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EFFECT OF METABOLITES PRODUCED BY BENEFICIAL FUNGI ON THE PLANT METABOLOME, PHYSIOLOGY AND AGRONOMIC PERFORMANCE

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1. Introduction

It has been estimated that global agricultural production is annually reduced by 31-42% due to plant pathogens and pests (Agrios, 2008). Currently, the use of chemical pesticides is the most common method to protect crops from pathogens, but these products may have negative effects on both the environment and consumers. Indeed, synthetic pesticides pollute the atmosphere, damage the environment, leave harmful residues, and can lead to the development of resistant strains of target pathogens (Naseby et al., 2000). A reduction or elimination of synthetic pesticide applications in agriculture is highly desirable. One of the most promising means to achieve this goal is the use of biocontrol agents (BCAs), or the integration of BCAs with reduced doses of chemicals for the control of plant pathogens (Chet and Inbar, 1994; Harman and Kubicek, 1998). The most applied BCAs are microbial antagonists of important plant pathogens, including bacteria (such as *Bacillus*, *Pseudomonas* and *Enterobacter*), numerous yeasts (such as *Pichia guillermondii*, *Candida sake*, *C. pulcherrima*, *Cryptococcus laurentii* and *C. flavus*), and fungi (including *Acremonium breve*, *Trichoderma* spp. and *Gliocladium* spp.). The use of BCAs provides numerous benefits compared to other methods, such as the control of pesticide-resistant pathogens, the absence of toxic effects on crops, reduced health risks for farmers, no impact on beneficial fauna, etc. However, possible disadvantages related to the use of BCAs are the specificity towards the microbial target and the difficulty in developing effective formulations. Furthermore, abiotic and biotic factors like weather, pressure and competition of the indigenous microflora may reduce the performances of biocontrol agents.

Various strains of the filamentous genera *Trichoderma* spp. are among the most successfully applied biocontrol agents in the world. These fungi show enormous potentials also in different industrial applications (enzyme production, bioremediation, etc.). Recently, several studies have analysed the interactions between *Trichoderma* spp., crop plants, phytopathogens and soil community, thus improving our

understanding on the mechanisms and molecular factors involved (Lu et al., 2004; Marra et al., 2006; Woo et al., 2006).

1.1. Plant defences against pathogens

Plants have developed a complex system of defence mechanisms based on structural and biochemical defences to protect themselves from pathogens. Structural defences act as physical barriers that inhibit the pathogen from gaining entrance and spreading through the plant, while biochemical defences consist in producing substances that are either toxic to the pathogen or create conditions that inhibit the pathogen growth.

The plant surface is the first line of defence against pathogens. They must adhere and penetrate it to cause infection. Some structural defences are present in the plant even before the pathogen comes in contact with the plant, for instance, the wax and cuticle that cover the epidermal cells, the structure of the epidermal cell walls, the size, location and shapes of stomata and lenticels, and the presence of tissues made of thick-walled cells that hinder the advance of the pathogen into the plant.

Although structural characteristics may tool up the plant against attacking pathogens, it is clear that the resistance depends not so much on its structural barriers as on the substances produced in its cells before or after infection.

Constitutive biochemical defences as fungitoxic exudates and substances with antifungal activity (such as phenolic compounds, caffeic acid and catechol), glycosides (saponins, phenolic glycosides) and glucosinolates) prevent pathogen penetration into the plant.

Also after infection, the plants are able to produce defence-related substances, such as phytoalexin, ROS (Reactive Oxygen Species), PR proteins (Pathogenesis-Related proteins), etc.

After pathogen attack, plants quickly generate ROS that are chemically reactive molecules containing oxygen (i.e. oxygen ions and peroxides). ROS are natural product of aerobic metabolism and have important roles in plant cell signalling and homeostasis, controlling processes such as growth, development, response to biotic and abiotic

environmental stimuli, activation of the Hypersensitive Reaction (HR) and the Induced Systemic Resistance (ISR).

The production of PR proteins in plants is generally the result of biotic and abiotic stresses. These proteins include antifungal chitinases, glucanases, thaumatins, and oxidative enzymes, such as peroxidases, polyphenol oxidases and lipoxygenases. The transgenic expression of one or more constitutive PR proteins determined an increased resistance in plant (Broglie *et al*., 1991; Zhu *et al.*, 1994; Guido *et al.*, 1995), thus confirming their involvement in ISR.

Low-molecular-weight compounds with antimicrobial properties called phytoalexins can also accumulate in plants after pathogen attack. Phytoalexins are synthesized *ex novo* by plants and accumulate rapidly around the area of pathogen infection. They include chemically different compounds, such as terpenoids, glycosteroids and alkaloids, which display a broad spectrum of inhibitory activity.

A common feature of the plant defense system is the hypersensitive response (HR) that is characterized by the rapid death of cells at the site of infection, thus inhibiting the pathogen growth. As a result of this localized response, the whole plant develops a systemic acquired resistance (SAR) against subsequent infection by the same or other pathogens.

The type and the effects of defence mechanisms that plants use to contrast pathogen attacks may vary according to the specific host-pathogen combination, the age of plant, the organ or tissue attacked, the nutritional status of the plant, as well as the environmental conditions.

1.2. The plant immune system

The plant immune system is based on the innate immunity of each cell and on systemic signals produced at the sites of infection (Jones and Dangl, 2006).

Resistance (R) proteins in plants can be activated indirectly by effectors, which are molecules produced by pathogenic organisms that contribute to their virulence. Plant R proteins are able to recognize these effectors indirectly by monitoring the integrity of host cellular targets and the action of these effectors. There are, essentially, two branches of the plant immune system. The first exploits the presence of transmembrane receptors (PRRs, Pattern Recognition Receptors) and microbe/pathogen-associated molecular patterns (MAMPs/PAMPs), such as the flagellin (Zipfel and Felix, 2005). The second acts primarily within the cell, using polymorphic protein compounds (i.e. proteins containing NB-LRR,Nucleotide Binding Leucine Rich Repeat domain) encoded by R genes (Dangl and Jones, 2001). Interestingly, effectors produced by pathogens from diverse kingdoms are recognized by NB-LRR proteins, and activate similar defence responses. Disease resistance mediated by NB-LRR proteins is effective against pathogens that can live only on living tissue of the host (biotrophs) but not against pathogens that kill the host tissue during colonization (necrotrophs).

The zigzag model (Figure 1.1) described by Jones and Dangl (2006) explains the evolution of plant immune system. During the first phase, microbe/pathogen-associated molecular pattern (MAMP/PAMP)-triggered immunity (MTI/PTI) is invoked by recognition of conserved molecular patterns endemic to the invading pathogen that activate host basal defense responses. In the second phase, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). During the third phase, the effector-triggered immunity (ETI) is subsequently engaged as a host response to suppression of its basic defences. ETI is predicated upon recognition of one or more pathogen-derived ‘effectors’. This type of response is more intense than PTI’s and determines the triggering of the HR. In the phase four, natural selection drives pathogens to avoid ETI either by shedding or diversifying the recognized effector gene, or by acquiring

additional effectors that suppress ETI. Natural selection results in new R specificities so that ETI can be triggered again.

