation using the Solanum proteome data obtained

from the EMBL-EBI Gene Ontology Annotation (GOA) proteome database (https://www.ebi.ac.uk/GOA/proteomes). The GOA database was downloaded and imported into the R environment. Subsequently, a custom database was constructed using the mgsa package version 1.48.0 (Bauer and Gagneur, 2023). Furthermore, within the same protein clusters, with clusterProfiler, we performed enrichment analysis of over-represented Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa *et al.*, 2017) among the differentially expressed proteins (DEPs). This analysis was performed using the "enrichKEGG" command, which automatically retrieved annotated pathways for S. lycopersicum ('sly') from the KEGG database (https://www.genome.jp/kegg/catalog/org_list.html). Statistical significance for enriched KEGG pathways was determined by considering a P-value <0.05 after applying the Benjamini–Hochberg correction for multiple testing.

3.2.6.2 Statistical analysis

The statistical analysis was performed with the R environment, version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). The datasets include only proteins that were found to be differentially expressed with an adjusted p-value <0.05 after applying the Benjamini–Hochberg correction for multiple comparisons. The heatmaps were generated for each comparison based on the Gower's distance metric analysis applied to the centered $\log_2$ peak intensity values. Significantly enriched GO terms and enriched KEGG pathways were identified by applying a P-value cutoff of 0.05, followed by the Benjamini–Hochberg correction for multiple testing.

3.2.7 Leaf hormonomic analysis

A leaf hormonomic analysis was performed in the same samples on which we have performed the proteomic analysis, i.e. control plants, colonized with *B. bassiana* or with *T. harzianum* alone*,* colonized with the *B. bassiana*-*T. harzianum* consortium, infected with *B. cinerea* and infested with the *M. euphorbiae* and on plants pre-colonized with the individual beneficial microbes or with the consortium and subsequently subjected to stress by *B. cinerea* or *M. euphorbiae*.

The concentration levels of phytohormones in the plant samples were analysed by ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC–MS/MS) according to protocol by Šimura *et al*, 2018. Briefly, 15 mg of fresh weight (FW) of homogenized material were weighed into microtubes. Each treatment was represented by three independent replicates. The samples were extracted in 1 mL of 60% acetonitrile (v/v) with four zirconium oxide beads and stable isotope labelled internal standards. The mixture of internal standards in the particular concentrations was described previously (Šimura *et al.,* 2018). The samples were shaken in a Retsch MM400 bead mill (Retsch, Haan, Germany) for 5 min at 27 Hz, sonicated for 3 min, and incubated for 30 min in a refrigerator. After the centrifugation at 20,000 rpm for 10 min at 4 °C (Allegra 64R benchtop centrifuge, Beckman Coulter, Brea, CA, USA), the supernatants were loaded onto an Oasis® HLB 30 mg/1 cc extraction cartridges (Waters, Milford, CT, USA). The cartridges were washed with 0.5 mL 60% acetonitrile (v/v) and 0.5 mL 30% acetonitrile (v/v). Both fractions (load and wash in total volume 2 mL) were collected into glass tubes and evaporated to dryness using a SpeedVac concentrator (RC1010 Centrivap Jouan, ThermoFisher, Waltham, MA, USA). The dry samples were dissolved in 40 µL of 25% (v/v) ACN and 5 µL of the sample was injected onto an Acquity UPLC CSH C18 RP 150 × 2.1 mm, 1.7 µm chromatographic column (Waters, Milford, CT, USA). Acquity UPLC I-Class System (Waters, Milford, CT, USA) coupled to a triple quadrupole tandem mass spectrometer (Xevo TQ-XS) equipped with electrospray ionization (Waters, Manchester, UK) was used for the separation and identification of targeted plant hormones. The quantities (pmol per g FW) were calculated by isotope dilution method (Rittenberg & Foster, 1940).

3.2.7.1 Hormonomics data analysis

The raw data of absolute quantification of targeted hormones (pmoles/g of plant tissue) were processed using R Statistical Software (v4.3.1 R Core Team 2023). Initial data were transformed by scaling the hormone quantities for comparative analysis. Significant differences among treatments were determined using the Kruskal-Wallis test, with p-values adjusted using the Benjamini-Hochberg method. Subsequently, data were aggregated by

treatment and hormone, and the means were calculated to assess treatment effects. Heatmaps were generated with the pheatmap R package (v1.0.12, Kolde, R. 2012) to visualize the patterns of hormone abundance across treatments, utilizing the scaled mean hormone data.

4 Results and Discussion

4.1 *Beauveria bassiana* promotes plant growth and increases defense responses against *Botrytis cinerea*

During the first part of this thesis work I have been studying the molecular mechanisms that regulate the tomato-*B. bassiana* interaction. The results obtained have been published recently (Proietti *et al.,* 2023).

Beneficial fungi are often associated with plant physiological and metabolic reprogramming that can promote growth and strengthen the defense barriers. Here, we investigated the molecular events regulating the tomato-*B. bassiana* interaction at the leaf level by conducting molecular analysis on *B. bassiana* root-colonized and control (not colonized) plants.

4.1.1 *Beauveria bassiana* induces plant growth and triggers plant proteome reprogramming over time

Successful fungal colonization was confirmed as described under ‘Plant growth and colonization by *B. bassiana’* paragraph (3.1.2), and *B. bassiana* was reisolated from roots, stems, and leaves as previously observed by Wei *et al.* (2020). Beneficial effects of *B. bassiana* colonization on plant growth (stem height and leaf area) were observed at all time points analysed, with a greater effect at T4 as shown in figure 10.

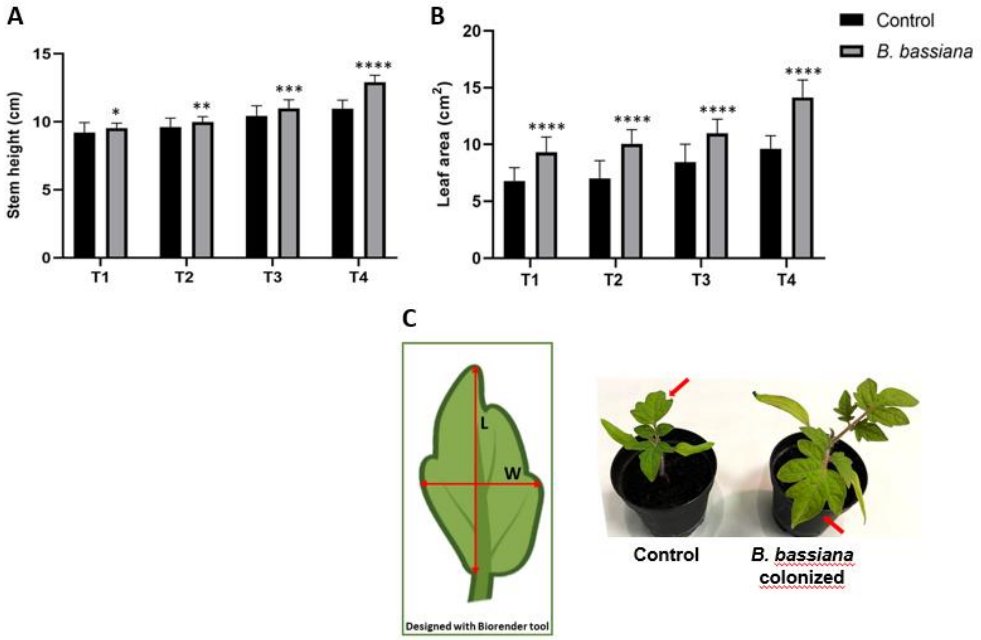

Figure 10. Growth indexes of tomato plants colonized by *B. bassiana* or under control (not colonized) conditions. (A) Stem height. (B) leaf area (LA) measured in each considered time point. (C) LA was calculated on the widest leaf (indicated with the arrow) with the following formula: LA= L x W, where LA (leaf area); L (leaf length); W (leaf width). Asterisks above the bars indicate significant differences between *B. bassiana*-colonized vs control plants (Two-way analysis of variance, Šídák's test; n=30; ****<0.0001; ***<0.0004, **<0.005, *<0.02) (From Proietti *et al.*, 2023).

Plant proteome changes induced over time by *B. bassiana* were investigated by a combination of high-throughput profiling proteomics techniques and bioinformatics tools, identifying proteins across the different samples. We performed a principal component analysis (PCA) on log_2-transformed quantification values of all detected proteins (6783 unique proteins) to understand global proteome changes induced by *B. bassiana* treatment at all time points. The PCA plot (Fig. 11) shows clear separation of the four protein clusters related to the analyzed time points with largest distance between T1 and T4 and smallest distances between T2 and T3, and T1 and T3.

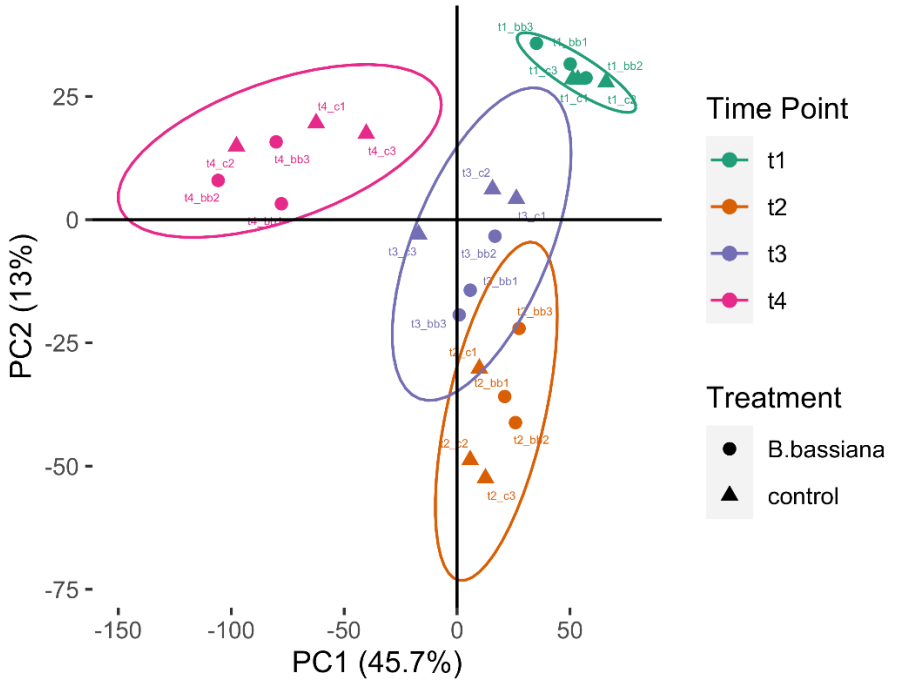

Figure 11. PCA plot showing the global proteome changes across all samples. The convex hulls grouping samples from the same time point were drown with 95% confidence. Samples belonging to control and *B. bassiana*-colonized plants are represented on the plot by triangles and circles, respectively (From Proietti *et al.,* 2023).

Different distributions of samples belonging to different time points may reflect the different physiological stages of the plants. However, within the same time point, comparisons between *B. bassiana*-treated plants and controls show a trend of continuous increase in distances between groups, over the time series. The PCA analysis results were confirmed and more precisely described with a proteome-wide comparative analysis of the protein abundance changes over time in both *B. bassiana*-colonized and control plants. Fold change (on a logarithmic scale) profiles between all detected proteins over all time points are summarized in a hierarchal clustering heatmap plot (Fig. 12A). This analysis shows that proteome reprogramming started in the early stage of root colonization by *B. bassiana* and continued until the last time point (Fig. 12A). A set of proteins with pronounced changes in abundance due to *B. bassiana* colonization was selected for each time point according to their significance (FDR adjusted *P*-value <0.05, *y*-axes) and fold change ($|\log_2$ fold change$|$ >1, *x*-axes) in comparison with the respective control (Fig. 12B).

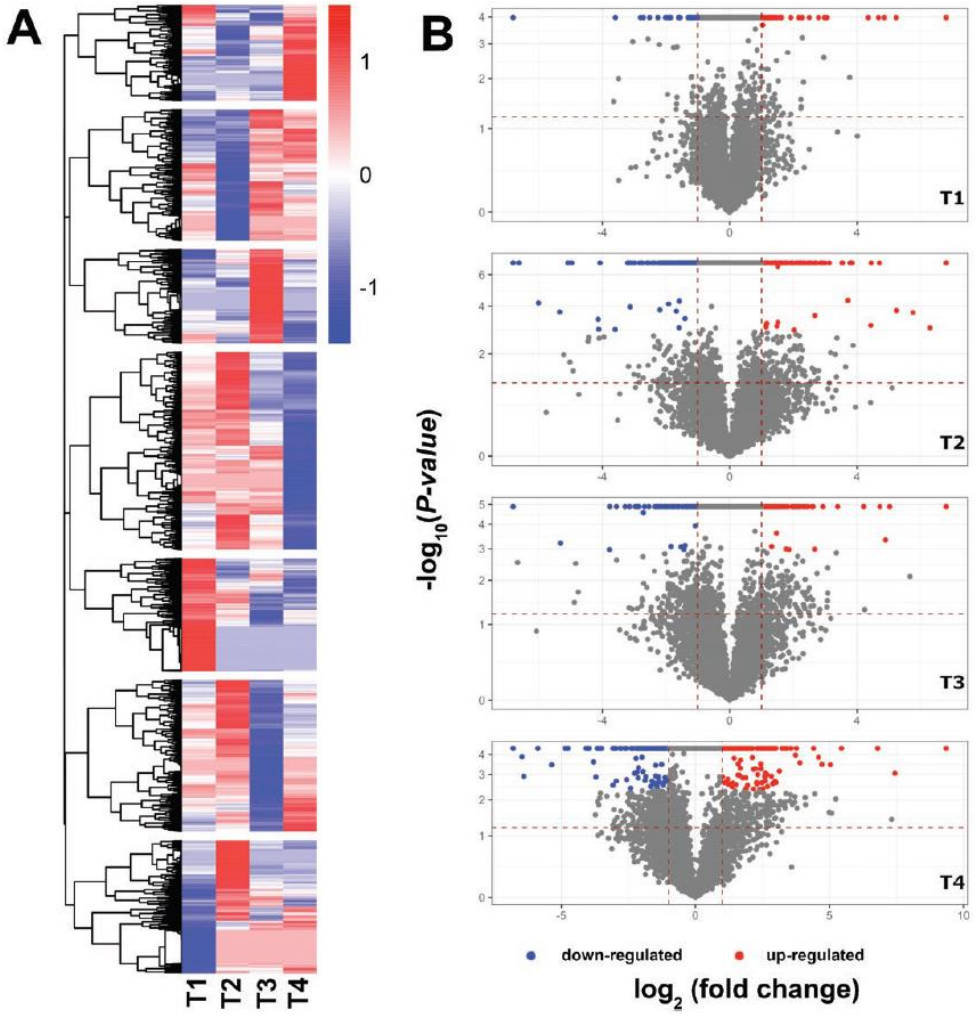

Figure 12. Proteome reprogramming after *B. bassiana* colonization. (A) Proteome-wide hierarchical clustering analysis at all time points. Red and blue colors represent *z*-scores of scaled fold changes of up- and down-regulated proteins of *B. bassiana*-treated plants versus control, respectively. The analysis highlighted the formation of seven clusters reflecting distinct expression patterns for each time point. (B) Volcano plots showing the results of the differential expression analysis for each time point. *x*-Axes display $\log_2$ fold change values comparing *B. bassiana*-treated plants versus control. Red and blue dots on the plot represent all significant up- and down-regulated proteins, respectively (FDR<0.05), after Benjamini–Hochberg test correction for multiple comparisons (From Proietti *et al.*, 2023).

A total of 198 characterized proteins of interest at the four time points were identified. In particular, we detected 7, 30, 18, and 48 proteins up-regulated, with a $\log_2$ fold change value greater than 1, and 1, 18, 28, and 48 proteins under-expressed with a $\log_2$ fold change value less than −1, at T1, T2, T3, and T4, respectively. GO analysis (Yu *et al.*, 2012) of selected proteins identified the biological processes (Fig. 13A), the cellular component (Fig. 13B), and the molecular function (Fig. 13C) enriched during the plant response to *B. bassiana* colonization over time, as further discussed below.

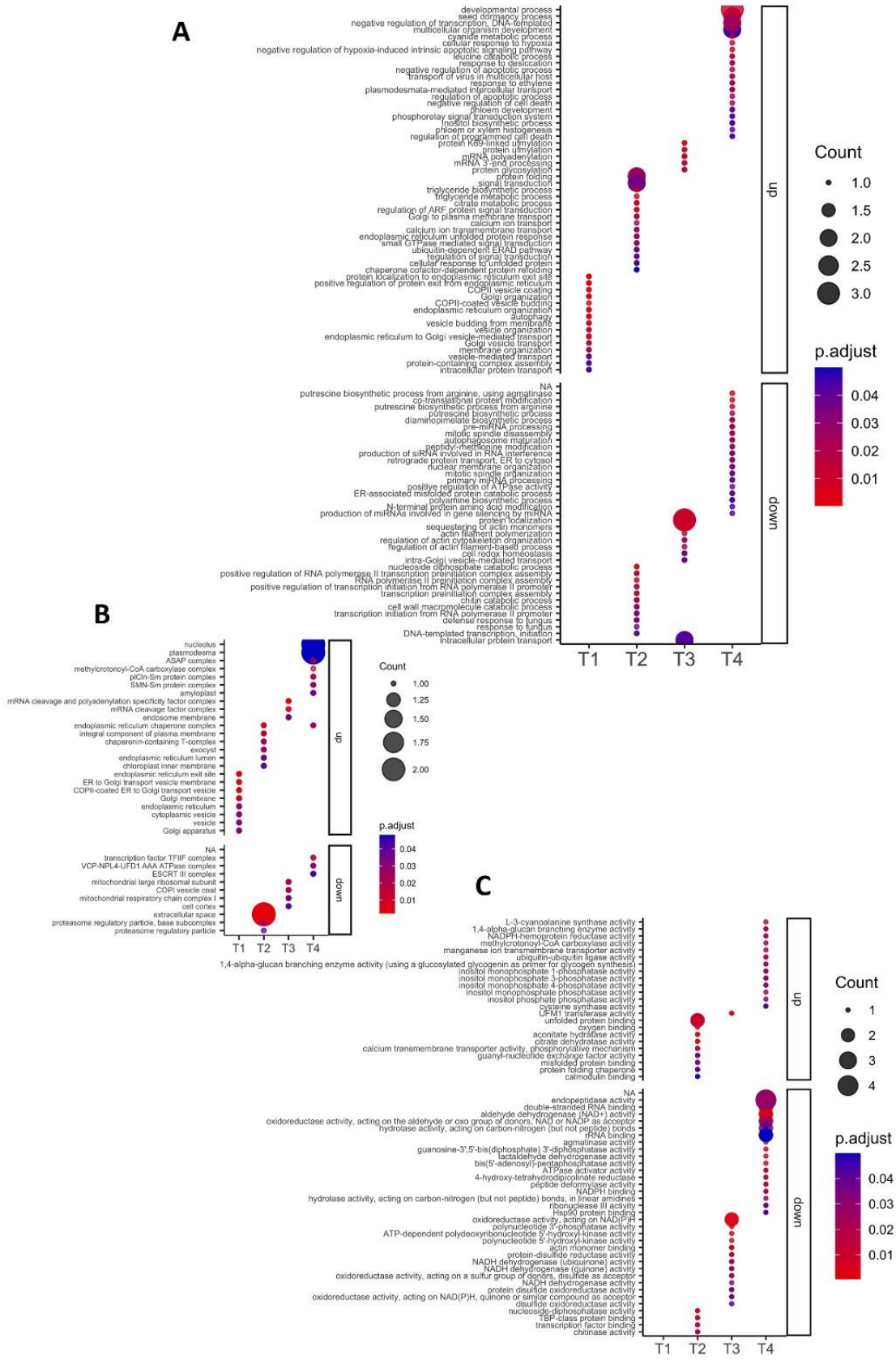

Figure 13. Dotplots of enriched GO terms over time. Different plots represent GO terms enriched for each ontological category. (A) Biological processes; (B) cellular component; (C) molecular function. Dots are colored with a red–blue gradient based on the *P*-values adjusted by the Benjamini-Hochberg test correction for multiple comparisons (*P*adj). Size of dots (counts) represents the number of DEPs up- or down-regulated that significantly enrich a specific GO term (From Proietti *et al.*, 2023).

Protein names are those used in UniPROT. For biological processes and cellular components, only upregulated proteins were found at the first time point (T1). These proteins were related to protein transport from the endoplasmic reticulum to the Golgi apparatus such as “transport protein sec16” (A0A3Q7HKB9) (Fig. 13A, B). The induction of this protein could be related to the entry mechanism of microbes into the host cell, which is preceded by the formation of a pre-infection structure at the entry site, thereby facilitating the targeting of vesicles towards this site, especially in symbiotic fungi (Ivanov *et al.*, 2010). In T2, proteomic analysis revealed changes triggered by *B. bassiana* colonization in a consistent number of significant proteins involved in defense response, protein folding, and signal transduction (Fig. 13). Among the down-regulated processes, we found those involved in response to fungus and chitin catabolism. A marker of these down-regulated processes is the protein “acidic 26 kDa endochitinase” (Q05539). Plants possess endochitinases with powerful antifungal activity and that have been reported as pathogenesis-related proteins induced by herbivores or phytopathogens (Neuhaus, 1999). In *Allium porrum* it has been reported that chitinases are down-regulated after the full establishment of symbiosis with the mycorrhizal fungus *Glomus versiforme* (Spanu *et al.*, 1989). Host penetration and colonization can elicit strong defense responses, and thus symbiotic fungi need to rewire the plant’s warning system to effectively suppress defense responses during the initial entry and proliferation phases (Shen *et al.,* 2018). It is tempting to speculate that the reduction of defense responses observed at T2 reflects the establishment of a symbiotic relationship in the early stages of interaction rather than defense. This hypothesis is further corroborated by the up-regulation of calcium ion (Ca^{2+}) transport in which a “cyclic nucleotide-binding domain-containing protein” (A0A3Q7HNZ3) is involved (Fig. 13). It has been suggested that the cyclic nucleotide gated channel (CNGC) is one of the most important calcium conduction channels. Several studies pointed out that Ca^{2+} flux across the plasma membrane is an early signaling step in establishing symbiosis and immunity, as documented in *Lotus japonica*, *Medicago troncatula*, and Arabidopsis (Zipfel and Oldroyd, 2017; Moscatiello *et al.*, 2018; Dietrich *et al.*, 2020; Yuan *et al.*, 2022). While at the first two time points all protein

changes probably reflect establishment of the plant-fungus symbiosis, in the last two time points growth and development-related processes were observed, such as up-regulation of processes involved in protein synthesis and folding or in providing energy to cells. For instance, the "methylcrotonyl coenzyme A (CoA) carboxylase complex" and the "apoptosis- and splicing-associated protein (ASAP) complex" were up-regulated at T4 (Fig. 13 B, C). "3-Methylcrotonyl-CoA carboxylase" (MCC) (A0A3Q7ESD1) is an essential enzyme for the catabolism of the branched-chain amino acid leucine, which prevents the accumulation of toxic intermediates and provides energy to the cell (Tomassetti *et al.*, 2018). In most organisms, this complex leads to the formation of products controlling different metabolic pathways, such as the TCA cycle (Tomassetti *et al.*, 2018). Besides, the ASAP complex is involved in the regulation of mRNA processing and apoptosis. It binds to RNA in a sequence-independent manner and is recruited into the exon junction complex before or during the splicing process (Chen *et al.*, 2019). These two up-regulated processes suggest that, following the establishment of the plant‑fungus symbiosis, there is a general reprogramming of the metabolic landscape towards energy production that can contribute to plant growth promotion. Among other T4 up-regulated proteins related to plant growth and development, there were those involved in "inositol monophosphatase activity" (Fig. 13C), including "inositol-1-monophosphatase" (A0A3Q7FZW9), involved in inositol synthesis. Inositol monophosphatase is developmentally regulated, with the highest levels detected in *Solanum lycopersicum* plant tissues undergoing rapid cell divisions (Gillaspy *et al.*, 1995). Finally, among the up-regulated significant proteins, there was the "cysteine synthase" (A0A3Q7IDI1) involved in "cysteine synthase activity" (Fig. 13C). Sulfur is a macronutrient essential for plant growth and development. Sulfate, the most abundant form of sulfur in nature, is absorbed by plants and reduced and assimilated into cysteine (Wirtz and Hell, 2006). Cysteine is required to synthesize proteins and metabolites, being indispensable for growth and development. Cysteine also plays an essential role in plant immunity and redox signaling. It is involved in the detoxification of cyanide, which is essential for root hair development and plant response to pathogens, as demonstrated in Arabidopsis (Romero *et al.*, 2014). On the

other hand, at the later time points down-regulation of proteins involved in redox signaling suggests that *B. bassiana* does not generate oxidative stress levels harmful for the plant (Fig. 13C). To further deepen our understanding of the functions regulated by *B. bassiana* in tomato plants, we carried out a KEGG pathways enrichment analysis on significant proteins (Fig. 14).

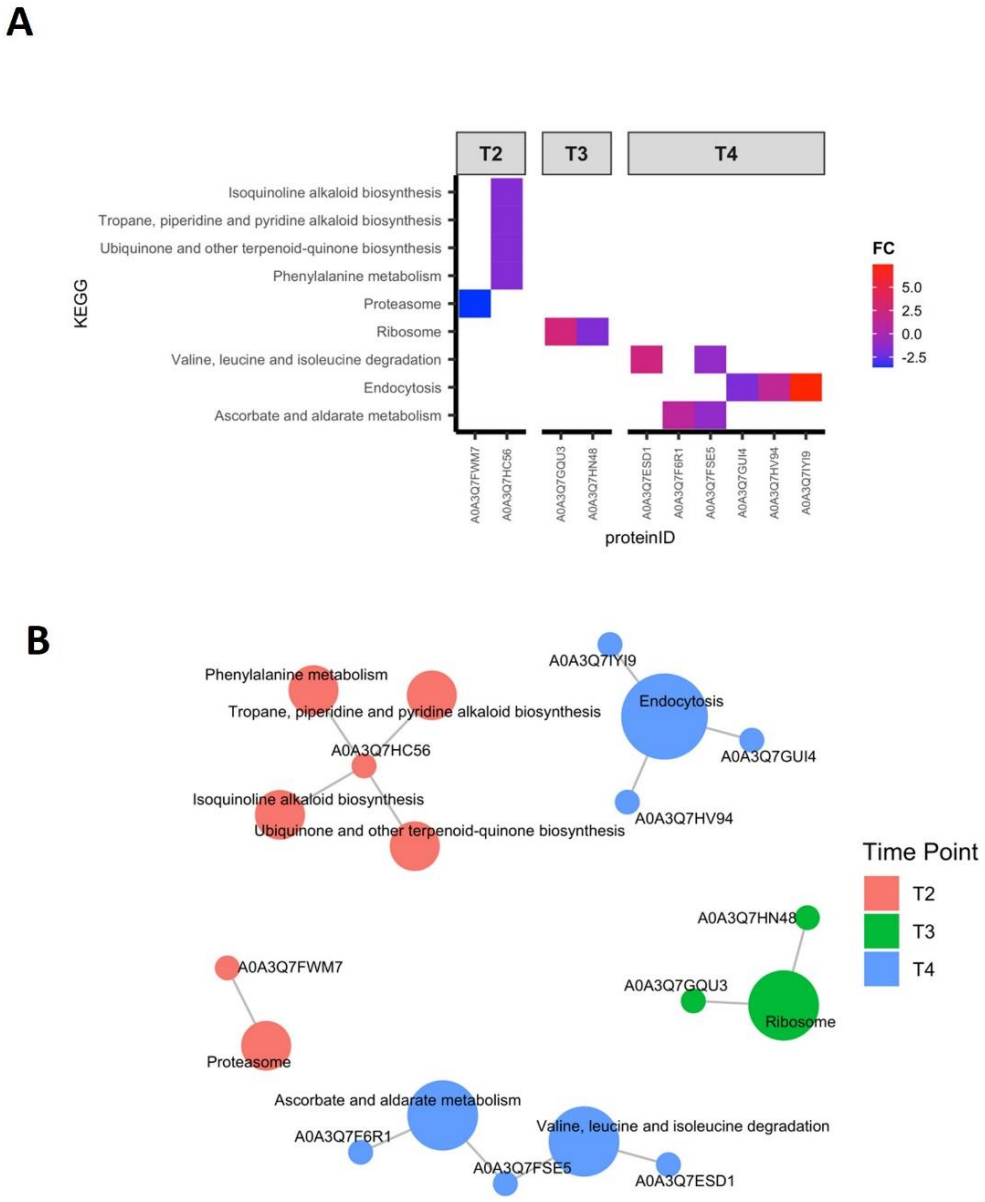

Figure 14. KEGG analysis over time. (A) Heatmap of significantly enriched KEGG pathways (P-values adjusted by the Benjamini–Hochberg test <0.05) at the different time points. Cells are colored by a red–blue gradient based on $\log_2$ of the fold change (FC) value of DEPs considering the ratio *B. bassiana*-colonized versus control plants for each time point. (B) Cnetplot of the enriched KEGG pathways for the different time points. The Cnetplot network graph highlights the contribution of one or more proteins to a KEGG pathway as well as the contribution of a single protein to multiple pathways (From Proietti *et al.,* 2023).

It is noteworthy mention that, since KEGG annotation is also based on orthologs, the results of this analysis are not species-specific, and may rather

be generalized (Kanehisa *et al*., 2015). KEGG analysis further revealed down-regulation of "aminotran_1_2 domain-containing" (A0A3Q7HC56), a tyrosine aminotransferase at T2 (Fig. 14 A, B). Tyrosine aminotransferases have been implicated in hijacking phenylalanine and tyrosine to secondary metabolite pathways, such as the production of antioxidants that scavenge free radicals and protect plants from various stresses (Parthasarathy *et al.*, 2018). In most plants, tyrosine aminotransferase also catalyses the transamination of tyrosine to 4-hydroxyphenylpyruvate, which serves as a precursor for the formation of numerous specialized metabolites with functions in plant defense (rosmarinic acid, dhurrin and benzylisoquinoline alkaloids), electron transport (plastoquinone and ubiquinone), and structural support (lignin) (Schenck and Maeda, 2018; Parthasarathy *et al.*, 2018; Xu *et al.*, 2019). It is noteworthy that plastoquinone and ubiquinone are two important prenylquinones, playing essential roles in the biosynthesis and metabolism of important chemical compounds involved in the stress response in several plant species, including tomato (Liu and Lu, 2016). Moreover, at T2 we found the down-regulation of "AAA domain-containing protein" (A0A3Q7FWM7), highly similar to the 26S Proteasome Regulatory Subunit 7 (source UniProt), included in the "proteasome" term, and at T3, the up-regulation of "small subunit ribosomal protein S4e" (A0A3Q7GQU3) included in the "ribosome" term (Fig. 14A, B). The 26S proteasome regulatory subunit 7 is a subunit of the protease complex responsible for the degradation of a wide range of intracellular proteins, especially those modified with polyubiquitin chains (Yang *et al.*, 2004). Small subunit ribosomal protein S4e (also known as 40S ribosomal protein S4) is a structural constituent of ribosomes with protein and RNA binding activity. Ribosomal proteins play pivotal roles in translation efficiency and ribosome stability, and participate in important cellular processes, such as DNA repair, apoptosis, and regulation of gene expression, which are prominent for sustaining plant life (Song *et al.*, 2015). The down- and up-regulation of these two proteins, respectively, suggest that protein degradation is inhibited, with accumulation of proteins regulating growth and developmental pathways. Furthermore, maspardin (A0A3Q7HV94), included in the "endocytosis pathway", was found to be upregulated at T4 (Fig. 14A, B). Maspardin was

first identified in humans but has also been reported in plant species like sweet potato and rice (Liu *et al.*, 2014; Wang *et al.*, 2022). This protein is a member of the AB hydrolase superfamily, which has been found to localize in intracellular endosomal/*trans*-Golgi vesicles and is likely involved in protein transport and sorting (Zeitlmann *et al.*, 2001). Accordingly, it has been reported that intracellular colonizers trigger massive vesicular membrane trafficking events (Leborgne-Castel and Bouhidel, 2014). Taken together, these data indicate that plant-fungus symbiosis likely takes place at an early stage (T1 and T2), when defense-related processes are down-regulated. At the two last time points (T3 and T4), following a well-established plant-fungus relationship, processes related to vesicular trafficking, growth, and development are boosted, with a consequent beneficial outcome for plant health. To obtain a more comprehensive picture of the physiological rewiring at later time points, the impact of *B. bassiana* was further explored.