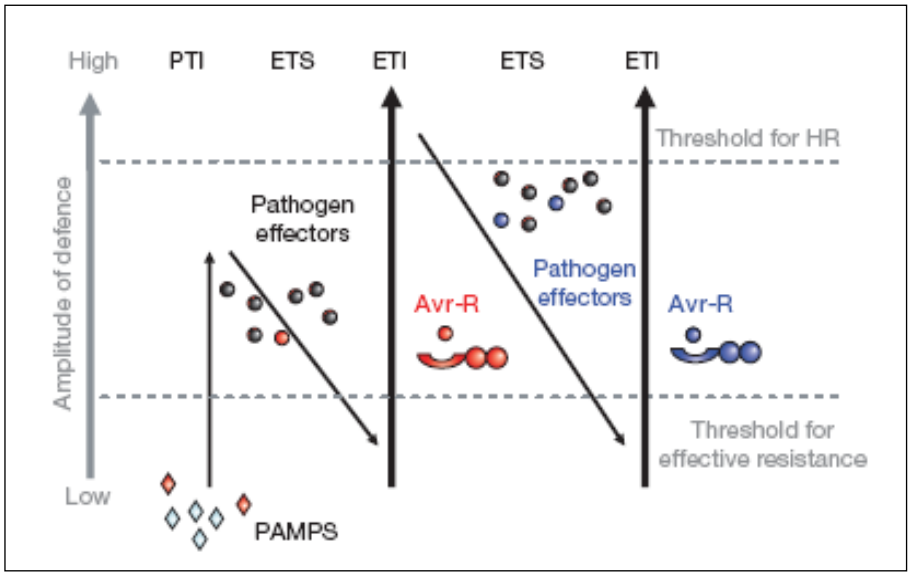

Figure 1.1 A zigzag model illustrates the quantitative output of the plant immune system. In this scheme, the ultimate amplitude of disease resistance or susceptibility is proportional to [PTI – ETS1ETI]. In phase 1, plants detect microbial/pathogen-associated molecular patterns (MAMPs/ PAMPs, red diamonds) via PRRs to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue)—these can help pathogens to suppress ETI. Selection favours new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI. (Jones and Dangl; 2006).

1.3. Biological control

Actually plant diseases caused by fungi, bacteria, viruses and nematodes are mainly controlled by using synthetic pesticides. However the prolonged use of chemicals selects resistant strains and species among pathogens; this may cause new outbreaks of the former "controlled/eliminated" disease. Today costumers ask for agricultural products that have to be healthy, safe and environmental friendly, that means with no chemical residues and having low impact on men and environment. Therefore, the interest of a growing number of agricultural industries turned for biologically and environmentally acceptable alternative methods of disease control, such as biological control or biocontrol.

Biological control is a method to control pest populations by using natural enemies and typically involves an active human role. Biological control methods include:

- control of pathogen populations through actions on soil and environment;
- exploitation of the host plant resistance;
- control of the infection by using microorganisms with antagonistic activity(Gabriel and Cook, 1991).

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*.

In addition to being involved in the processes of decomposition of organic matter, the removal of toxic substances and participation in the nutrient cycle, these organisms are able to suppress plant diseases caused by soil-borne pathogens and to stimulate plant growth (Kubicek and Harman, 1998).

Bacilli: bacteria belonging to the genus *Bacillus* are Gram-positive, rod-shaped, aerobic or facultative anaerobes. They are ubiquitous but the soil is considered their habitat. The bacilli are able to adapt and live in environments characterized by extreme conditions of pH, temperature and salinity; they can behave as saprophytes degrading living and non-living organic matter. Bacilli are often antagonistic against other microorganisms (Table

1) through the production of metabolites, antibiotics.These BCA may be also pathogenic for animals and insects (Whipps, 2001).

B. subtilis, B. mycoides and *B. cereus* produce several antibiotics including polymyxins, difficidin, subtilisin, etc., which are active against both bacteria and fungi.

Table 1. Antagonistic bacteria used as biological control agents.

ANTAGONISTIC BACTERIA	PATHOGEN	PROTECTED PLANT
***Bacillus*spp.**	*G. graminis*var. *tritici*, *R. solani*	Wheat
B. subtilis	*F. oxysporum* f. sp. *ciceris*	chickpea
P. aureofaciens	*P. ultimum*	tomato
***A. radiobacter*K84**	*A. tumefaciens*	Fruit trees, ornamental plant
S. plymuthica	*P. ultimum*	cucumber

Actinomycetes: are Gram-positive bacteria. They have similar morphology to that of filamentous fungi. They show different types of metabolism, both aerobic and anaerobic. The actinomycetes are known to produce secondary metabolites biologically active (with antibacterial and antifungal activity). The most important are: actinomycin, streptomycin, tetracycline, kanamycin and antifungal substances such as candicidin and nystatin (Hornby, 1990).

In addition, the actinomycetes produce substances that may inhibit or promote the growth of other microorganisms, such as vitamins, hormones and siderophores. When colonize the rhizosphere, they can behave both as antagonists against other microorganisms, as well as promoters of plant growth.

Pseudomonads: many strains of *Pseudomonas* spp. are antagonist against phytopathogenic agents and increase the resistance in plant. Some *Pseudomonas* strains isolated from suppressive soil produce antibiotic compounds (Bloem et al., 2005) such as 2,4-diacetilfloroglucinolo, a metabolite with antifungal activity. Furthermore, *P.*

fluorescens, P. chlororaphis, P. aureofaciens and *P. syringae* are identified as biocontrol agents against both bacteria and phytopathogenic fungi.

Agrobacteria are both pathogenic species (*A. tumefaciens*), and non-pathogenic species (*A. radiobacter*). *A. tumefaciens* cause the crown gall (the formation of tumors) in over 140 species of dicotyledons, a disease spread around the world causing serious problems, especially in nurseries of fruit trees and ornamental plants, making the plants infectedunmarketable. All chemical or physical methods applied to control the disease are proved to be unsatisfactory. The best results are obtained with biological control, using the non-pathogenic K84 strain of *A. radiobacter*. This produce sagrocyn 84 that is an antibiotic specific to control pathogenic strains of *A. tumefaciens*.

Fungi: Many fungi live only into the soil, where they colonize plant tissue fragments and interact with plant roots, other fungi, bacteria or soil community (Kubicek and Harman, 1998). Fungi are able to grow and spread in the soil through the formation of hyphae. The interaction that these microorganisms develop with plants and the microbial community can be different. Fungi can be obligate parasites, if they need an association with the plant for the duration of their life cycle, or not obligate parasites, if they need plant for only part of their life cycle, while during the rest of their life are saprophytes. Several species of *Trichoderma* can be used as biocontrol agents against many plant pathogens (Harman et al., 2004a; Woo et al., 2006; Benitez et al., 2004; Vinale et al., 2008).

1.3.1. Mechanisms of action of biocontrol agents

Many fungal and bacterial antagonistic microorganisms control different plant diseases and promote plant development. The biocontrol activity against phytopathogens can be expressed through different mechanisms of action: parasitism, antibiosis, competition, the induction of plant resistance and the plant growth promotion (PGP). (Benitez et al., 2004; Harman et al., 2004).

Parasitism is a interaction between the antagonist and the pathogen. The antagonist establishes an intimate association with the pathogen. This mechanism involves a phase of physical contact with the host. This interaction is called mycoparasitism when both partners are fungi. Mycoparasites produce cell wall-degrading enzymes (CWDEs), including endochitinases, β-N-acetylhexosaminidases (N-acetyl-β-D-glucosaminidases), chitin-1,4-β-chitobiosidases, proteases, endo- and exo-β-1,3-glucanases, endoβ-1,6-glucanases, lipases, xylanases, mannanases, pectinases, pectin lyases, amylases, phospholipases, RNAses, DNAses, etc. (Lorito, 1998).CWDEs allow mycoparasites to penetrate into other fungi and extract nutrients for their own growth (Inbar and Chet, 1992). However, many so-called mycoparasites produce also antibiotics which may first weaken the fungus they parasitize.Among the examples of parasitic fungi there are: *Trichoderma* spp., which are able to attack many different pathogenic fungi (Chet, 1987), *Sporidesmium sclerotivorum*, which parasitizes the sclerotia of *Sclerotinia minor* (Fravel et al., 1992), and *Verticillium biguttatum* that attacks *R. solani* (Van den Boogert et al., 1990).