4.1.2 *Beauveria bassiana* promotes protein synthesis

It is well documented that beneficial microbes have an important impact on biosynthetic pathways, particularly on protein synthesis, positively affecting plant health (Etalo *et al.,* 2018). During the symbiosis process, modification, degradation, and biosynthesis of peptides and proteins are critical for maintaining cell functions (Song *et al.*, 2015). Based on data reported in the literature and on our results at T3, we analyzed the impact of *B. bassiana* colonization on protein synthesis rate. A Western Blotting (WB)-SUnSET assay was conducted to assess the rate of protein synthesis in tomato leaf protoplasts isolated from *B. bassiana*-colonized and control plants at T3. The SUnSET assay is a non-radioactive method used to estimate the level of newly synthesized proteins. It has been widely used in animal cell research, but was only recently introduced, and is still little used, in plant cells assays (Van Hoewyk, 2016). This technique consists in treatment with the antibiotic puromycin, a structural analog of tyrosyl-tRNA. The incorporation of puromycin into nascent polypeptides leads to translation termination and the formation of peptidyl-puromycin products (Van Hoewyk, 2016). Newly synthesized proteins containing puromycin can be measured by western-blot by using an anti-puromycin antibody. Here, the WB-SUnSET assay was

optimized in performance on leaf protoplasts of tomato plants. The protein synthesis rate is calculated based on the intensity of the reference protein band in samples colonized by *B. bassiana* and not colonized (Fig. 15A). Foldchange is reported in Fig. 15C.

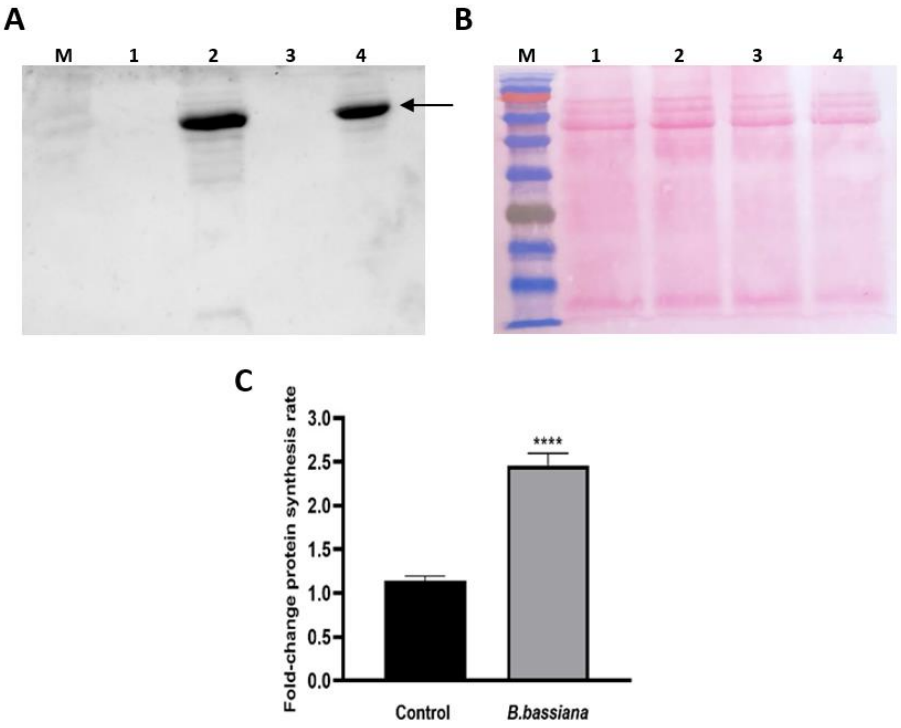

Figure 15. Protein synthesis analysis by WB-SUnSET. A: Representative image of the WB-SUnSET of newly synthesized protein in *B. bassiana*-colonized tomato plant at 12 days after the second colonization and control (T3). Leaf protoplasts were treated or not with 10 µg ml-1 puromycin for 60min. Protein molecular weight markers (M), *B. bassiana* not treated (1) and treated with puromycin (2), control not treated (3) and treated with puromycin (4). Western blot was performed with anti-puromycin monoclonal antibody using control and *B. bassiana*-colonized total protein extracts (40 µg) from five-week-old plant leaves. B: Ponceau S staining was used to normalize protein loading. Fold-change of protein synthesis rate is given by the ratio of the protein band in lane 2 (*B. bassiana*-treated sample) and protein band in lane 4 (control sample) and normalized to the Ponceau stained band. C: Fold-change protein synthesis rate. The data shown are means of three biological replicates. Error bars represent SEM. Asterisks indicate the statistically significant difference between control and *B. bassiana*-colonized samples (*t*-test; ****$P<0.0001$) (From Proietti *et al.,* 2023).

As shown, the rate significantly increased by twice in *B. bassiana*-colonized plants compared with controls. Our results confirmed that *B. bassiana* positively impacts the protein synthesis rate in the later stages of plant colonization. As a consequence, it can be hypothesized that an increase in protein biosynthesis induced by *B. bassiana* may contribute to improving plant health, providing energy and structural material for the living system.

4.1.3 *Beauveria bassiana* affects the profile of plant hormones and related compounds

Plant hormones are involved in plant growth and development by inducing an array of cellular, morphological, and physiological processes (Weyers and Paterson, 2001). They are also involved in defense mechanisms against biotic and abiotic stresses (Aerts *et al.*, 2020; Waadt *et al.*, 2022). We performed analysis of leaf hormone and related compounds on plants colonized and not colonized by *B.bassiana,* at T4. By using targeted HPLC-MS/MS we detected 26 hormones and related compounds that correlated to growth and defense. Among the detected compounds 10 were significantly affected by *B. bassiana* colonization with respect to controls (Fig. 16).

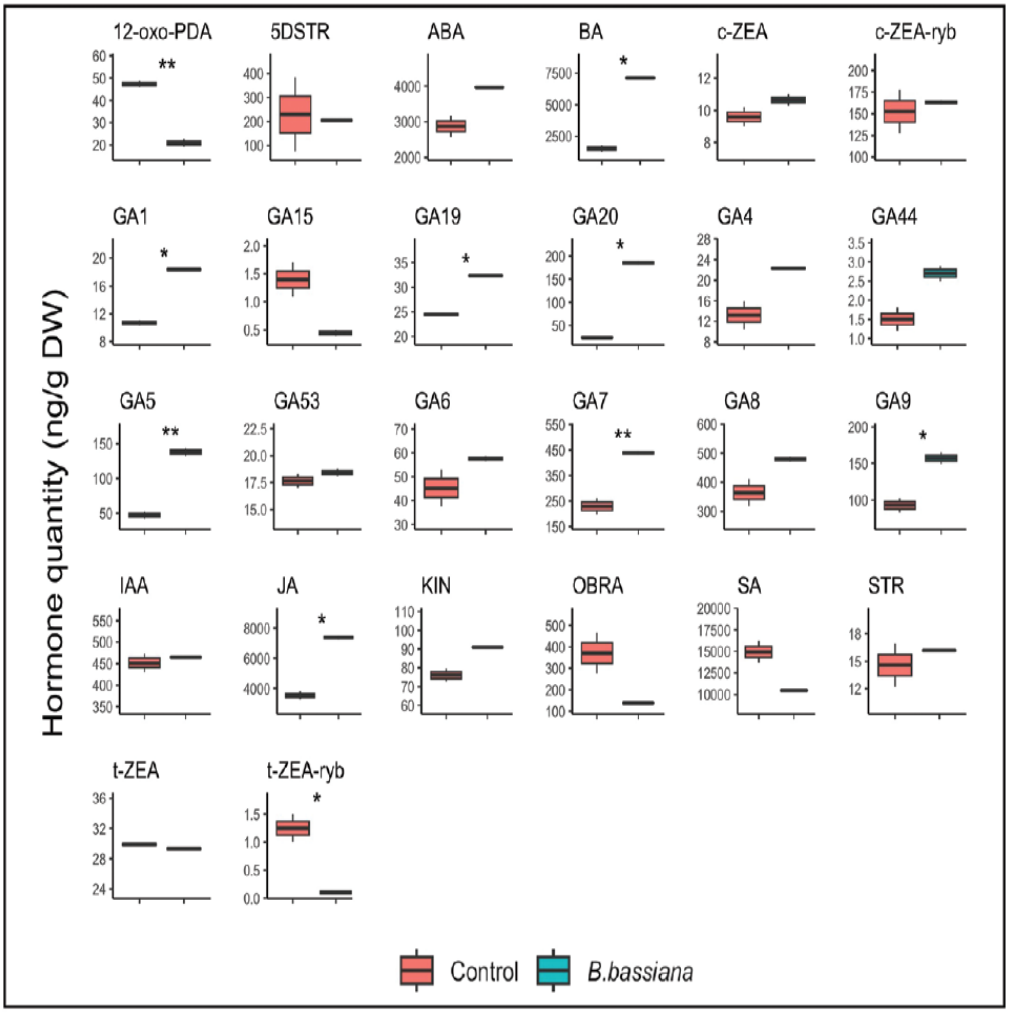

Figure 16. Hormones and related compounds identified by HPLC-MS/MS in plants colonized by *B. bassiana* and not colonized (control). Boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (second quartile). Whiskers denote the lowest and the highest values within 1.5×IQR from the first and third quartiles, respectively. Asterisks indicate significant differences between

B. bassiana colonized vs control plants (*t*-test; Benjamini–Hockberg FDR correction; **P*<0.05; ***P*<0.01). Physico-chemical determinations were performed in three biological replicates. 5DSTR, 5 deoxy-strigol; 12-oxo-PDA, 12-oxo-phytodienoic acid; ABA, abscisic acid; BA, benzoic acid; c-ZEA, *cis*-zeatin; c-ZEAryb, *cis*-zeatin riboside; GA1, gibberellin A1; GA4, gibberellin A4; GA5, gibberellin A5; GA6, gibberellin A6; GA7, gibberellin A7; GA8, gibberellin A8; GA9, gibberellin A9; GA15, gibberellin A15; GA19, gibberellin A19; GA20, gibberellin A20; GA44, gibberellin A44; GA53, gibberellin A53; IAA, indole-3-acetic acid; JA, jasmonic acid; KIN, kinetin; OBRA, orobanchol; SA, salicylic acid; STR, strigol; t-ZEA, *trans*-zeatin; t-ZEA-ryb, *trans*-zeatin riboside (From Proietti *et al.,* 2023).

We observed up-regulation of growth-related hormones like gibberellin (GA) precursors and their active forms, as well as of related compounds with defense functions, such as benzoic acid and JA. GAs are a group of diterpenoid growth hormones strongly associated with growth promotion, including stem elongation and germination. The role of GAs in developing and maintaining plant-beneficial microbe symbioses is an emerging area of research (McGuiness *et al.*, 2019). GA1 and GA4 are the major bioactive GAs and are more abundant than GA3 and GA7 in many plant species (Daviere and Achard, 2013). In our experimental conditions, we found several active GAs (GA1, GA5, GA7, GA19) as well as some GA precursors (GA9, GA20) significantly up-regulated in *B. bassiana*-colonized plants compared to controls. Some proteins found to be up-regulated by *B. bassiana* colonization may be linked to the gibberellin pathway. Among them, NADPH-cytochrome P450 reductase (A0A3Q7H9M0) is the essential redox partner of multiple cytochrome P450s involved in the biosynthesis of a variety of secondary metabolites, including GAs (Bak *et al.*, 2011). Another *B. bassiana* up-regulated protein involved in gibberellin production, is the "C_2H_2-type domain-containing protein" (A0A3Q7HYW7). This protein belongs to the histone deacetylase HD2 family, and recently emerged as a mediator of the switch from cell division to expansion in the root tip through repressing the transcription of GA2ox (Li *et al.*, 2017). The enzyme GA2ox2 catalyses the transition of GA9 and GA20 into further gibberellins (Israelsson *et al.*, 2005). This observation could explain the observed GA9 and GA20 accumulation following *B. bassiana* colonization. An up-regulated hormone-related compound involved in important plant development and survival processes is benzoic acid (BA). Among the key functions provided by BA and its derivatives are growth regulation, repellency, and pollinator attraction (Qualley *et al.*, 2012). BA has been documented to influence several

physiological processes, including anthocyanin production, chlorophyll biosynthesis, ion uptake, root elongation, and enzyme activity (Maffei *et al.*, 1999). Additionally, Windisch *et al.* (2021) showed that lettuce roots secrete benzoic acid as a defensive metabolite against fungal pathogens. Taking all the above data into account, we can argue that BA accumulation in our samples may be related to a defensive role. Moreover, it is worthwhile remember that BA is the immediate precursor of SA (Chen *et al.*, 2009), reinforcing the hypothesis of its defensive role. However, there is an apparent contradiction in our dataset since BA is up-regulated while SA is down-regulated. We could hypothesize that when plants recognize a colonizing microorganism, either a rhizobacterium or a fungus, as a biotrophic pathogen they suddenly activate the SA pathway. This may have occurred in the early stages of *B. bassiana* colonization, and we may have missed the moment when SA peaked. Later, the microorganism responds by repressing SA by specific effectors to establish an interaction with the future host (Pozo *et al.*, 2015). Indeed, it has been reported that during a well-established symbiosis the SA pathway is repressed, the levels of JA and JA precursors increase, and there is an up-regulation of JA-responsive genes (Bastias *et al.*, 2021). The antagonism between SA and JA is often observed in dicots, rather than monocots, and in response to (a)biotic stresses (De Vleesschauwer *et al.*, 2013; Tamaoki *et al.*, 2013). This fine-tuned regulation of SA and JA signaling pathways by beneficial colonization could also play a role in increasing plant resistance to pests (Bastias *et al.*, 2021).

4.1.4 *Beauveria bassiana* protects plants from *Botrytis cinerea*-induced oxidative stress

The fungal necrotrophic pathogen *B. cinerea* can affect all aboveground parts of tomato plants, both in the greenhouse and in the field. In recent years, many efforts have been made to develop an effective solution for the biological control of this disease in tomato (Bouaoud *et al.*, 2017; Minchev *et al.*, 2021). It is widely recognized that jasmonates are effective against necrotrophic pathogens (Antico *et al.*, 2012). The upregulation of JA by *B. bassiana* in our dataset prompted us to investigate whether *B. bassiana*-colonized plants are more resistant to *B. cinerea* infection. Plants colonized with *B. bassiana* and

subsequently infected with *B. cinerea* displayed less severe symptoms than plants infected with *B. cinerea* only (Fig. 17).

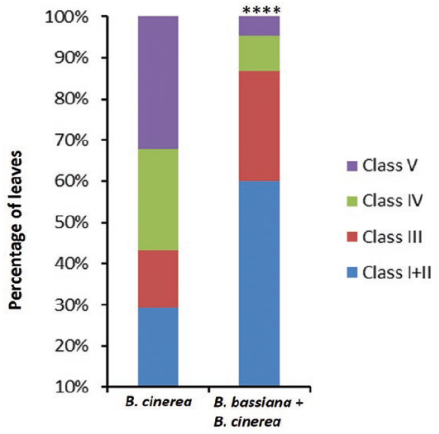

Figure 17. Distribution of disease symptoms of leaves from plants colonized by *B. bassiana* and infected with *B. cinerea* compared with *B. cinerea* infected plants, 3 d after inoculation. The bars indicate the frequency distribution of disease symptoms. Disease rating is expressed as the fraction of leaves falling into five classes: I, only spot (no lesion); II, lesion smaller than 3 mm; III, lesion bigger than 5 mm; IV, lesion smaller than 1 cm; V, disease symptoms visible on the whole leaf. The percentage of leaves in each class was calculated per plant (n=10). Asterisks indicate statistically significant difference between *B. bassiana* + *B. cinerea* and *B. cinerea* samples ($\chi 2$ test; P<0.0001). Data shown are means of three biological replicates (From Proietti *et al.,* 2023).

It is well known that necrotrophic pathogens like *B. cinerea* can produce or induce the production of ROS in plants (Lopez-Cruz *et al.*, 2016). Increased ROS levels in Arabidopsis and tomato increased *B. cinerea* colonization and necrotrophic development (Govrin and Levine, 2000; Kuzniak and Skłodowska, 2004). On the other hand, local ROS production enhanced resistance to *B. cinerea* in tomato (Asselbergh *et al.*, 2007). It has been recently pointed out that the endophyte-mediated plant antioxidant system may play an important role in alleviating stress damage by controlling ROS (Omoarelojie *et al.*, 2021; Sahu *et al.*, 2022). Additionally, arbuscular mycorrhizal colonization relieves oxidative stress induced by abiotic cues, monitored as a decrease of lipid peroxidation and activity of ROS scavenging enzymes (Molina *et al.*, 2020).

To evaluate the impact of oxidative stress in response to *B. cinerea* infection in the presence or absence of *B. bassiana*, we measured ROS after treating plants with a ROS-sensitive dye as 2′,7′-dichlorofluorescein diacetate ($DCFH_2$-DA) (Proietti *et al.*, 2019; Falconieri *et al.*, 2022). $DCFH_2$-DA diffuses into the cytoplasm through the plasma membrane and is deacetylated

by intracellular esterase before being oxidized by ROS to form the green fluorescent dye 2′,7′- DCF. Originally, it was considered that H_2O_2 was the only ROS capable of converting $DCFH_2$ to DCF, but recent studies showed that other ROS such as hydroxyl radical, hydroperoxides, and peroxynitrite may also oxidize $DCFH_2$, albeit with considerably lower sensitivity than H_2O_2 (Proietti *et al.*, 2019; Falconieri *et al.*, 2022). Results revealed abundant green spots marking the presence of ROS in leaf samples infected by *B. cinerea* compared with those also colonized by *B. bassiana* (Fig. 18).

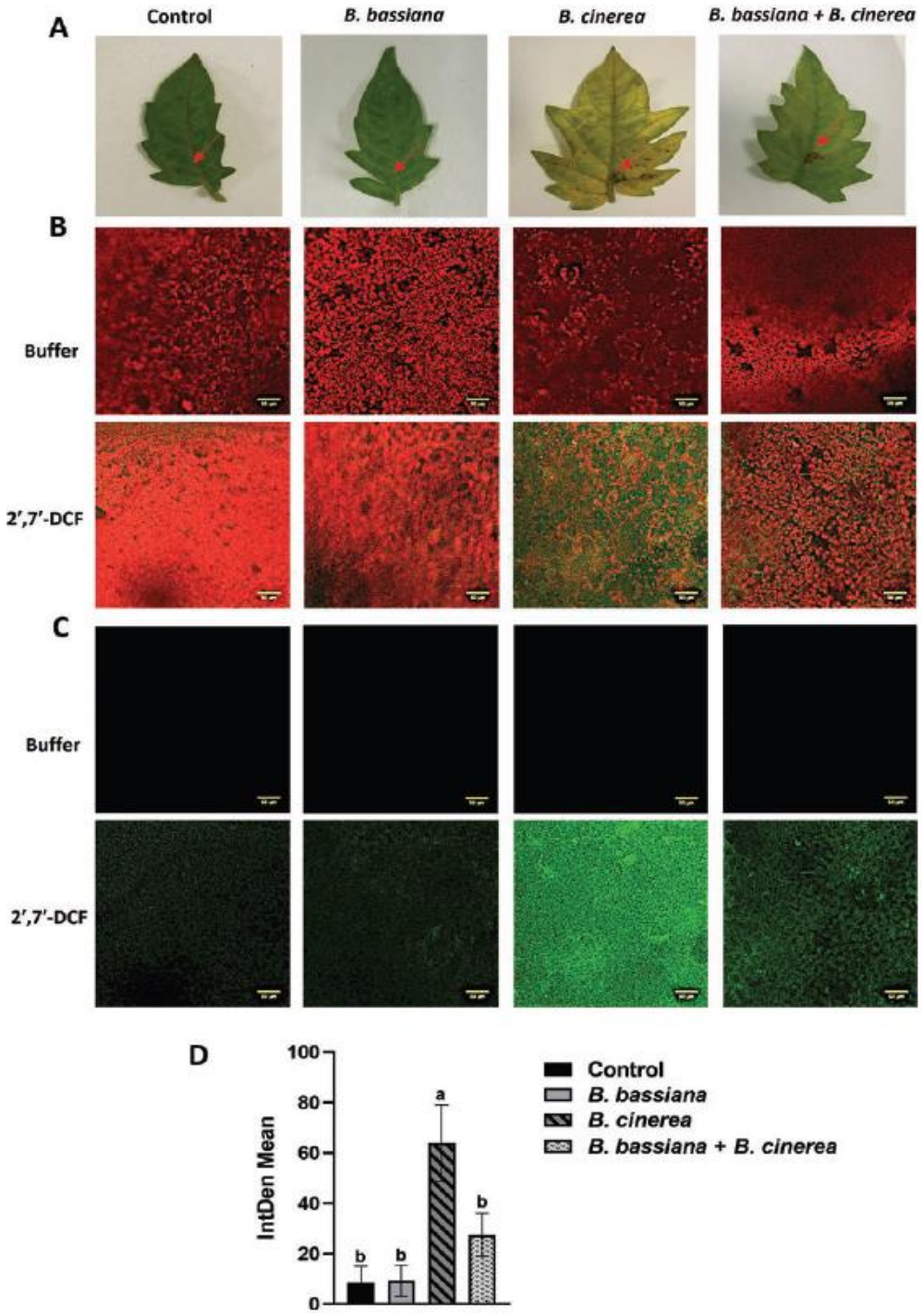

Figure 18. Detection of ROS in tomato leaves. (A) Representative leaves related to: (i) control, (ii) *B. bassiana* colonized plants, (iii) *B. cinerea* infected plants, and (iv) *B. bassiana* colonized + *B. cinerea* infected plants. Arrow indicates the site of inoculation of PDB droplet (i; ii) or *B. cinerea* droplet (iii; iv), further investigated for ROS assay. (B) Detection of ROS was carried out by using 2′,7′-DCFH2-DA or buffer (negative technical control). Fluorescence was observed under an LSM 710 confocal microscope with Plan Neofluar 20/1.30 objective. Laser excitations line was used, i.e. 488 nm for probe detection (green) and 561 nm for chlorophyll autofluorescence (red). Scale bar: 50 μm. Representative merged images are shown. (C) Green fluorescence only is shown. (D) Quantification of green fluorescence detected by using 2′,7′-DCFH2-DA in all four tested conditions, performed by ImageJ, version 1.53s. The integrated density mean from three biological replicates is reported (one-way ANOVA, Tukey's multiple comparison test: $P < 0.05$) (From Proietti *et al.*, 2023).

Additionally, we measured lipid peroxidation (through the TBARS assay) and the activity of SOD, CAT, and APX enzymes (Fig. 19).

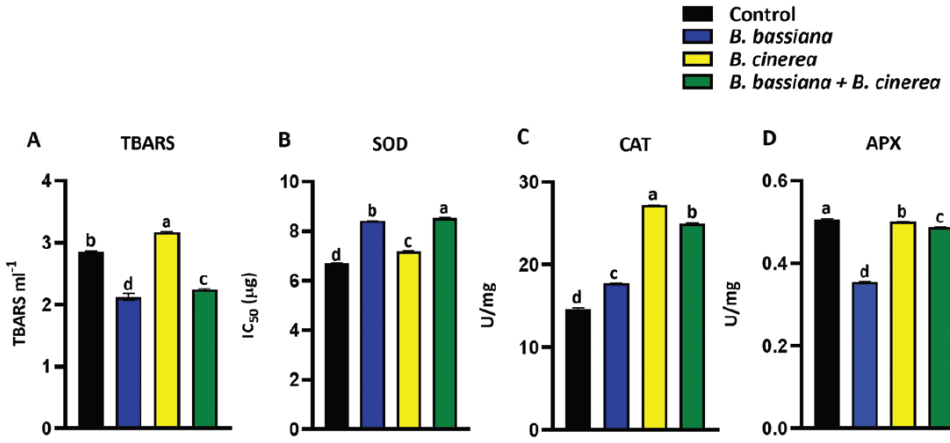

Figure 19. Lipid peroxidation and antioxidant enzyme activities in tomato leaves. (A) TBARS; (B) superoxide dismutase (SOD); (C) catalase (CAT); (D) ascorbate peroxidase (APX). Different letters indicate significant differences between treatments (one-way ANOVA, Tukey's multiple comparison test: $P<0.05$). Data shown are means of three biological replicates. (From Proietti *et al.,* 2023).

Lipid peroxidation is a harmful process in plants, altering membrane characteristics, inducing protein breakdown, and reducing ion transport capacity, ultimately leading to cell death (Yamamoto *et al.*, 2001). One of the most commonly used methods to detect lipid peroxidation is the TBARS assay. Figure 19A shows that TBARS were significantly lower in *B. bassiana* + *B. cinerea* plants than in plants infected by *B. cinerea* only. This means that lipid peroxidation induced by the fungal pathogen was counteracted by the presence of the endophyte, reinforcing the idea of a protective effect exerted by *B. bassiana*. The activities of SOD, CAT, and APX are frequently assayed as an indicator of oxidative stress in different living models (Marrocco *et al.*, 2017; Liu *et al.*, 2018). SODs are the first line of defense against ROS within cells. These enzymes are members of the metal-enzyme family, which catalyses the dismutation of superoxide anions (O_2^-) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). Catalases typically convert two molecules of H_2O_2 to water and O_2. APX is the other major H_2O_2-scavenging enzyme in plant cells, removing H_2O_2 via the ascorbate‑glutathione cycle (Omoarelojie *et al.*, 2021). In our work, the IC_{50} of SOD in *B. bassiana* + *B. cinerea*-treated plants was higher than in *B. cinerea*-infected plant, implying that less protein extract is required to achieve 50% of formazan inhibition.

This suggests that SOD activity in *B. cinerea*-infected samples is higher than in *B. bassiana* + *B. cinerea*-treated plants (Fig. 19B). In our experiment, *B. cinerea* infection induced both CAT and APX activity, indicating that oxidative stress developed after pathogen attack. However, when infected plants were colonized by *B. bassiana*, APX and CAT activities decreased (Fig. 19C, D). It is thus plausible that *B. bassiana* implements molecular strategies to enhance resistance to *B. cinerea*, leading to slower pathogen fungal growth and less leaf area affected by the infection, in turn reducing the oxidative damage. In conclusion, in this first part of the study, we detected key molecular pathways perturbed by *B. bassiana* colonization related to primary and secondary metabolism, likely responsible for a well-established tomato plant-fungus symbiosis and plant health promotion. Analysis of plant hormones and related compounds highlighted the impact of *B. bassiana* on growth- and defense related substances. Finally, we showed that *B. bassiana* is effective against *B. cinerea* infection, reducing plant oxidative stress. A summary of the sequential activation of responses elicited in tomato plants colonized by *B. bassiana* is shown in the working model of Fig. 20.

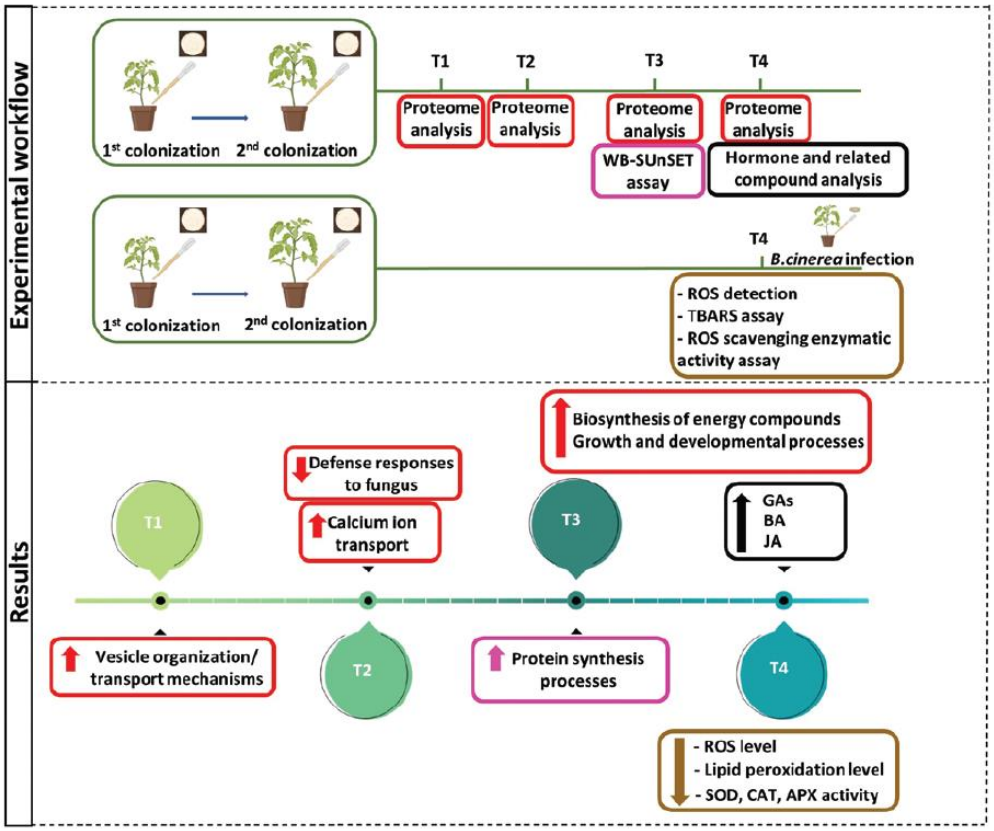

Figure 20. Summary of the experimental workflow and the main results obtained. In the experimental workflow box, the approaches used are distinguished by different colors. In the results box, the same color code is used. Arrows indicated up-regulated or down-regulated processes/compounds. Briefly, tomato plants have been colonized twice with *B. bassiana* and a time course leaf proteome analysis was performed at 5, 7, 12, and 19 d after the second colonization (T1, T2, T3, and T4, respectively). Results highlighted up-regulation of vesicles organization (T1); down-regulation of defense response to fungus and up-regulation of calcium ion transport (T2); and up-regulation of biosynthesis of energy compounds and of growth and developmental processes (T4). Up-regulation of protein synthesis has been shown at T3 by a WB-SUnSET assay. Analysis of hormones and related compounds at T4 showed among others up-regulation of gibberellins (GAs), benzoic acid (BA), and jasmonic acid (JA). At T4, plants colonized by *B. bassiana* are more resistant to *B. cinerea* infection, likely impacting oxidative stress. Reactive oxygen species (ROS) detection, a thiobarbituric acid reactive compounds (TBARS) assay, and a ROS scavenging enzymatic activity assay showed a decreased ROS and lipid peroxidation level and decreased superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) activities. Created with BioRender.com (From Proietti *et al.*, 2023).

4.2 *Beauveria bassiana* and *Trichoderma harzianum* modulate plant molecular network reprogramming activating different defense strategies to cope with pathogens

In the second part of the thesis work, we focused on dissecting the tripartite interaction existing between beneficial microbes-tomato-biotic stress, which will increase our knowledge on the sophisticated way in which beneficial

microbes promotes plant health and strengthen defense barriers. Molecular analyses were conducted on leaves of control plants, colonized with *B. bassiana* or with *T. harzianum* (strain T22) alone, colonized with the *B. bassiana*-*T. harzianum* consortium, infected with *B. cinerea* and infested with the *Macrosiphum euphorbiae* aphid and on plants pre-colonized with the individual beneficial microbes or with the consortium and subsequently subjected to stress by *B. cinerea* or *M. euphorbiae*.