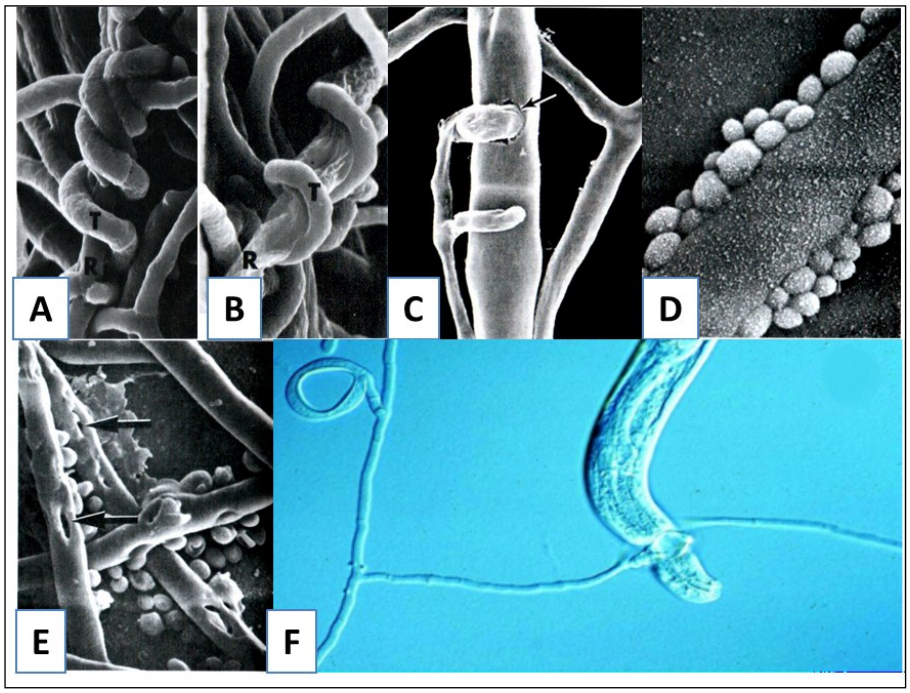

Figure 1.2 Electron microscope images of some examples of parasitism [A] Effect of parasitization of *Trichoderma harzianum* (T) on *Rhizoctonia solani* (R) after 2 days and [B] after 6 days; [C] hypha of *Pythium* which penetrates a hypha of *Phytophthora*; [D] the yeast *Pichia*

***guilliermondi* on a hypha of *Botrytis* and [E] on a hypha of *Penicillium;* [F] the fungus *Arthrobotrys dactyloides* while traps a nematode (photoes by Agrios, 1998).**

Antibiosis consists in the production by antagonistic fungi and bacteria of metabolites that inhibit the growth and development of pathogenic microorganisms. The most important antibiotic producers are: fluorescent *Pseudomonas*, bacteria able to produce phenazines, which were the first antibiotics to be clearly implicated in biocontrol activity. The fungal antibiotics, gliovirin and gliotoxin produced by different strains of *T. virens* (Bisset, 1991), are very important.

Competition. Microorganisms compete with each other for space and nutrients (such as: carbon, nitrogen, oxygen and iron). Nutrient competition is likely to be the most common way by which one organism limits the growth of another. Some fungi and bacteria produce molecules called siderophores which are efficient in chelating Fe^{3+}. Individual strains can have their own particular siderophores and receptors which can bind Fe^{3+} in such a way that the iron becomes inaccessible to other microorganisms, including pathogens. Siderophores production appears to be important to the survival of microorganisms through elimination of microbial competition for nutrient sources, which are usually very limited in soil (Velusamy et al., 2006). In some cases, siderophore production and competitive success in acquiring Fe^{3+} is the mechanism by which biocontrol agents control plant diseases (Benitez et al., 2004).

Induction of plant resistance. Among the biocontrol mechanisms, the induction of plant resistance has received considerable attention in the last few decades. Kloepper et al. (1992) defined the induced disease resistance as 'the process of active resistance dependent on physical or chemical barriers of the host plants, activated by biotic or abiotic agents (inducing agents)'.

The induction of plant defence responses mediated by the antagonistic fungus *Trichoderma* spp. has been well documented (De Meyer et al., 1998; Yedidia et al., 1999; Hanson and Howell, 2004; Harman et al., 2004). Various plants, both mono- and dicotyledonous, showed increased resistance to pathogen attack when pre-treated with *Trichoderma* spp. (Harman et al., 2004). Plant colonization by *Trichoderma* spp.

reduced disease symptoms caused by one or more different pathogens, both at the site of inoculation (induced localized acquired resistance, LAR), as well as when the biocontrol fungus was inoculated at different times or sites than the pathogen (induced systemic resistance or ISR). The induction of plant resistance by colonization with some *Trichoderma* species is similar to that elicited by rhizobacteria, which enhance the defence system but do not involve the production of pathogenesis-related proteins (PR proteins) (Van Loon et al., 1998; Stacey and Keen, 1999; Harman et al., 2004). The major differences are that PR proteins, such as chitinases, β-1,3glucanases, proteinase inhibitors and one or two other rarer types, have not been universally associated with bacterially induced resistance (Hoffland et al., 1995), and the salicylic acid (a known inducer of SAR) is not always involved in the expression of ISR, but this is dependent by the bacterial strain - host plant combination (Pieterse et al., 1996; de Meyer et al., 1999; Chen et al., 1999). Moreover, the ISR mediated by bacteria may also require ethylene responsiveness at the site of inoculation (Knoester et al., 1999).

Changes that have been observed in plant roots exhibiting ISR include: (1) strengthening of epidermal and cortical cell walls and deposition of newly formed barriers beyond infection sites, including callose, lignin and phenolics (Duijff et al., 1997; Jetiyanon et al., 1997); (2) increased levels of enzymes such as chitinase, peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase (Chen et al., 2000); (3) enhanced phytoalexin production (van Peer et al., 1991; Ongena et al., 1999); (4) enhanced expression of stress-related genes (Timmusk and Wagner, 1999).

In a recent work Alfano et al. (2007) investigated at a molecular level the plant genes involved in induction of resistance mechanisms by using a high-density oligonucleotide microarray approach. Interestingly, *Trichoderma*-induced genes were associated with biotic or abiotic stresses, as well as RNA, DNA, and protein metabolism. In particular, genes that codify for extensin and extensin-like protein were found to be induced by the BCA, but not those codifying for proteins belonging to the PR-5 family (thaumatin-like proteins), which are considered to be the main molecular markers of SAR.

Moreover, an ISR effect was also induced by the formation of mycorrhizae that are a symbiotic and mutual association between a non-pathogenic or weakly pathogenic fungus and the living cells of the plant root (Agrios, 1998). The induction of resistance

in plant could be due probably to an early conditioning of the host, called "priming", which activates the tissues making them more willing to respond to the attack of a pathogen. In fact, the colonization of the roots by mycorrhizal fungi is able to protect the tomato plants from infection of *Phytophthora parasitica* and promote the accumulation of phytoalexin, riscitin and solavetivone in potato seedlings infected by *Rhizoctonia* (Yao et al., 2003).