4.2.1 Beneficial microbes hijack plant proteome programming

A comparative proteomic analysis was performed on all samples described above. All data were analyzed in order to understand the impact of the individual beneficial microbe or the consortium in response to stress compared to plants subjected to stress but not colonized by the beneficial microbes. For each comparison, we conducted a principal component analysis (PCA) on $\log_2$-transformed quantification values of proteins that were differentially expressed in at least one treatment, with the goal of understanding global proteome changes induced by the various treatments. Subsequently, we generated a hierarchical clustering heatmap plot based on the expression levels of these differentially expressed proteins. Within each cluster identified by hierarchical clustering, top enriched Gene Ontology (GO) terms related to biological processes were annotated on the right side of the corresponding protein cluster. Furthermore, within the same clusters, we identified enriched KEGG metabolic pathways for each comparison.

4.2.1.1 *T. harzianum*-*B. bassiana* consortium vs *T. harzianum* and vs *B. bassiana*

This first comparison allows to understand how plants responds to *B. bassiana*-*T. harzianum* consortium treatment compared to individual beneficial microbe treatment.

The PCA plot (Fig. 21A) shows clear separation of the clusters related to *B. bassiana* alone and *B. bassiana*-*T. harzianum* consortium treatment; in contrast, those related to control and *T. harzianum* alone treatment have a

smallest distance. This sample distribution may suggest that the colonization with *T. harzianum* does not cause any effect on the plant.

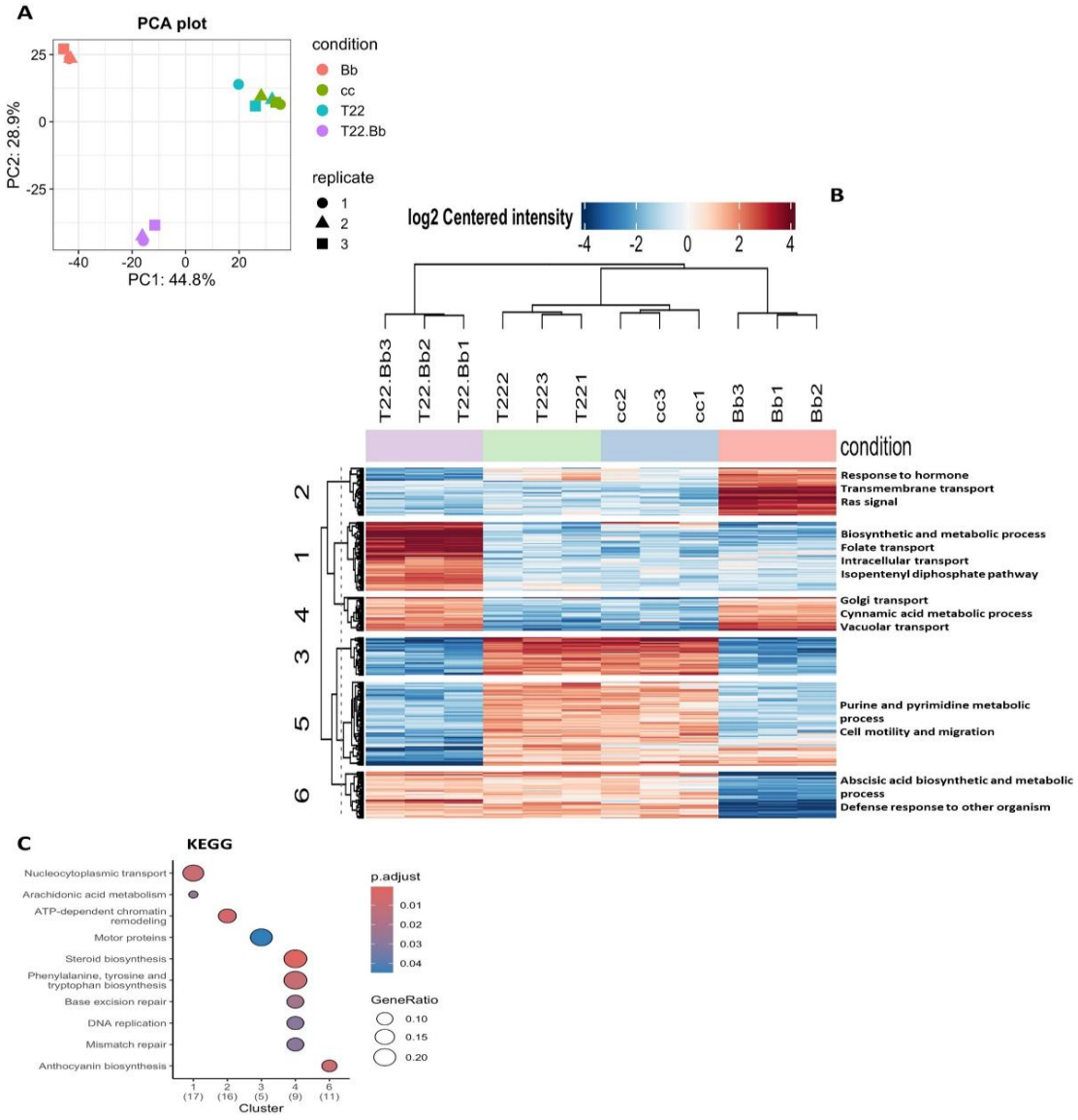

Figure 21. Comparisons between differentially expressed proteins induced by *T. harzianum* (T22), *Beauveria bassiana* (Bb), and the combined treatment of *T. harzianum* and *B. bassiana* (T22.Bb). In all cases, the differentially expressed proteins (DEPs) induced by these treatments are compared to the DEPs of untreated control plants (cc). A: Plot of principal component analysis (PCA) on log2-transformed quantification values of differentially expressed proteins (DEPs) in at least one treatment. Each treatment is represented by different colors, while replicates are denoted by distinct shapes. B: Heatmap plot of hierarchical clustering analysis displaying differentially expressed proteins clustered by expression patterns of the log2-transformed quantification values. Right annotations to each cluster show top enriched Gene Ontology (GO) biological processes. C: Dot plot representing enriched KEGG metabolic pathways within each cluster (displayed on the y-axis) identified by hierarchical clustering of DEPs. Each dot on the plot is colored on a blue-red scale based on the P value adjusted by the Benjamini-Hochberg method for controlling the false discovery rate (FDR) for multiple comparisons. The size of the dots corresponds to the gene ratio, indicating the proportion of proteins in the enriched pathway relative to the total number of proteins (genes) in that pathway. Larger dots signify a higher gene ratio and a more significant enrichment.

The PCA analysis results were confirmed with a comparative proteome analysis which clearly shows marked differences between the various samples (Figure 21B). In particular, treatment with the *B. bassiana* - *T. harzianum* consortium up-regulates the biosynthetic and metabolic pathways that support plant growth and development and also leads to the synthesis of secondary metabolites such as isoprenoids and phenylpropanoids (i.e. cinnamic acid) which play a pivotal role in defense against herbivores and pathogens. Furthermore, vesicular transport processes are also upregulated, probably due to the contribution of *B. bassiana*, already described in Proietti *et al.,* 2023. This finding could confirm a well-established communication between the plant and the beneficial microbes. In addition, biosynthetic and metabolic processes linked to ABA and defense responses against other organisms are also activated. These very interesting data are confirmed in several studies in which an increase in ABA was reported in tomato leaves treated with *T. harzianum,* leading to greater adaptation of the plants to resistance against abiotic and biotic stresses (Alwhibi *et al.,* 2017). Moreover, KEGG pathway analysis shows that in the consortium-treated plants there is an upregulation of "Arachidonic acid metabolism" that is involved in defense responses in Solanaceous plants (Savchenko *et al.*, 2010) (Figure 21C). As mentioned above, *T. harzianum* and control samples have the same expression pattern; in particular, in this treatment the metabolic pathways involving purines and pyrimidines with energy functions and cell motility that underlie growth and development processes are highly sustained. Finally, it is evident that *B. bassiana* could upregulate several processes including those related to hormonal responses or transmembrane transport and the RAS signal transduction pathway for the activation of genes involved in cell growth and differentiation (Krysan and Colcombet, 2018).

4.2.1.2 *T. harzianum* + *B. cinerea* vs *T. harzianum* and vs *B. cinerea*

The effect of *T. harzianum* colonization on *B. cinerea* infected plants compared to *T. harzianum* or *B. cinerea* samples has been deeply investigated. As shown in figure 22A, the PCA plot shows the clustering of the samples related to *T. harzianum* colonized and control samples, while those related to *T. harzianum* + *B. cinerea* and *B. cinerea* alone are distant. The heatmap plot

(Figure 22B) indicate that when the plant is infected with *B. cinerea*, several intracellular signal transduction pathways and responses to external stimulus are activated: this does not occur under the other conditions.

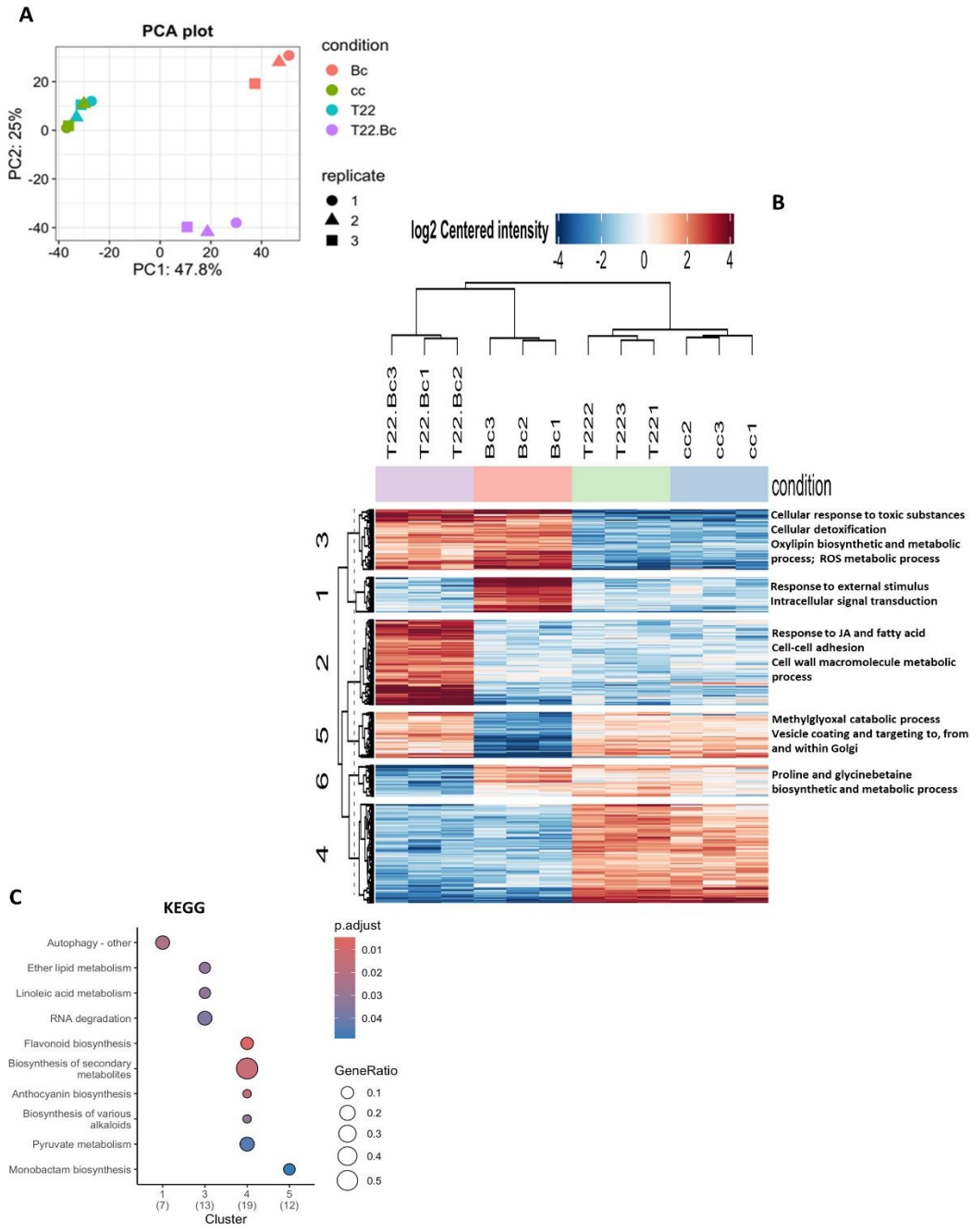

Figure 22. Comparisons between differentially expressed proteins induced by *T. harzianum* (T22), *Botrytis cinerea* (Bc) and *T. harzianum* on plants infected by *Botrytis cinerea* (T22.Bc). In all cases, the differentially expressed proteins (DEPs) induced by these treatments are compared to the DEPs of untreated control plants (cc). A: Plot of principal component analysis (PCA) on log2-transformed quantification values of differentially expressed proteins (DEPs) in at least one treatment. Each treatment is represented by different colors, while replicates are denoted by distinct shapes. B: Heatmap plot of hierarchical clustering analysis displaying differentially expressed proteins clustered by expression patterns of the log2-transformed quantification values. Right annotations to each cluster show top enriched Gene Ontology (GO) biological processes. C: Dot plot representing enriched KEGG metabolic pathways within each cluster (displayed on the y-axis) identified by hierarchical clustering of DEPs. Each dot on the plot is colored on a blue-red scale based on the P value adjusted by the Benjamini-Hochberg method for controlling the false discovery rate (FDR) for multiple comparisons. The size of the dots corresponds to the gene ratio, indicating the proportion of proteins in the enriched pathway relative to the total number of proteins (genes) in that pathway. Larger dots signify a higher gene ratio and a more significant enrichment.

Furthermore, in *B. cinerea* infected plants, there is the activation of a series of processes in response to detoxification, including ROS, and toxic substances. However, when the plant is pre-colonized with *T. harzianum* and then infected with *B. cinerea*, the beneficial microbe induces the response to JA and fatty acids which is not detected in *B. cinerea* infected sample. This data is also confirmed by KEGG analysis in which the linoleic acid metabolism is upregulated in these samples (Figure 22C). In *T. harzianum* + *B. cinerea* treatment the metabolic processes related to cell wall macromolecules are upregulated, corroborating the strategy adopted by *T. harzianum* that involves the synthesis of enzymes capable of degrading cell wall to prevent the proliferation of the pathogen (Wiest *et al.,* 2002; Ghasemi *et al.,* 2020). This beneficial fungus secretes various enzymes that degrade the cell wall, including cellulases and hemicellulases that contribute to the breakdown of complex carbohydrates in the fungal cell wall, destroying its structure and integrity, leading to a weakening of the pathogen's ability to invade plant tissues. In fact, this protein cluster is up-regulated only in the *T. harzianum* + *B. cinerea* treatment and down-regulated in the other ones (Figure 22B). Furthermore, *T. harzianum* also allows the detoxification of methylglyoxal, a compound that is toxic to the plant if accumulated in high amounts. Finally, another interesting data is represented by proline and glycinebetaine metabolism in *B. cinerea* and in a smaller quantity in *T. harzianum* samples: these compounds act as signal molecules and in antioxidant defense.

4.2.1.3 *B. bassiana* + *B. cinerea* vs *B. bassiana* and vs *B. cinerea*

The effect of *B. bassiana* colonization on *B. cinerea* infected plants compared to *B. bassiana* or *B. cinerea* treated plants has been also investigated. The PCA plot shows the clustering of the *B. cinerea* and *B. bassiana* + *B. cinerea* samples, as reported in figure 23A. When the plant experiences stress, such as *B. cinerea* infection, it triggers autophagy mechanisms. Moreover, processes that result in the biosynthesis and metabolism of terpenoid or carotenoid compounds are activated in response to stress (Figure 23B). Figure 23C shows that this data is also confirmed by the KEGG pathway analysis. An increase in the production of carotenoids as a consequence of the

activation of the plant defense biosynthetic pathways is normally associated with an induction of systemic resistance demonstrated by greater control of pathogens (Kaur *et al.,* 2022).

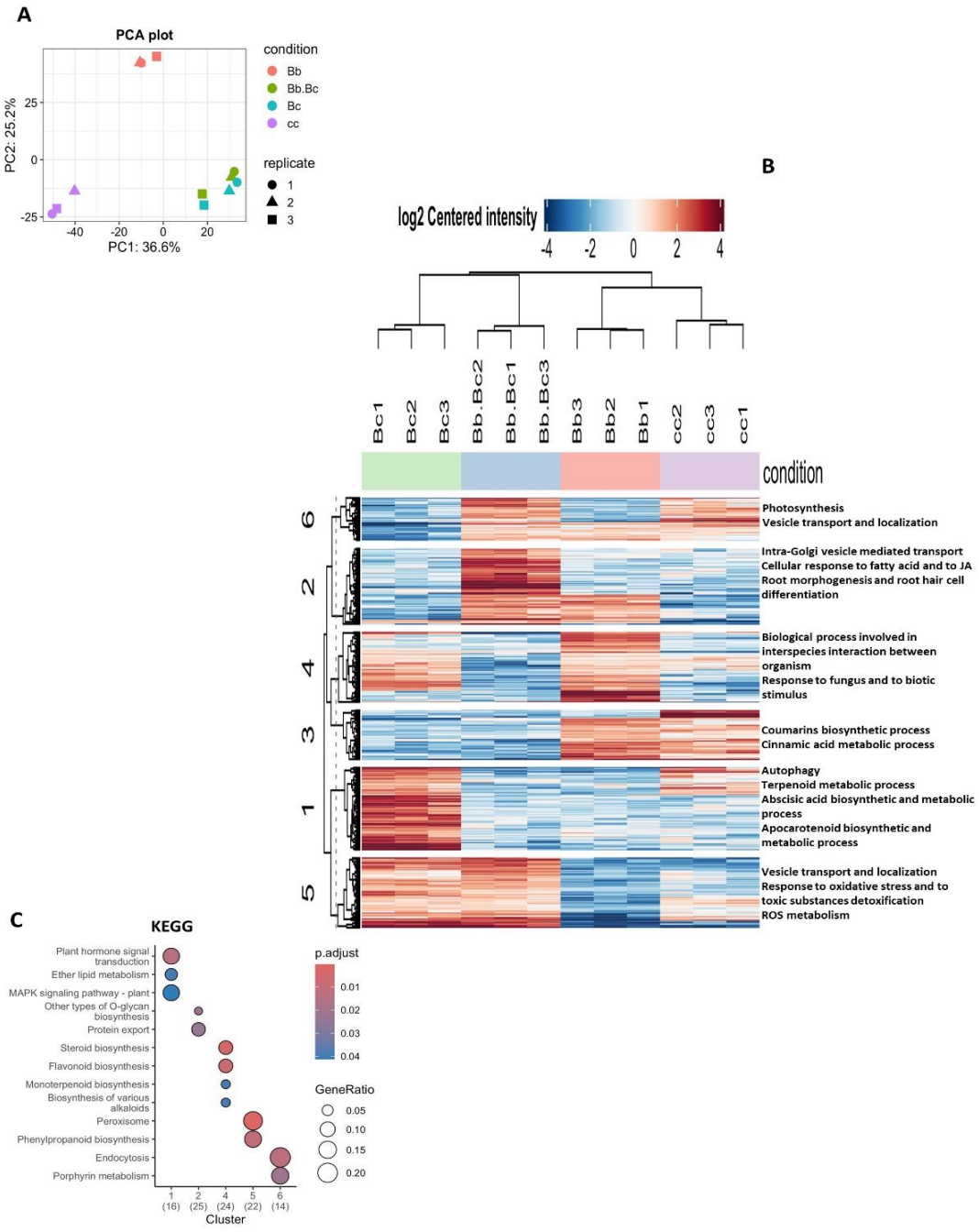

Figure 23. Comparisons between differentially expressed proteins induced by *Beauveria bassiana* (Bb), *Botrytis cinerea* (Bc), and *B. bassiana* in plants infected by *Botrytis cinerea* (Bb.Bc). In all cases, the differentially expressed proteins (DEPs) induced by these treatments are compared to the DEPs of untreated control plants (cc). A: Plot of principal component analysis (PCA) on log2-transformed quantification values of differentially expressed proteins (DEPs) in at least one treatment. Each treatment is represented by different colors, while replicates are denoted by distinct shapes. B: Heatmap plot of hierarchical clustering analysis displaying differentially expressed proteins clustered by expression patterns of the log2-transformed quantification values. Right annotations to each cluster show top enriched Gene Ontology (GO) biological processes. C: Dot plot representing enriched KEGG metabolic pathways within each cluster (displayed on the y-axis) identified by hierarchical clustering of DEPs. Each dot on the plot is colored on a blue-red scale based on the P value adjusted by the Benjamini-Hochberg method for controlling the false discovery rate (FDR) for multiple comparisons. The size of the dots corresponds to the gene ratio, indicating the proportion of proteins in the enriched pathway relative to the total number of proteins (genes) in that pathway. Larger dots signify a higher gene ratio and a more significant enrichment.

Sathasivam *et al.,* 2021 demonstrated that these molecules have an antioxidant action and contribute to ABA production which is one of the biosynthetic processes that is upregulated in this sample. However, when the plant is pre-colonized with *B. bassiana* and then infected with *B. cinerea*, different processes are activated to defend the plant and support its growth.
In fact, the beneficial microbe facilitates the activation of the JA pathway, enhancing the ability to respond to oxidative stress, toxic substances and ROS metabolism. Additionally, it upregulates the vesicular transport mechanism. Another interesting data is represented by the induction of biosynthetic processes in *B. bassiana* samples, particularly in the production of coumarins which has been reported to play a role in plant-beneficial microbes communication (Stringlis *et al.,* 2019). Furthermore, phenylpropanoid pathway derived compounds, including coumarins, are often involved in structural or chemical defenses. For example, the cell wall-fortifying compounds lignin, cutin and suberin form structural barriers that inhibit pathogen invasion (Doblas *et al.,* 2017).
Noteworthy, the activation of biological processes related to interactions between different organisms and the responses to the fungus, which could be a signal of an interaction between the plant and the fungus, is observed in both *B. cinerea* and *B. bassiana* treatments (Figure 23B).

4.2.1.4 *T. harzianum-B. bassiana* consortium + *B. cinerea* vs *T. harzianum + B. cinerea,* vs *B. bassiana + B. cinerea,* vs *T. harzianum-B. bassiana* consortium and vs *B. cinerea*

The goal of this analysis is to explore the response of the *B. bassiana-T. harzianum-B. cinerea* treatment compared to the single beneficial microbes (*T. harzianum* or *B. bassiana*) + *B. cinerea* and to *B. cinerea* alone. The PCA analysis reveals distinct separation among the samples, except for the control and the *T. harzianum* + *B. bassiana* consortium samples, likely due to the contribution of *T. harzianum* (Figure 24A). The trend of these latest treatments is also evident by the heatmap plot with the exception of cluster 3, where processes related to plant growth and development are increased in the *T. harzianum-B. bassiana* treatment compared to control one (Figure 24B).

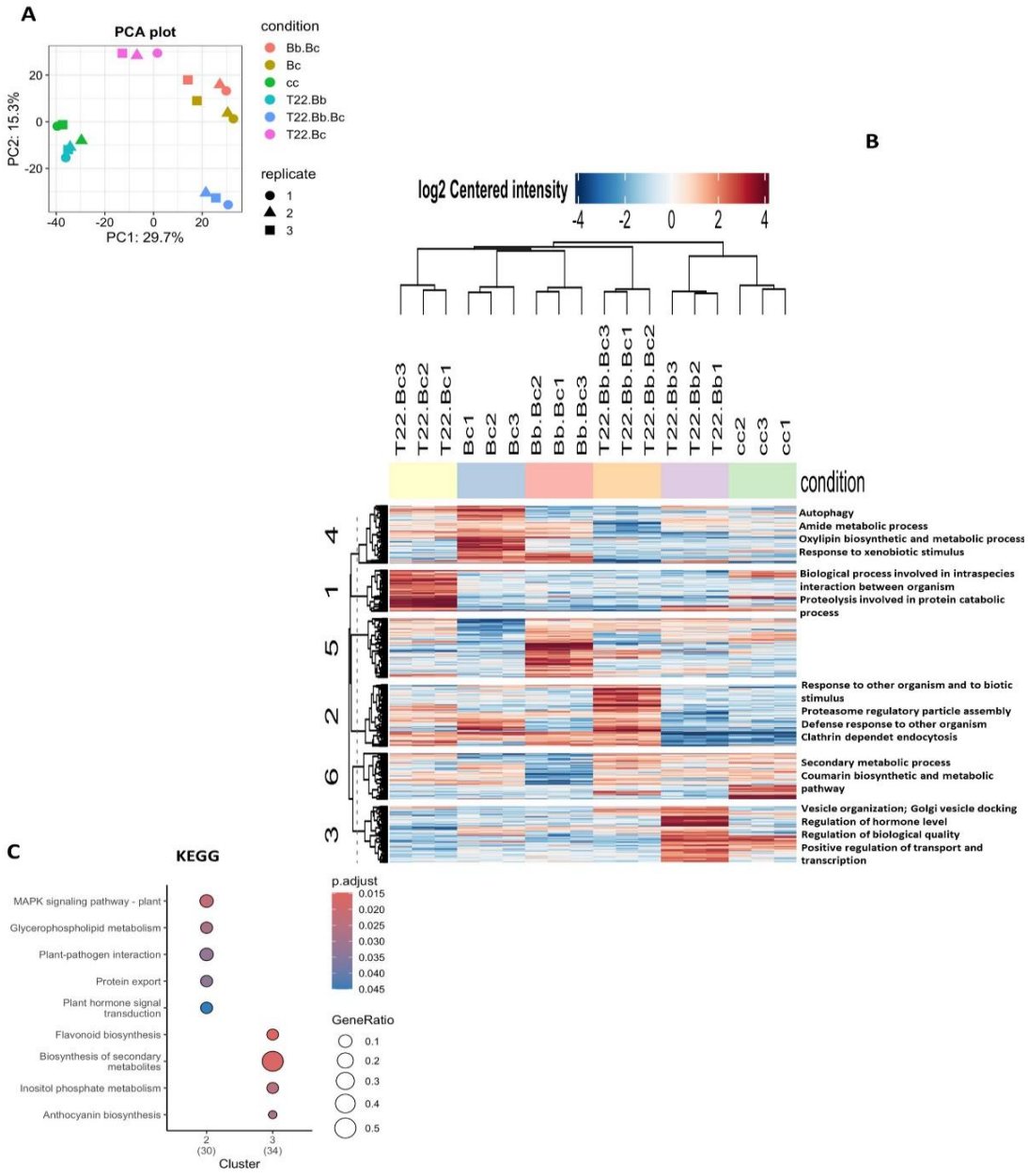

Figure 24. Comparisons between differentially expressed proteins induced respectively by *T. harzianum* (T22.Bc) or *B. bassiana* (Bb.Bc) and the effect of the combined treatment *T. harzianum* and *B. bassiana* (T22.Bb.Bc) on plants infected by *B. cinerea* (T22.Bc), *B. cinerea* alone (Bc), and the combined treatment of *T. harzianum* and/or *B. bassiana* (Bb.Bc) on plants infected with *B. cinerea*. In all cases, the differentially expressed proteins (DEPs) induced by these treatments are compared to the DEPs of untreated control plants (cc). A: Plot of principal component analysis (PCA) on log2-transformed quantification values of differentially expressed proteins (DEPs) in at least one treatment. Each treatment is represented by different colors, while replicates are denoted by distinct shapes. B: Heatmap plot of hierarchical clustering analysis displaying differentially expressed proteins clustered by expression patterns of the log2-transformed quantification values. Right annotations to each cluster show top enriched Gene Ontology (GO) biological processes. C: Dot plot representing enriched KEGG metabolic pathways within each cluster (displayed on the y-axis) identified by hierarchical clustering of DEPs. Each dot on the plot is colored on a blue-red scale based on the P value adjusted by the Benjamini-Hochberg method for controlling the false discovery rate (FDR) for multiple comparisons. The size of the dots corresponds to the gene ratio, indicating the proportion of proteins in the enriched pathway relative to the total number of proteins (genes) in that pathway. Larger dots signify a higher gene ratio and a more significant enrichment.

It is evident that the *T. harzianum-B. bassiana-B. cinerea* treatment impacts on the increase of defense responses against other organisms and biotic stimuli, as also shown by KEGG pathway analysis highlighting the term "plant-pathogen interaction related processes" as upregulated in this sample (Figure 24C). This beneficial effect triggered by the microbial consortium is more pronounced compared to *T. harzianum* + *B. cinerea* or *B. bassiana* + *B. cinerea* samples, where a decreased expression of these processes is found. The beneficial involvement of these microorganisms in plant survival under stress conditions was further corroborated by the downregulation of processes related to xenobiotic stimuli, autophagy, or amidic metabolism, typically involved in plant defense. Conversely, in *B. cinerea* infected samples, these processes have a boost, consistent with a stress scenario.

4.2.1.5 *T. harzianum* + *M. euphorbiae* vs *T. harzianum* and vs *M. euphorbiae*

The effect of *T. harzianum* colonization on *M. euphorbiae* infested plants compared to *T. harzianum* or *M. euphorbiae* samples has been investigated. The PCA plot (Figure 25A) reveals clustering of *T. harzianum* and control samples, evident also by the heatmap plot (Figure 25B), while it is showing clear separation of the other treatments.

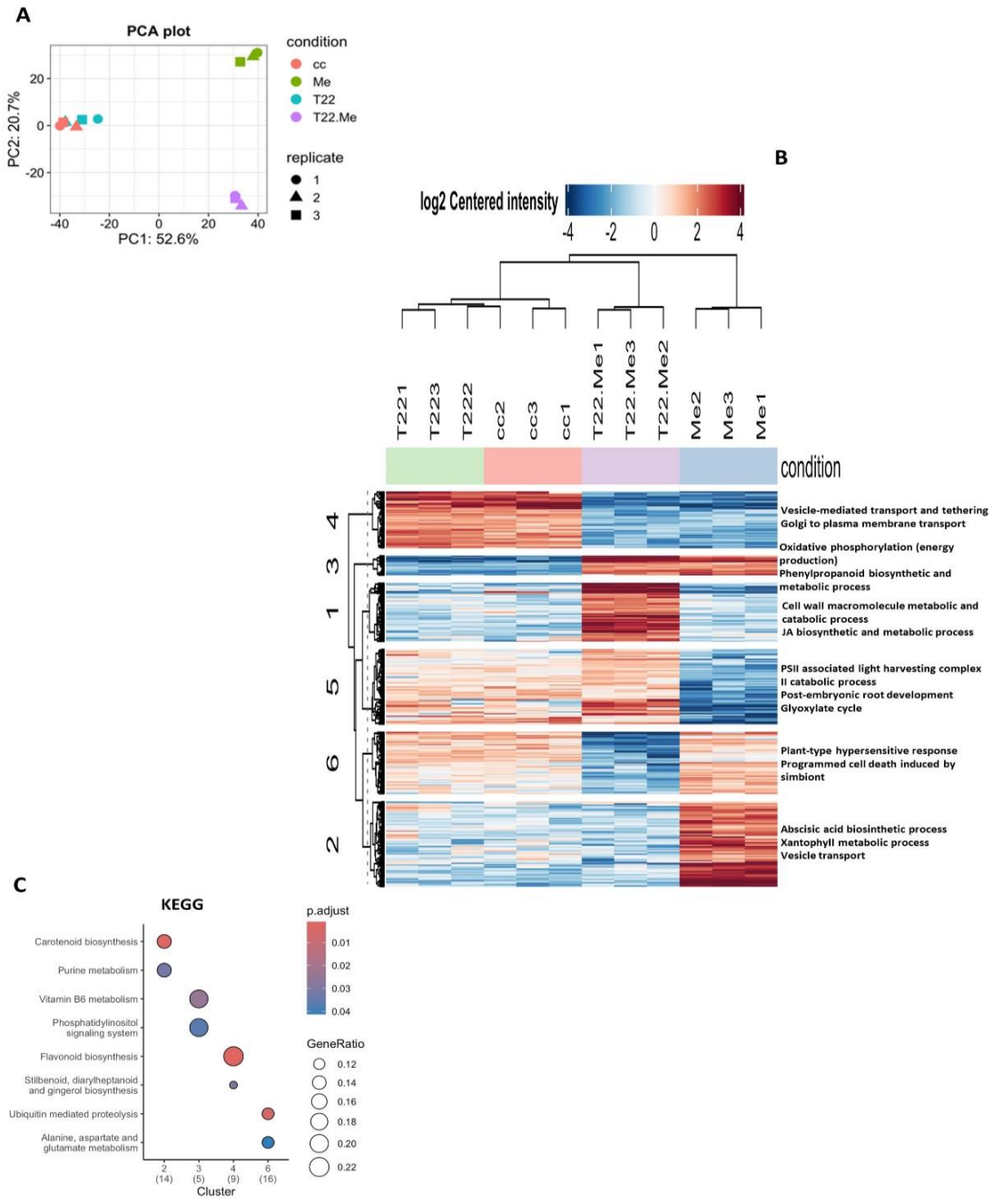

Figure 25. Comparisons between differentially expressed proteins induced by *T. harzianum* (T22), the aphid *M. euphorbiae* (Me), and the effect of *T. harzianum* on plants infested by *M. euphorbiae* (T22.Me). In all cases, the differentially expressed proteins (DEPs) induced by these treatments are compared to untreated control plants (cc). A: Plot of principal component analysis (PCA) on log2-transformed quantification values of differentially expressed proteins (DEPs) in at least one treatment. Each treatment is represented by different colors, while replicates are denoted by distinct shapes. B: Heatmap plot of hierarchical clustering analysis displaying differentially expressed proteins clustered by expression patterns of the log2-transformed quantification values. Right annotations to each cluster show top enriched Gene Ontology (GO) biological processes. C: Dot plot representing enriched KEGG metabolic pathways within each cluster (displayed on the y-axis) identified by hierarchical clustering of DEPs. Each dot on the plot is colored on a blue-red scale based on the P value adjusted by the Benjamini-Hochberg method for controlling the false discovery rate (FDR) for multiple comparisons. The size of the dots corresponds to the gene ratio, indicating the proportion of proteins in the enriched pathway relative to the total number of proteins (genes) in that pathway. Larger dots signify a higher gene ratio and a more significant enrichment.