Plant growth promotion. Many saprotrophic fungi, particularly certain isolates of *Trichoderma* species, can promote plant growth (Whipps, 1997; Inbar et al., 1994). For example, *Trichoderma harzianum* 1295–27 was able to solubilize phosphate and micronutrients making them available for the plant and thus supporting its growth (Altomare et al., 1999). In addition several fungal biocontrol agents, including some *Trichoderma* species, binucleate *Rhizoctonia* isolates and *Pythium oligandrum*, can stimulate plant growth in the absence of pathogens (Chang et al., 1986; Windham et al., 1986; Shivanna et al., 1996; Wulff et al., 1998; Harris, 1999). Furthermore, the ability to colonize seed or root surface or the endophytes attitude have been frequently considered highly desirable traits for biocontrol agents (Kleifeld and Chet, 1992; Harman and Bjôrkman, 1998). The relationship between rhizosphere colonization and biocontrol activity has been clearly demonstrated in numerous biocontrol fungi such as *Trichoderma* species, non-pathogenic Fusaria, *P. oligandrum*, *Verticillium biguttatum*, and *Talaromyces flavus* (Ahmad and Baker, 1988; Couteadieret al., 1993; van den Boogert and Velvis, 1992; Al-Rawahi and Hancock, 1997; Lo et al., 1996; Tjamos and Fravel, 1997; Nagtzaam and Bollen, 1997; Björkmanet al., 1998).

Among the microorganisms that establish beneficial interactions with the plant there are PGPR (Plant-Growth-Promoting Rhizobacteria), or beneficial bacteria, not symbionts that inhabit the rhizosphere. This term is commonly referred to bacteria belonging to the genera *Pseudomonas, Serratia, Bacillus* and *Azospirillum*. PGPR were classified, according to the beneficial effect that determine the plant, in two groups: those involved in the metabolism of nutrients (bio-fertilizers and phytostimulants) and biocontrol agents of plant pathogens (Bashan and Holguin,1998). PGPR-acting as biofertilisers are able to fix nitrogen making it usable by the plant and thus causing an increase in growth

even when the quantities of nitrogen in the soil are scarce. They are also responsible for the increased availability of nutrients in the soil (particularly phosphate); many rhizobacteria and rhizofungi, in fact, solubilize poorly soluble phosphates by the release of chelating organic acids (Vessey, 2003). The rhizobacteria phytostimulants, represented primarily by members of the genus *Azotobacter* and *Azospirillum*, promote directly plant growth through the production of phytohormones (auxins,cytokinins, gibberellins) rather than through the activity of nitrogen-fixation

The biocontrol activity shown by some PGPR against soil-borne pathogens is due to mechanisms that determine a reduction of the saprophytic growth of pathogens and the frequency of infection, competition for nutrients, colonization of habitats, stimulation of the systemic resistance (ISR) in the host plant and/or production of antifungal metabolites. The rhizobacteria biocontrol agents better characterized belong to the genus *Pseudomonas*, the majority of which produces metabolites toxic, including phenazine, pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG), pyoluteorin and cyclic lipopeptides (Haas and Keel, 2003). The synergy between the action of lipodepsipeptides of *P. syringae* pv. *syringae* and lytic enzymes (CWDEs) of the antagonistic fungus *Trichoderma atroviride* strain P1 can play a key role in the antagonism of the rhizobacterium, supporting the hypothesis that a more effective control of the disease is obtained by using a combination of several biocontrol agents (Fogliano et al., 2002; Woo et al., 2002).

1.4. *Trichoderma*

Trichoderma spp. are filamentous fungi commonly found in the soil community that are facultative saprophytes. They belong to a group of largely asexually reproducing fungi that includes a wide spectrum of micromycetes ranging from very effective soil colonizers with high biodegradation potential to facultative plant symbionts that colonize the rhizosphere. Many strains of *Trichoderma* have not been associated with a sexual state and are believed to be mitotic and clonal. Species of *Hypocrea* and closely related genera in the *Hypocreales* have anamorphs referable to *Trichoderma* (Gams and Bissett, 1998).

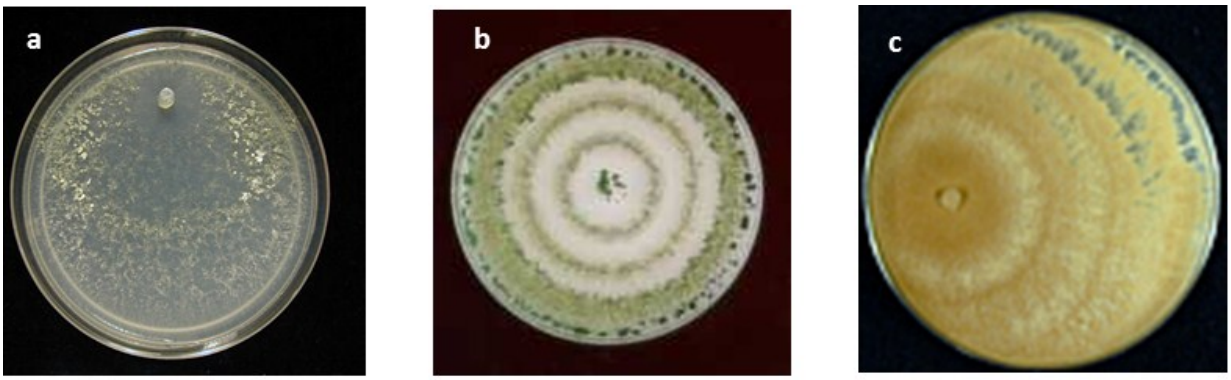

Figure 1.3 Examples of *Trichoderma* cultures grown in *Petri* dishes.(a) *T. atroviride*; (b) *T. viride*; (c) *T. harzianum*.

Trichoderma species use the competition for nutrients and/or space, the antibiosis and/or the mycoparasitism to control different phytopathogens. In addition, new mechanisms have been found in some species and strains of *Trichoderma*, such as: the inactivation of the enzymes of the pathogen, the detoxification of antibiotic substances or antimicrobial compounds produced and released by the fungus host and/or by the microflora of the soil, or in an indirect stimulation of defense mechanisms of the host plant (Benítez et al., 2004; Elad, 2003; Harman etal., 2004).

Trichoderma colonizes the root plant protecting by penetration of other pathogens (Elad et al., 1999; Elad and Kapat, 1999; Yedidia et al., 1999). Therefore, *Trichoderma* is avirulent having developed a relationship with plants of a symbiotic more than parasitic nature (Harman et al., 2004).

1.4.1. *Trichoderma*-plant interaction

Some *Trichoderma* species are able to colonize root surfaces and cause substantial changes in plant metabolism (Harman et al., 2004). The main effects of this beneficial interaction are:

- promotion of plant growth;

- increased nutrient availability;
- induction of disease resistance

Numerous experiments showed that crop productivity increased up to 300% after treatments with *Trichoderma* spp. and promotion of plant growth was clearly detectable on different plant species (examples in Figure 1.4 and 1.5).

Figure 1.4 Pepper plants treated with *Trichoderma* spp.

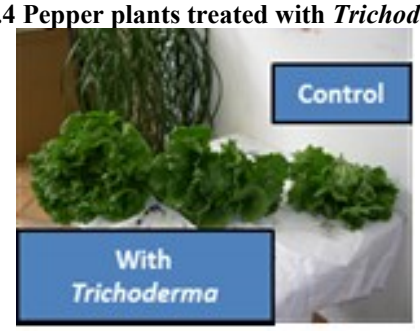

Figure 1.5 Lettuce plants treated with *Trichoderma* spp.

A yield increase was also observed when plant seeds were exposed to *Trichoderma* conidia that were separated from them by cellophane, suggesting that *Trichoderma* metabolites can affect the plant growth (Benıtez et al., 2004).

Trichoderma spp. produce organic acids, such as gluconic, citric and fumaric acids, which decrease soil pH and allow the solubilisation of phosphates, micronutrients and mineral cations (like iron, manganese and magnesium), useful for plant metabolism, especially in neutral or alkaline soils (Benitez et al., 2004).