The heatmap plot displays well-defined clusters, particularly those related to vesicular transport and tethering mechanisms processes, which are very similar in *T. harzianum* and control samples. In the *T. harzianum* + *M. euphorbiae* treatment, there is an interesting upregulation of cell wall macromolecule catabolic and metabolic process, as previously observed in the *T. harzianum* + *B. cinerea* treatment. This finding is highly interesting as several studies have demonstrated that plants under aphid stress could implement strategies, such as the production of wall-modifying enzymes, as a defense mechanism (Silva-Sanzana *et al.,* 2020). In fact, De Vos *et al.*, (2005) studied the transcriptional profile of Arabidopsis plants challenged by various attackers, such as necrotrophic and biotrophic pathogens, a chewing caterpillar, thrips and aphids, revealing that plants upregulated the expression of attacker-specific genes, that in the case of under-study aphid corresponded to Pectin Methylesterase Iinhibitor 13 (PMEI13). Afterwards, Silva-Sanzana *et al.* (2019) characterized the role of PMEI13 during aphid infestation, showing that aphids significantly preferred *pmei13* mutants as host compared to wildtype genotypes probably due to phloem and nutrients facility access. These results are particularly interesting considering that an increase in total PME and in other cell wall-modifying enzyme activities could lead to the production of oligogalacturonides (OGs) which elicit plant local defense responses such as the production of callose deposits and hydrogen peroxide (Will and van Bel, 2008). *T. harzianum* colonization seems to assist the plant in this function, since other treatments exhibit a downregulation of these processes. Furthermore, *T. harzianum* upregulates phenylpropanoid biosynthetic pathway in response to *M. euphorbiae*, compounds which play crucial roles in various physiological processes in plants, such as defense responses against pathogens and insects, as previously reported also by Coppola *et al*. (2019). Additionally, colonization with this beneficial microbe led to an upregulation of the glyoxylate pathway, which sustains plant growth and development. As demonstrated by KEGG analysis in figure 25C, *M. euphorbiae* upregulates carotenoid and ABA biosynthetic processes, which are found downregulated in other conditions, confirming the absence of stress in those treatments. Finally, in this last sample, the GO biological process related to hypersensitive response is activated, a rapid localized cell death

occurring at the pathogen penetration point and associated with disease resistance. This is accompanied by programmed cell death processes induced by the symbiont, which however are found downregulated in the double treatment *T. harzianum* + *M. euphorbiae.*

4.2.1.6 *B. bassiana* + *M. euphorbiae* vs *B. bassiana* and vs *M. euphorbiae*

The effect of *B. bassiana* colonization on *M. euphorbiae* infested plants compared to *B. bassiana* or *M. euphorbiae* samples has been also investigated. PCA plot (figure 26A) shows clear separation of the various treatments reflecting on delineated protein clusters as demonstrated by heatmap plot in figure 26B.

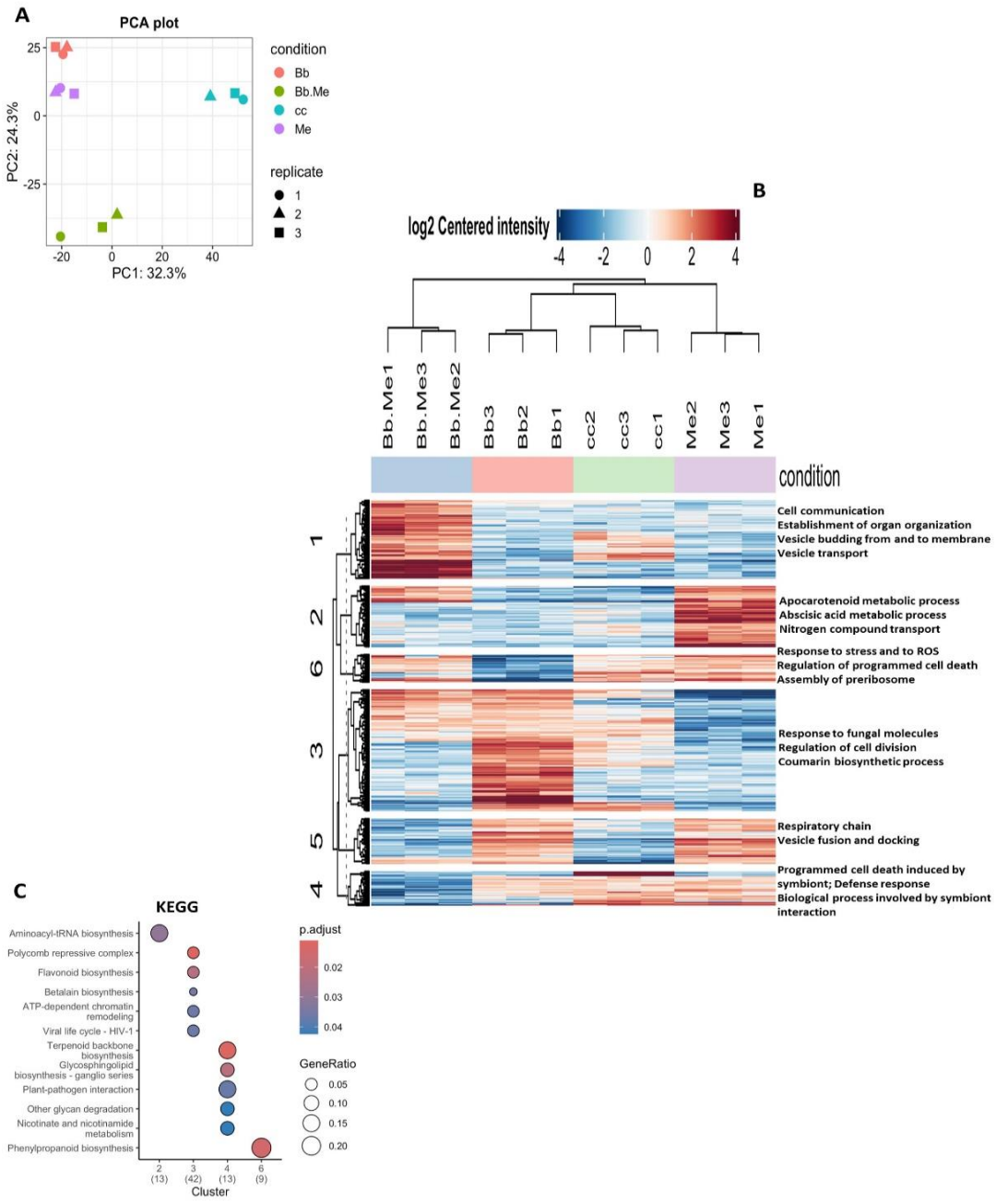

Figure 26. Comparisons between differentially expressed proteins induced by *B. bassiana* (Bb), *M. euphorbiae* (Me) and the effect of *B. bassiana* (Bb) on plants infested by *M. euphorbiae* (Bb.Me). In all cases, the differentially expressed proteins (DEPs) induced by these treatments are compared to untreated control plants (cc). A: Plot of principal component analysis (PCA) on log2-transformed quantification values of differentially expressed proteins (DEPs) in at least one treatment. Each treatment is represented by different colors, while replicates are denoted by distinct shapes. B: Heatmap plot of hierarchical clustering analysis displaying differentially expressed proteins clustered by expression patterns of the log2-transformed quantification values. Right annotations to each cluster show top enriched Gene Ontology (GO) biological processes. C: Dot plot representing enriched KEGG metabolic pathways within each cluster (displayed on the y-axis) identified by hierarchical clustering of DEPs. Each dot on the plot is colored on a blue-red scale based on the P value adjusted by the Benjamini-Hochberg method for controlling the false discovery rate (FDR) for multiple comparisons. The size of the dots corresponds to the gene ratio, indicating the proportion of proteins in the enriched pathway relative to the total number of proteins (genes) in that pathway. Larger dots signify a higher gene ratio and a more significant enrichment.

In *B. bassiana* + *M. euphorbiae* samples, there is an overexpression of processes involved in cell communication and vesicle transport to and from the membrane. It has been reported that vesicular trafficking is one of the mechanisms that plants can use to regulate the outcome of stress, as the plasma membrane is among the organelles most exposed to oxidative stress damage and therefore needs to be constantly recycled (Levine, 2002). This process is probably most favoured by *B. bassiana* colonization since in *M. euphorbiae* sample this term is downregulated (Figure 26B). However, in *M. euphorbiae* there is a boost of processes involved in the biosynthesis of ABA or compounds with defense activity, which we expect in this stressed sample and which are downregulated in the other ones. In addition, the aphid activates stress and ROS responses, together with programmed cell death, processes that are downregulated in *B. bassiana* + *M. euphorbiae* samples, in which probably these aspects are mitigated by the colonization with *B. bassiana,* making the plant more resistant. Finally, *B. bassiana* colonization results in an enhancement of all processes associated with plant growth and development. These aspects are also underlined by KEGG pathway analysis that shows the upregulation of pathway related to "Betalain biosynthesis" and "Polycomb repressive complex" (26C). Betalains are a group of pigments found in some plants responsible for the red, purple and yellow colors in various fruits and vegetables; betalains metabolism in plants is multifaceted contributing to plant UV protection, stress responses, cellular stability and defense against pathogens (Sadowska-Bartosz and Bartosz, 2021). Moreover, the Polycomb Repressive Complex (PRC) is a conserved group of protein complexes that plays a crucial role in the regulation of gene expression in plants. It is a key player in the epigenetic regulation of gene expression with significant implications for plant growth, development and responses to environmental stimuli (Godwin and Farrona, 2022; Baile *et al.,* 2022). The precise targets and functions of PRCs can vary in different plant species and under different conditions, highlighting their versatility in regulating diverse biological processes. These observations are in line with results of previous proteomic analysis, confirming the positive impact of this beneficial microbe on various physiological processes linked to plant growth and development.

4.2.1.7 *T. harzianum-B. bassiana* consortium + M. euphorbiae vs *T. harzianum* + *M. euphorbiae,* vs *B. bassiana* + *M. euphorbiae* and vs *M. euphorbiae*

Finally, to obtain a more comprehensive picture of the physiological events that regulate the *B. bassiana-T.harzianum-M. euphorbiae* interactions, the impact of this tripartite interaction compared to *T. harzianum* + *M. euphorbiae, B. bassiana* + *M. euphorbiae* and *M. euphorbiae* alone was further explored. The PCA plot indicated in figure 27A shows that samples related to *M. euphorbiae*, *B. bassiana* + *T.harzianum* + *M. euphorbiae* and *T. harzianum* + *M. euphorbiae* are clustered, while *B. bassiana* + *M. euphorbiae* and control samples have clear separation.

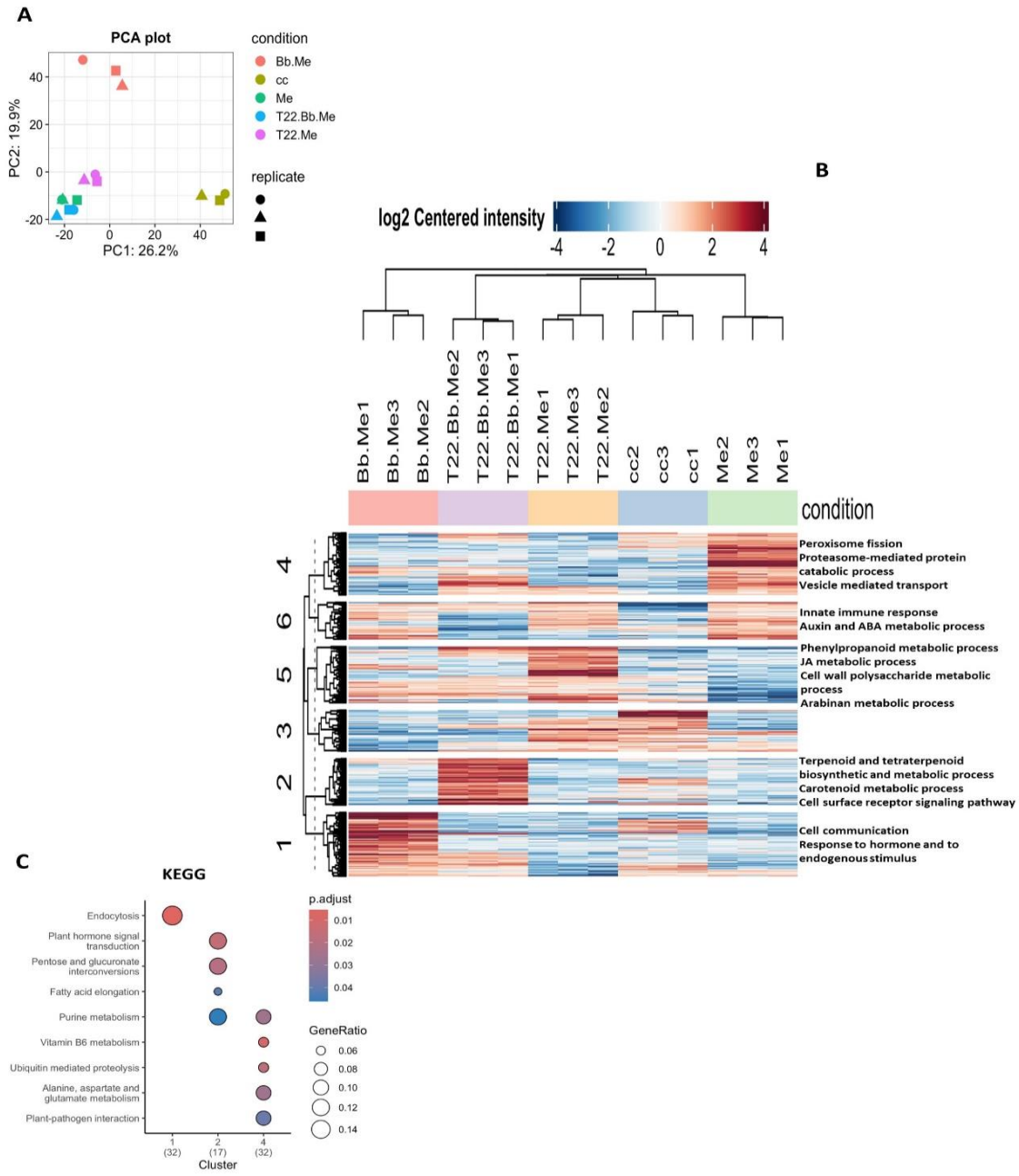

Figure 27. Comparisons between differentially expressed proteins induced respectively by *T. harzianum* (T22.Me) or *B. bassiana* (Bb.Me) and the effect of the combined treatment *T. harzianum* and *B. bassiana* (T22.Bb.Me) on plants infested by *M. euphorbiae* and *M. euphorbiae* alone (Me). In all cases, the differentially expressed proteins (DEPs) induced by these treatments are compared to the DEPs of untreated control plants (cc). A: Plot of principal component analysis (PCA) on log2-transformed quantification values of differentially expressed proteins (DEPs) in at least one treatment. Each treatment is represented by different colors, while replicates are denoted by distinct shapes. B: Heatmap plot of hierarchical clustering analysis displaying differentially expressed proteins clustered by expression patterns of the log2-transformed quantification values. Right annotations to each cluster show top enriched Gene Ontology (GO) biological processes. C: Dot plot representing enriched KEGG metabolic pathways within each cluster (displayed on the y-axis) identified by hierarchical clustering of DEPs. Each dot on the plot is colored on a blue-red scale based on the P value adjusted by the Benjamini-Hochberg method for controlling the false discovery rate (FDR) for multiple comparisons. The size of the dots corresponds to the gene ratio, indicating the proportion of proteins in the enriched pathway relative to the total number of proteins (genes) in that pathway. Larger dots signify a higher gene ratio and a more significant enrichment.