Iron is an essential nutrient due to its required metabolic function. As a transition metal, its redox properties allow it to exist in two oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}) for the donation and acceptance of electrons, respectively. Therefore, sufficient

iron supply is a necessity for survival. Although iron is one of the most abundant elements on earth, bioavailability is low in aerobic environments (in the presence of oxygen and at neutral pH), primarily because ferric iron reacts with oxygen to form insoluble ferric hydroxides. To maintain iron homeostasis regulated strategies for the careful control of iron uptake, utilization, and storage have evolved in different organisms (Expert, 2009).

Some antagonistic microorganisms react to limiting iron conditions by using a high-affinity iron uptake system based on the release of Fe^{3+}-chelating molecules, called siderophores. Although siderophores have an important function in many phytopathogens, their production by microorganisms can be beneficial to plants for two reasons: i) siderophore formation can solubilize iron unavailable for the plant (Prabhu et al. 1996); ii) siderophore production by non-pathogenic microorganisms can also suppress growth of pathogenic microorganisms by depriving the pathogens of iron (Leong J., 1986).

A primary method of pathogen control occurs through the ability of *Trichoderma* to reprogram plant gene expression. They can also induce systemic and localized resistance to a variety of plant pathogens.

The ISR effect, triggered by different strains of *Trichoderma,* determine in plant the production of defense metabolites, such as enzymes involved in the biosynthesis of phytoalexins, or compounds related to the oxidative stress, or even PR-proteins. *Trichoderma* strains produce different classes of compounds able to induce resistance in plants, including:

- proteins with enzymatic or other functions,
- homologues of proteins encoded by the avirulence (*Avr*) genes,
- oligosaccharides and other low-molecular-weight compounds that are released from fungal or plant cell walls by the activity of *Trichoderma* enzymes,
- secondary metabolites (peptaibols, pyrons, etc.)

Trichoderma may affect plant defense against pathogen attack by increasing the immunity activated by MAMPs (MTI) and reducing the susceptibility triggered by effectors (ETS) (Figure 1.6).

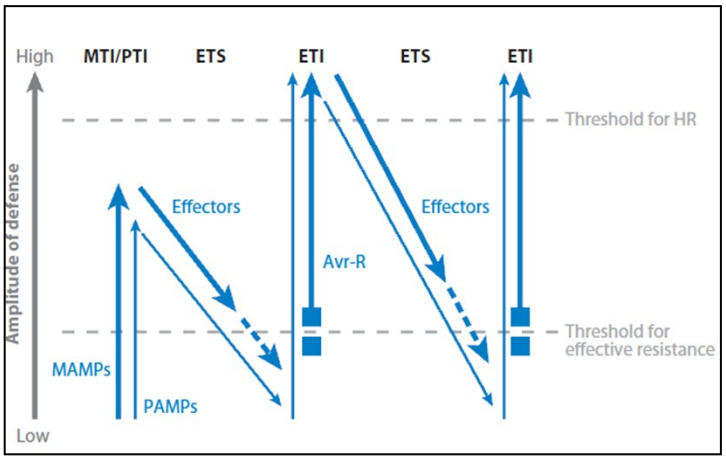

Figure 1.6 Changes in the amplitude of plant defense against pathogen attack caused by effective biocontrol strains of *Trichoderma*, as indicated by using the zigzag model proposed by Jones & Dangl (thin blue arrows). Thick blue arrows indicate the plant response in the presence of *Trichoderma*. MAMPs, microbe-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; MTI, MAMPs-triggered immunity; PTI, PAMPs-triggered immunity; ETS, effector-triggered susceptibility; ETI, effector-triggered immunity; HR, hypersensitive response. *Trichoderma* spp. are able to increase the level of the first response (MTI>PTI) by producing a variety of MAMPs. They also contrast the action of pathogen effectors that cause ETS , thus limiting the loss of resistance and therefore keeping the plant response to a level above or just below the effective threshold (<ETS). *Trichoderma* can also improve ETI by causing a faster response (priming) or activate defense by producing compounds that are specifically recognized (Avr-R) by plant receptors and elicit defense mechanisms. Modified from Jones &Dangl. (Lorito et al. 2010.)

In fact, strains of *Trichoderma* are able to increase plant defense responses more than pathogens (MTI> PTI), by producing various types of MAMPs (Navazio et al., 2007). Some strains are also able to respond to pathogen effectors that interfere with the MTI, for example by inhibiting the pathogenicity factors or by controlling the dispersion and nutrition of pathogens. This reduces the susceptibility caused by effectors (ETS), limits the loss of resistance and maintains the response of the plant to a level above or just below the effective threshold. *Trichoderma* can also improve the ETI by activating a faster defense response (priming), or by releasing the compounds that are specifically recognized by receptors plant cells (Avr-R), as it happens for pathogen effectors (Lorito et al., 2010).

1.4.2. Novel approaches to study the *Trichoderma*-plant interaction: metabolomics

Trichoderma-plant interactions have been extensively studied using a variety of analytical approaches, including genomics, transcriptomics, proteomics, metabolomics, etc. (Lloyd et al., 2003; Figure 1.9).

Expressed sequence tag (EST) sequencing and mRNA profiling using either microarrays (Kehoe et al., 1999) or serial analysis of gene expression (SAGE) (Velculescu et al., 1995) allow a comprehensive analysis of the transcriptome of an organism, cell or tissue. Advances in mass spectrometry have enabled the analysis of cellular proteins and metabolites (proteome and metabolome, respectively) on a scale previously unimaginable. The cumulative utilization of these technologies has advanced the fields of functional genomics (Holtorf et al., 2002; Oliver et al., 2002; Somerville and Somerville, 1999) and systems biology (Ideker et al., 2001; Kitano, 2000).

Functional genomics decipher the function of unknown genes. The absence of a single database pushes to compare the functions of genes revealed through the similarity with the nucleotide sequences of genes of known function with the use of traditional empirical methods.

Proteomic analysis of biotechnologically important fungi has developed significantly only in the last decade, with relatively few cases studied compared with the numerous species whose genome has been sequenced.

Although the transcriptome represents the delivery mechanism of a translational code to the cellular machinery for protein synthesis, increases in mRNA levels do not always correlate with increases in protein levels (Gygi et al., 1999). Furthermore, once translated a protein may or may not be enzymatically active. Due to these factors, changes in the transcriptome or the proteome do not always correspond to alterations in biochemical (i.e. metabolic) phenotypes. In the absence of existing database information, transcript or protein profiling often yield only limited information. Based on the above limitations, profiling the metabolome may actually provide the most

"functional" information of the "omics" technologies. Metabolomics (comprehensive analysis in which all the metabolites of an organism are identified and quantified) has emerged as a functional genomics methodology that contributes to our understanding of the complex molecular interactions in biological systems. As such, metabolomics represents the logical progression from large-scale analysis of RNA and proteins at the systems level.