As demonstrated by the heatmap plot in fig. 27B, *B. bassiana* + *T.harzianum* + *M. euphorbiae* results in an enhancement of processes related to the synthesis of terpenoid defense compounds. This includes various aromatic compounds also known for their repellent properties against insects. In fact, several studies have highlighted that plant-emitted VOCs display attractive, repellent, masking or anti-feeding properties that may alter insect behavior (Dardouri *et al.,* 2021; Dieudonnè *et al.,* 2022). Plants attacked by an herbivore may modulate their metabolism and emit an olfactory mixture that repels the pest, attracts its natural enemies, and/or induces defense reactions in surrounding plants (Mofikoya *et al*., 2019; Dieudonnè *et al.,* 2022). Probably, this effect seems to be attributed to the *B. bassiana-T. harzianum* consortium, since in *B. bassiana* + *M. euphorbiae* and *T. harzianum* + *M. euphorbiae* treatments, these processes are downregulated. In fact, there is no rare evidence that beneficial microbes have the potential to upregulate the production of terpenoid compounds in plants (Su *et al.,* 2023). The interaction between plants and beneficial microbes can stimulate plant defense mechanisms and secondary metabolite production, including terpenoids. This phenomenon is often part of a mutualistic relationship where both the plant and the beneficial microbe benefit (Su *et al.,* 2023). Plant colonization with *B. bassiana* + *T. harzianum* consortium also appears to mitigate the stress caused by aphid, comparing *B. bassiana* + *T. harzianum* + *M. euphorbiae* treatment to the *M. euphorbiae* one. Aphid-induced stress leads to an increase in proteasome-dependent protein catabolism and peroxisome fission, organelles responsible for detoxifying ROS (Figure 27B). Furthermore, this stress condition activates processes involved in ABA and auxin metabolism, which are also expressed in *B. bassiana* + *M. euphorbiae* and *T. harzianum* + *M. euphorbiae* samples but decrease in *B. bassiana* + *T.harzianum* + *M. euphorbiae*, suggesting an enhancement of the defense mechanisms of the consortium compared to the single beneficial microbe treatment. Additionally, the triple treatment increases processes that result in the activation of “Cell surface receptor signalling pathway” that is involved in the recognition of an extracellular molecule leading to a sequence of molecular events that transmit the signal from outside to inside the cell, ultimately inducing a cellular response involved in plant development,

immune response and cell survival. Interestingly, KEGG pathway analysis (Fig. 27C) demonstrates the activation of the "Plant hormone signal transduction" pathway for the *B. bassiana* + *T.harzianum* + *M. euphorbiae*. In fact, during plant-microbe interactions, the modulation of the hormonal signaling pathways allow plants fine-tune their responses to different types of microbes (pathogens, symbiotic or beneficial microbes). The outcome is a dynamic and context-dependent integration of hormonal signals that contribute to the plant ability to perceive, respond to, and establish relationships with various microbes in its environment. As previously observed, *T. harzianum* significantly contributes to the activation of the phenylpropanoid pathway and to the metabolism of cell wall polysaccharides, while *B. bassiana* appears to be involved in hormone response and endogenous stimulation, in addition to its role in cell communication. Taken together, this data demonstrates that the contribution of the individual beneficial microbe is crucial for the plant in stress management, activating well-distinct pathways; nevertheless, *B. bassiana*-*T. harzianum* consortium appears even more crucial, seemingly acting in a synergistic way in dealing with biotic stresses.

4.2.2 *Beauveria bassiana* and *Trichoderma harzianum* trigger changes in the hormonal signaling network to balance plant growth and defense

The association between plants and beneficial microbes involves a complex interplay of hormonal signaling pathways, facilitating mutualistic relationships that benefit both the plant and the microbe. Here, we performed a leaf hormonomic analysis in the same samples in which we have performed the proteomic analysis, i.e. control plants, colonized with *B. bassiana* or with *T. harzianum* alone, colonized with the *B. bassiana*-*T. harzianum* consortium, infected with *B. cinerea* and infested with the *M. euphorbiae* and on plants pre-colonized with the individual beneficial microbes or with the consortium and subsequently subjected to stress by *B. cinerea* or *M. euphorbiae*. By using targeted HPLC-MS/MS we detected 56 hormones and related compounds involved in growth and defense, belonging to Cytokinins (CKs), Auxins (AUXs), Jasmonates (JAs), Salicylates (SAs) and Abscisates (ABAs)

families. Among the detected compounds 14 were significantly affected in the different treatment (Fig. 28), and therefore further discussed below.

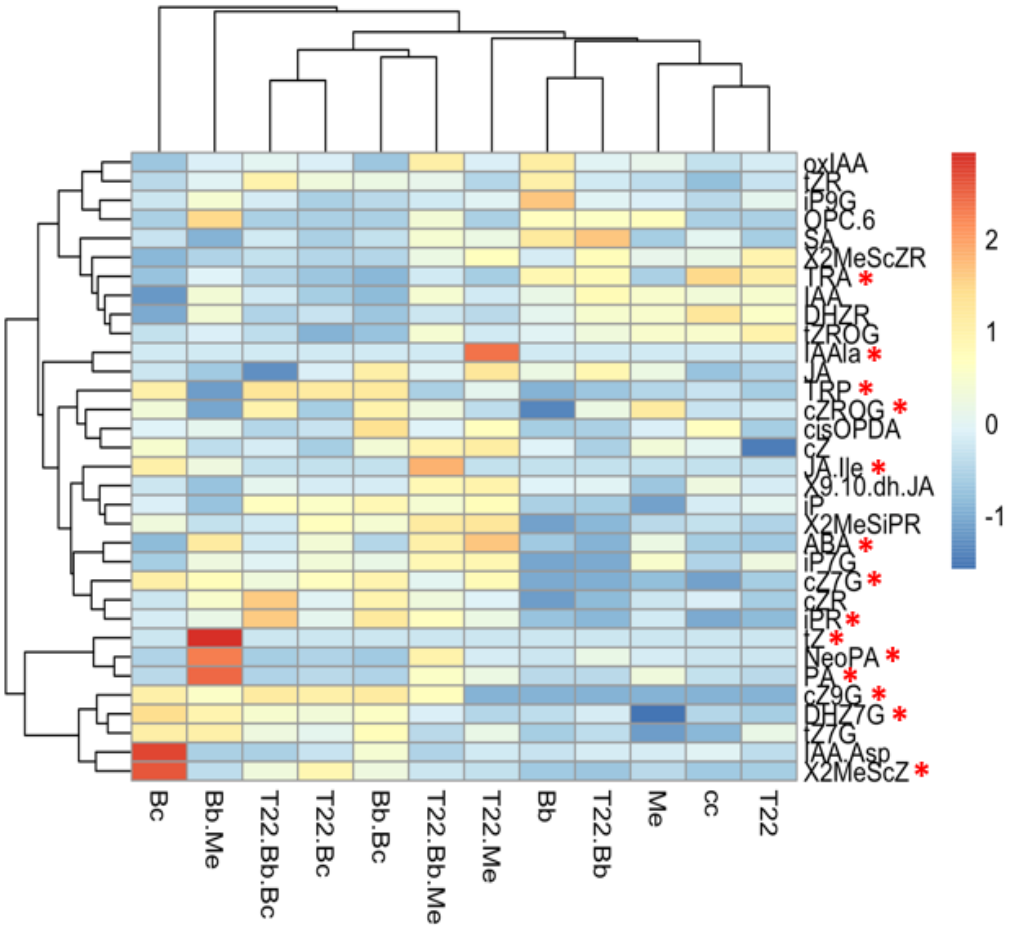

Figure 28. Heatmap depicting hierarchical clustering analysis of hormones and related compounds identified by HPLC-MS/MS across different treatments. The heatmap displays the mean of the centered scaled values of biological replicates for hormone quantitation, expressed in pmol/g of sample. Red asterisks next to hormone names serve as annotations, indicating differentially abundant hormones. The significance of abundance differences is determined by the Kruskall-Wallis test, and the P values are adjusted by the Benjamini-Hochberg (BH) method, controlling the false discovery rate for multiple comparisons.

CKs are adenine derivatives that act as phytohormones, have roles in a variety of developmental processes and include different compounds found naturally in plants. In Arabidopsis, these abundant family is divided into four classes: trans-Zeatin (tZ), isopentenyladenine (iP), cis-Zeatin (cZ), and dihydrozeatin (DZ). In addition, each CK class has several forms, such as a ribophosphate, riboside, base, O-glucoside and N-glucoside forms with diverse functions. In

general, the ribophosphate form is a precursor of bioactive CKs, the riboside form is a transported form with some bioactivity, the base form is the active canonical form, the O-glucoside form acts as an inactive but convertible storage form, and N-glucosides have historically been referred to as permanently inactive and irreversibly conjugated to a glucose molecule, although some exceptions exist (Hallmark *et al.,* 2020). 2MeS-CKs are iP- or Z-type CKs modified by the addition of a thiol group (–SH) at position 2 of the adenine ring and that is subsequent methylated. They are compounds with unknown biological activity and functions, especially in plants. In our work, among CKs, cZ7G accumulates in *B. cinerea* infected plants while in plants pre-colonized with the consortium and then infected with *B. cinerea* these hormones decrease. In contrast, iPR is downregulated in *B. cinerea* while its expression increases in *B. bassiana* + *B. cinerea* and in *B. bassiana* + *T. harzianum* + *B. cinerea*. It would appear that CKs are differently modulated in response to the necrotrophic pathogen. In particular, in *Arabidopsis thaliana* cZ and iP accumulated considerably in infected leaves, suggesting an important role in the plant response to *B. cinerea* (Li *et al.*, 2021). Recently, Gupta *et al.* (2020) revealed how CK promotes tomato resistance to *B. cinerea* through SA and ET dependent mechanism. Moreover, it has been recognized that CKs are hormones produced not only by the plant but also by beneficial microbes to induce resistance in plants against pathogen infections (Akhtar *et al.,* 2019). Therefore, this last aspect could suggest a synergistic effect in counteracting the pathogen invasion in plant pre-colonized with a beneficial consortium and then infected with the necrotrophic pathogen. According to Morrison *et al.* (2015), other CKs may increase after plant-fungus interaction as the 2-methylthio cytokinins. The and the accumulation of two of this hormones family, 2-Methylthio-Zeatin riboside (2MeScZR) and 2-Methylthio-Zeatin (2MeSZ), during pathogen invasion could suggest their involved in promoting tissue proliferation around the site of infection. Another CK statistically affected is transZeatin (tZ) that has almost the same expression in all samples except for *B. bassiana* + *M. euphorbiae*. It has been reorted that tZ is one of the hormones that decreases in pea plant infested with the specialist pea aphid as a consequence of the suppression of plant defenses exerted by the insect (Swiegers *et al.,* 2022). Therefore, in our study the

upregulation of this hormone could be due to the positive effect of *B. bassiana*. Moreover, in response to *B. cinerea*, also Tryptophan (TRP) is perturbated. Tryptophan metabolism is a crucial biochemical pathway in plants that involves the conversion of tryptophan, an essential amino acid, into various metabolites. Tryptophan serves as a precursor for the biosynthesis of auxins, a class of phytohormones that regulate various aspects of plant growth and development. Indole-3-acetic acid (IAA) is the primary auxin derived from tryptophan. In our work, this amino acid is upregulated in response to *B. cinerea, T. harzianum* + *B. cinerea, B. bassiana* + *B. cinerea* and in *T. harzianum* + *B. bassiana* + *B. cinerea.* Rowe *et al.,* 2010 reported that tryptophan levels were upregulated in response to *B. cinerea,* participating to camalexin biosynthesis in planta. Camalexin is a specific secondary metabolite derived from tryptophan in plants, especially those of the Brassicaceae family. It is known for its antimicrobial properties and is produced in response to pathogenic infections. This evidence could explain its upregulation in response to *B. cinerea*, leading to the hypothesis that the beneficial microbes can activate this defense process. As shown in figure 28, treatment with *T. harzianum* + *B. bassiana* consortium also increases levels of JA-Ile in response to *M. euphorbiae,* revealing a further contribution of this beneficial microbes in the fight against this aphid. Finally, among other hormone families upregulated in our data set are the abscisates. Noteworthy, phaseic acid (PA) and neophaseic acid (neoPA), which are part of the regulatory mechanisms controlling ABA levels in plants contributing to its dynamic regulation in response to stress, are over produced in *B. bassiana* + *M. euphorbiae* and although less aboundant in *T. harzianum* + *B. bassiana* + *M. euphorbiae,* compared to *M. euphorbiae*. PA and neoPA are two important catabolites derived from ABA hydroxylation which is an important pathway for ABA inactivation and homeostasis maintenance. This aspect could suggest a role of the entomopathogen *B. bassiana* not only in the activation of the ABA response but also in maintaining its homeostasis. However, we found higher ABA levels and lower levels of PA and neoPA in *T. harzianum* + *M. euphorbiae* compared to *B. bassiana* + *M. euphorbiae* and *M. euphorbiae* samples. This is not surprising since it has been reported in the literature that *T. harzianum* stimulates a significant increase in ABA levels in

tomato leaves (Coppola *et al.,* 2019), promoting tomato defense against *M. euphorbiae*.

5 Conclusion and future perspective

In-dept understanding of the basic mechanisms controlling plant responses to beneficial microbes offers the opportunity to enhance and/or modulate their effects, such as biofertilization and/or the induction of natural defenses. Knowledge of these mechanisms is still incomplete, despite their importance for improving agriculture towards eco-compatibility and safeguarding consumer health. In this regard, the present research activity aimed to identify the molecular basis of plant response to beneficial fungi of the genus *B. bassiana* and *T. harzianum*, in terms of growth promotion and activation of natural defenses against biotic stresses.

In conclusion, the obtained novel results demonstrated:

- the ability of *B. bassiana* to reprogram plant proteome, affecting pathways related to primary and secondary metabolism and to plant growth and development;
- the potential of *B. bassiana* to influence plant hormones leading to the upregulation of growth- and defense-related hormones like GAs, BA and JA;
- the effect of *B. bassiana* in plants protection against *B. cinerea*-induced oxidative stress, probably regulating the plant antioxidant machinery;
- the involvement of *B. bassiana* and *T. harzianum* in the activation of different strategies in response to *B. cinerea* and *M. euphorbiae*; in particular the contribution of *T. harzianum* in activating the phenylpropanoid pathway and the cell wall polysaccharide metabolism and the involvement of *B. bassiana* in hormone response, endogenous stimulation and cell communication;
- the synergistic cooperation of these strategies in the *B. bassiana*-*T. harzianum* consortium with a common objective such as strengthening defense responses and support for plant growth.

Wrapping up, the results presented in this thesis are innovative as they fill a gap in the literature regarding the knowledge on plant molecular mechanisms modulated by the two beneficial microorganisms (*B. bassiana and T. harzianum*), used both individually and in consortium, on plant growth, and defense strategies. These results obtained pave the way for further studies

aimed at understanding the increasing importance of these beneficial microbes that can help build resilient agroecosystems and mitigate the environmental impact of traditional agricultural practices.

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