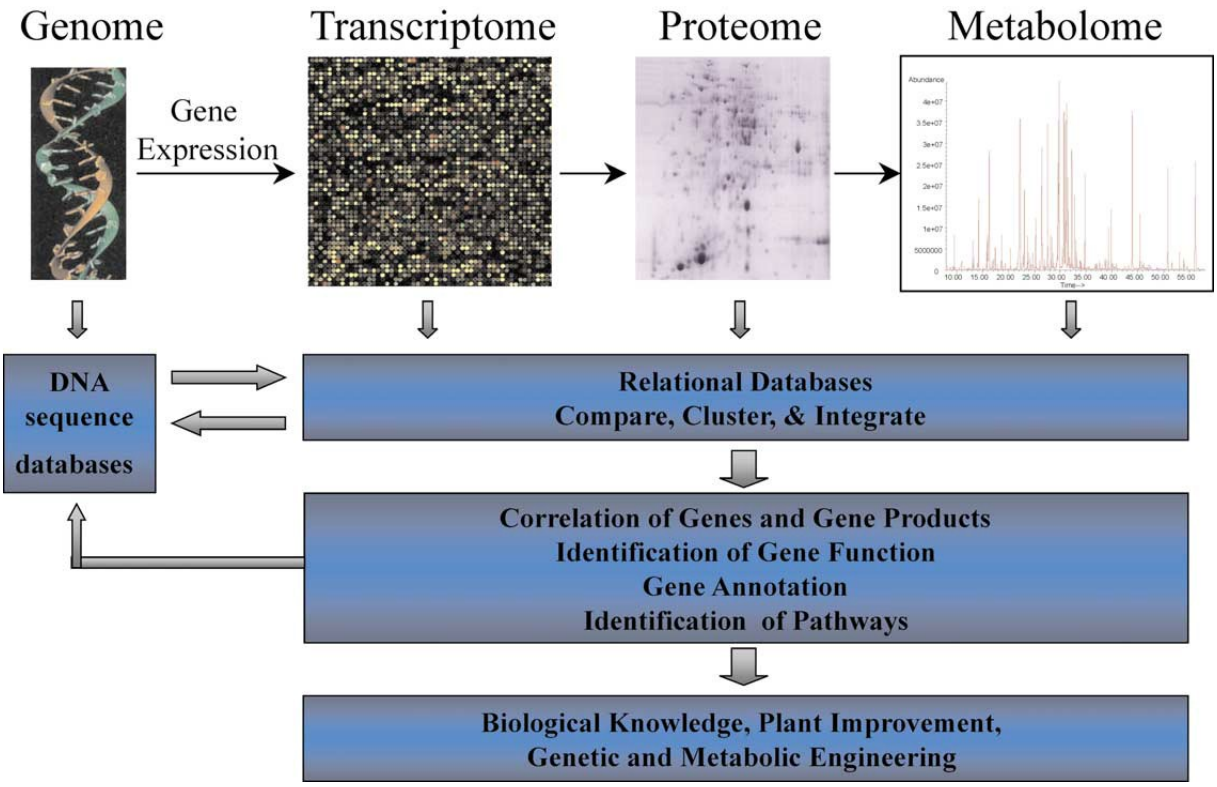

Figure 1.9 Typical techniques used to study functional genomics

Unfortunately, metabolomics is still in an infant state and many of the necessary tools are not available. These tools serve to align, visualize, and differentiate, components in large datasets. Individual components then need to be correlated and placed in metabolic networks or pathways.

Computer based applications are required that can differentiate whether or not samples are statistically similar or different and what the exact differences/similarities are. Ideally, this would be performed in a fully automated manner. For example, a system

should be able to automatically compare the UV, NMR, GC/ MS, LC/MS, or CE/MS profiles of a sample and immediately highlight the component(s) that are statistically different. The chemical identity of these components could then be related to the gene function or to the biological response of the system.

When a sample made up of a few hundred metabolites has to be processed, it is important to analyse the data carefully, in order to extrapolate the smallest differences. One of the most popular approaches to simplify the data include unsupervised methods such as principal component analysis (PCA). This approach summarizes the data based on many independent variables measured from the plant response, and groups them to smaller sets of derived variables which aids in determining their role in the metabolic processes of the plant.

1.5. Secondary metabolites (SMs)

Secondary metabolites (SMs) are generally defined as compounds that are not essential for the growth or survival of the producing organism. SMs tend to be more specialized, and are usually peculiar to only one organism or species. The production of these metabolites is tightly regulated and dependent on the immediate environment and developmental stage of the producing organism. While some secondary metabolites are designed to attract creatures that can pollinate their flowers or distribute their seeds, others protect the plant from the sun's radiation, or serve as 'chemical signals' that enable the plant to respond to 'environmental clues'. Others are defensive compounds, designed to deter or kill disease-causing organisms, potential predators or competitors.

Moreover, different species of the same family, and different isolates of the same species, can often produce significantly different compounds leading to the suggestion that secondary metabolites "express the individuality of species in chemical terms". On the other hand, widely separate species can produce the same class of secondary metabolite and sometimes even the same secondary metabolites.

In some cases, a SM may be essential for survival under particular environmental conditions; for example, siderophores, which are needed for growth at low iron concentrations. But in another case SMs production may have evolved for

communication with, or defense against, other microbes or multicellular organisms. There are many thousands of SMs known in the literature (i.e. pigments, siderophores and pheromones, antibiotics). Secondary metabolites are divisible into several characteristic groups (polyketides, terpenes, phenols, alkaloids) that reflect their origin and biosynthesis.

1.5.1. *Trichoderma* secondary metabolites

The study of *Trichoderma*'s mechanism has demonstrated that inhibiting properties against other fungi are probably due to the combined action of cell-wall degrading enzymes together with the capacity of *Trichoderma* to produce different SMs.
The production of SMs by the *Trichoderma* spp. is strain-dependent and includes antifungal substances belonging to different classes of chemical compounds. These compounds have been classified by Ghisalberti and Sivasithamparam (1991) into three main categories:

- volatile antibiotics;
- compounds soluble in water;
- peptaibols linear oligopeptides of 12–22 amino acids rich in amino-isobutyric acid, N-acetylated at the N-terminus and containing an amino alcohol (Pheol or Trpol) at the C-terminus (Le Doan et al., 1986; Rebuffat et al., 1989).

The different chemical structure of these substances suggests different mechanisms of action. The production of molecules of low molecular weight, non-polar and volatile (simple aromatic compounds, pyrones, butenolides ect.) determines the presence of high concentrations of antibiotics in soil ranging influence on the microbial community even at a long distance. In contrast, the short-distance may be associated with the production of antibiotics and polar peptaibols acting in the vicinity of the hyphae. Polar metabolites of high molecular weight could express their activity as a result of physical contact with the pathogen. As regards the peptaibols, given their amphiphilic nature, it is possible

that their activity is primarily associated with property like detergents. They influence the permeability properties of phospholipid bilayer and exert antibiotic activity against Gram-positive and Gram-negative bacteria. Furthermore, it has been shown that the peptaibols inhibit the action of the enzyme β glucan synthase and the enzyme chitin synthase of the fungus host, preventing the reconstruction of the cell wall of the pathogen and facilitating, at the same time, the destructive action of the chitinase.

Trichoderma strains seem to be an inexhaustible source of bioactive molecules (Sivasithamparam and Ghisalberti, 1998). Some of these compounds produce synergistic effects in combination with CWDEs, with strong inhibitory activity on many fungal plant pathogens (Lorito et al., 1996; Schirmböck et al., 1994). The potential of genes involved in biosynthetic pathways of antibiotics [e.g. polyketides (Sherman, 2002) and peptaibols (Wiest et al., 2002)] with applications in human and veterinary medicine is not been explored yet.

Based on the chemical properties the *Trichoderma* secondary metabolites are classified into the following main categories.

Pyrones

The pyrone 6-pentyl-2H-pyran-2-one (6-pentyl-α-pyrone or 6PP – Figure 1.7) is a common *Trichoderma* metabolite with a strong coconut aroma. 6PP, isolated from culture filtrate of different species (*T. viride, T. atroviride, T. harzianum, T. koningii*), showed antifungal activities towards several plant pathogenic fungi and a strong relationship was found between the production of this pyrone and its antagonistic ability (Scarselletti and Faull 1994; Worasatit et al. 1994). 6PP is also involved in plant growth promotion and induction of disease resistance (Vinale et al., 2008).

Koninginins

Koninginins are complex pyranes isolated from *T. harzianum*, *T. koningii*, and *T. aureoviride*. Koninginins A, B, D, E and G showed antibiotic activity towards the take-all fungus *Gaeumannomyces graminis* var. *tritici* (Almassi et al. 1991; Ghisalberti and Rowland 1993). Koninginin D also inhibits the growth of other important soil-borne

plant pathogens such as *Rhizoctonia solani*, *Phytophthora cinnamomi*, *Pythium middletonii*, *Fusarium oxysporum* and *Bipolaris sorokiniana* (Dunlop et al., 1989).

Viridins

The steroidal metabolite viridin is an antifungal metabolite isolated from different *Trichoderma* species (*T. koningii* - Beresteskii et al. 1976 - *T. viride* - Golder and Watson 1980 - *T. virens* - Singh et al. 2005). This compound prevents the germination of spores of *Botrytis allii*, *Colletotrichum lini*, *Fusarium caeruleum*, *Penicillium expansum*, *Aspergillus niger* and *Stachybotrys atra* (Brian and McGowan 1945; Ghisalberti 2002).

Nitrogen heterocyclic compounds

Harzianopyridone, a *T. harzianum* metabolite with a penta-substituted pyridine ring system with a 2,3-dimethoxy-4-pyridinol pattern, is a potent antibiotic compound active against *B.cinerea*, *R. solani* (Dickinson et al. 1989) *G. graminis* var. *tritici* and *P.ultimum* (Vinale et al. 2006).

T. harzianum produce also metabolites with pirrolidindione ring system named harzianic acid (Figure 1.7). This tetramic acid derivative showed antibiotic activity against *P.irregulare*, *Sclerotinia sclerotiorum* and *R. solani* (Vinale et al., 2009). A plant growth promotion on *Brassica napus* was also observed at low concentrations (Vinale et al., 2009).

Butenolides and hydroxy-lactones

Harzianolide and its derivatives, deydro-harzianolide and T39 butenolide, have been isolated from different strains of *T. harzianum* (Almassi et al. 1991; Claydon et al. 1991; Hanson et al., 1991; Ordentlich et al., 1992; Vinale et al., 2006). These metabolites showed antifungal activities against several plant pathogens (Almassi et al. 1991; Vinale et al., 2006).

A novel hydroxy-lactone derivative, named cerinolactone (Figure 1.7), has been recently isolated from culture filtrates of *T. cerinum*. *In vitro* tests with the purified

compound exhibited activity against *P. ultimum*, *R. solani* and *B. cinerea* (Vinale et al., 2011).

Diketopiperazines

Gliotoxin and gliovirin are two important *Trichoderma* secondary metabolite of this class of compounds. Strains of P group of *Trichoderma* (*Gliocladium*) *virens* produce the antibiotic gliovirin which is active against *P. ultimum* but not against *R. solani*. Strains of the Q group produce the gliotoxin which is very active against *R. solani* but less against *P. ultimum* (Howell, 1999). In seedling bioassay tests, strains of the P group are more effective biocontrol agents of damping off on cotton caused by *Pythium*, while those from the Q group are more effective as biocontrol agents of damping off incited by *R. solani* (Howell, 1991; Howell *et al*., 1993). These data clearly indicate the role of antibiotics production in biocontrol of the gliotoxin/gliovirin producers.

Peptaibols

Peptaibols are linear oligopeptides of 5-22 amino acids rich in α-amino isobutyric acid, N-acetylated at the N-terminus and containing an amino alcohol (Pheol or Trpol) at the C-terminus (Daniel and Filho, 2007). Lorito et al. (1996) demonstrated that peptaibols inhibited β-glucan synthase activity in the host fungus, while acting synergistically with *T. harzianum* β-glucanases. The inhibition of glucan synthase prevented the reconstruction of the pathogen cell wall, thus facilitating the disruptive action of β-glucanases. The most widely known peptaibol is the alamethicin produced by *T. viride*.

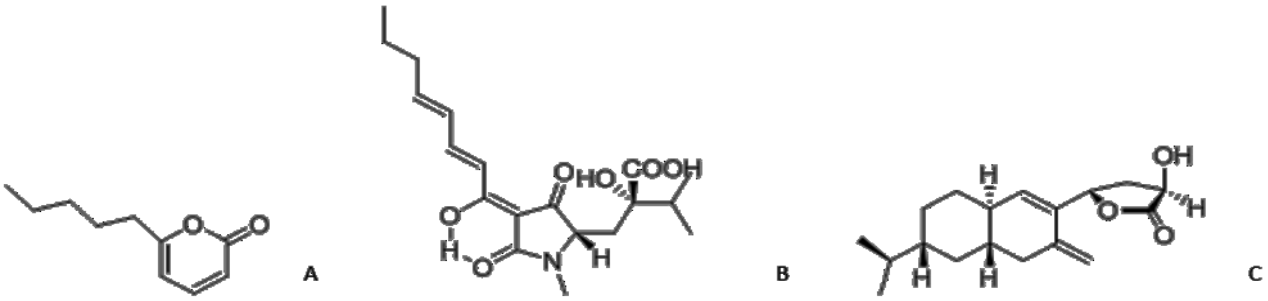

Figure 1.7 Structures of 6PP (A), HA (B), cerinolactone (C).

Hydrophobines

Another class of metabolites, isolated from fungi belonging to the *Trichoderma* genera, is the hydrophobines. The hydrophobin Hytra1 (fig. 1.8) is a part of the present thesis together with HA and 6PP. This protein is involved in many developmental processes including the formation of aerial hyphae, spores and fruiting bodies. Hytra1, purified from *Trichoderma longibrachiatum* MK1 culture filtrates, showed antimicrobial activity and was capable of inducing a strong Hypersensitivity Reaction (HR) and systemic acquired resistance (SAR) when infiltrated in tomato leaves. Hytra1 applied to the plant, could trigger plant defence reactions both locally and systemically. These results clearly demonstrated that Hytra1 from *Trichoderma* T22 is elicitor of plant defence response and is a key factor in the molecular dialog between *Trichoderma* spp. and tomato plants (Ruocco M. 2007; Ruocco M. 2008).

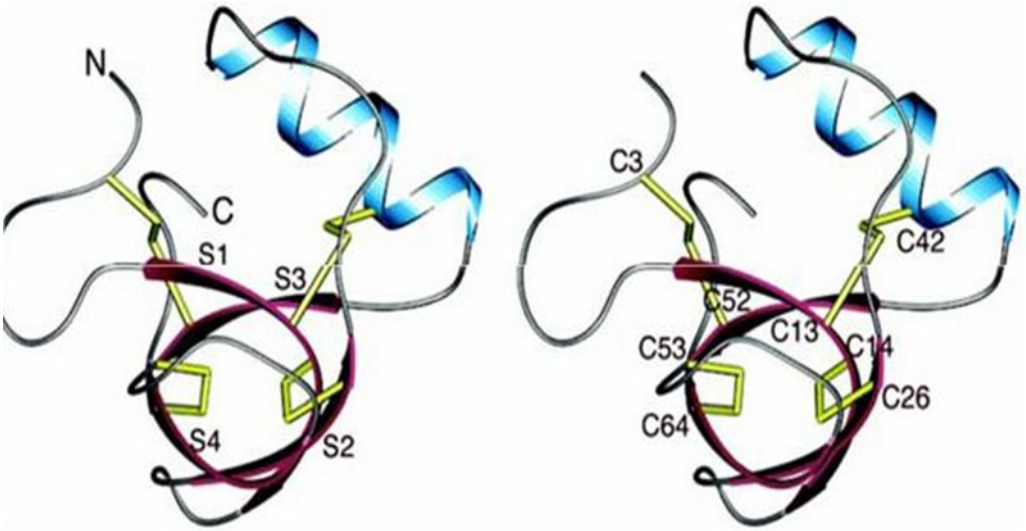

Figure 1.8 General structure of a hydrophobin

1.6. Commercial products used for biological control in agriculture

The use of antagonistic microorganisms to control plant pathogens, nematodes and weeds began over 50 years ago, and today there are several formulations of fungal and bacterial antagonists used as biopesticides.

To achieve commercial development, an antagonistic strain must meet several criteria: absence of toxicity and inability to produce unwanted side effects, adaptation and persistence in the environment field for at least one growing season, efficacy in different geographical areas, genetic stability and biological easy and inexpensive preparation, etc. (Fravel, 2005). After identification of the strain with the best features of biocontrol, this must produce a biomass sufficiently stable, even under adverse conditions, and application systems to ensure growth and antagonistic activity against plant pathogens must be developed.

The antagonistic fungi can be used in different ways. In general, the biomass of the fungal antagonist (cells, mycelium, spores) is treated and embedded in different matrices for the preparation of formulated granules, powders, liquids, etc.. Several studies have shown that the effectiveness of the product may depend on the type of formulation and mode of administration (Fravel, 2005).Today there are hundreds of products on the market based on antagonistic strains of bacteria, fungi or yeasts legally registered and used in organic agriculture. Approximately half of these products, of which some examples are reported in Table 2, is based on *Trichoderma* and *Gliocladium* species.

In addition to the products specifically registered for the protection of crops, there are on the market several formulations acting as bio-protectives, bio-fertilizers and bio-stimulants. The spread of these products is very wide due to the need to reduce the use of synthetic products for plant protection or to get the certification for organic products.

For the biological control of phytopathogenic fungi and bacteria, products based on bacterial antagonists, such as strains of *P. syringae*, can be used against *Penicillium expansum, Botrytis cinerea, Monilinia fructicola, Rhizopus stolonifer*, etc. *P. fluorescens* is effective in the control of diseases caused mainly by soil-borne fungi,

while various species of *Bacillus* are especially active on the leaf surface and have the characteristic of forming spores sufficiently resistant to be easily usable in commercial formulations.

Table 2.Some commercial products containing microbial antagonists.

Organism	Application or formulation	Recommended use, place and culture	Stated activities of the product	Commercial products
***Trichoderma* spp.**	**Granular**	**greenhouse crops, nurseries, indoor plants**	**Combating decay of seedlings, root rot**	**Soilgard 12G (Certis, USA)**
T. harzianum, T. virens* (=*T. lignorum*, *G. virens*), *B. subtilis	**Talc, seed treatment, dispersion, foliar spray, solution for dampening**	**Grape, cotton, bean, potato, tomato, tobacco, cereals**	**Control of powdery mildew and downy mildew, leaf decay, leaf drop**	**Combat (BioAg Corporation, USA)**
Different species of bacteria and fungi, including *Trichoderma* spp.	**Granules, spray**	**Soils**	**Competition for nutrients, suppression of pathogenicity**	**Nutri-Life 4/20 (Nutri-Tech, Australia). Not registered as pesticide.**
***Gliocladium* spp.**	**Granules**	**Horticulture, grasses**	**Growth promotion**	**Gliomix (Kemira Agro Oy, Finlandia; Fargro Ltd., UK)**
T. harzianum*, *T. koningii	**Seed treatment, wetting the soil during pre-sowing**	**greenhouse crops, nurseries, indoor plants**	**Control of *Pythium*, *Phytophtora*, *Rhizoctonia***	**Trichoderma (Euro Bio Consult, Holland)**

1.6.1. *Trichoderma* spp. in agriculture

The benefits of using *Trichoderma* in agriculture are multiple due to their ability to protect plants, enhance vegetative growth and contain pathogen populations under numerous agricultural conditions (Harman, 2000; Harman et al., 2004; Lorito et al., 2006). The biocontrol ability of *Trichoderma* can be attributed to numerous modes of antagonism against various disease causing agents and overall to the several beneficial effects for the plant.

In particular, *Trichoderma* spp. use the mycoparasitism to directly attack the pathogen but also it is able to colonize the roots or compete for nutrients thus excluding a pathogen from the plant roots or exudates. Moreover, these beneficial fungi produce secondary metabolites that inhibit the growth of the pathogens and/or induce plant resistance to their attack. *Trichoderma* species have the ability to create a suppressive environment by its interactions in the soil community to produce unfavourable ecological conditions that limit the development or multiplication of pathogenic populations.

These abilities represent the main reasons for the commercial success of products containing these fungal antagonists used in agriculture. In addition, a large volume of viable propagules can be produced rapidly and readily on numerous substrates at a low cost in diverse fermentation systems (Agosin et al., 1997). The living microorganisms, conserved as spores, can be incorporated into various formulations (liquid, granules or powder etc.) and stored for months without losing their efficacy (Jin et al., 1991; 1992; 1996).

Actually several commercial products based on microbes are registered worldwide and used for the control of bacterial and fungal diseases in field or in post-harvest. The use of beneficial microbes has several advantages, in particular:

- have a specific target;
- are not active on beneficial microorganisms;
- do not release toxic or harmful residues;
- reduce both chemical contaminants in food and the environment;

- limit the impoverishment of the soil in terms of microflora and organic matter;
- their production is low cost.

However when compared with chemical pesticides or fertilizers used in agriculture, these mixtures have some limitations. In particular the capacity of living microorganism to survive on plant surface is limited. Many chemical pesticides tend to not lose their effectiveness even after particularly lengthy periods (from a few days to a maximum of 4 weeks). On the other hand, the relative efficiency of BCAs is short. For some of them is limited to 12 hours (commercial preparation of Heliothis NPV). Equally important is the mode of preservation. In fact, bioformulation, if not stored correctly they lose the effectiveness.

Finally, a biocontrol agent needs to find favourable environmental conditions. It's possible that the microorganism may have difficulty in colonizing some soils or substrates as may be adversely affected by environmental factors or agronomic practices are not compatible (use of compounds based on sulfur, copper, etc.).

1.7. Biocontrol products: new perspectives

In addition to antagonistic microorganisms, biological control can use also molecules derived from microbial cultures, able to act against pathogens both directly by inhibiting the growth and possibly causing their death, and indirectly inducing a defence response in the plant. The degrading enzymes produced by antagonistic microorganisms represent an excellent alternative for the development of new products and strategies of defence against phytopathogenic fungi. They have the ability to synergize the effect of various synthetic fungicides. Thus, the enzyme mixtures can either be used as biological fungicides, and as adjuvant of chemical synthesis, allowing a significant reduction of the concentrations of chemical fungicides.

Trichoderma spp. are important producers of a large number of secondary metabolites, as described above. The use of anti-microbial compounds produced by fungal biocontrol agents has numerous advantages over the use of the whole "live" organisms in all aspects related to industrial production, commercialization and application. They

have the intrinsic characteristic of wide spectrum anti-microbial inhibitory activity that can be exploited. Their production can be readily manipulated and regulated at an industrial level. Indeed, several advantages are associated with the stability to the manufacturing processes downstream (drying or formulation), good shelf-life and the final product is stable, easy to store and transport. Furthermore, they are resistant to environmental conditions in the field variables (temperature, water, pH, light etc). All these conditions are much less restrictive than the use of the living microorganism for commercialization and use.

2. Aim of the work

Agricultural research has been oriented more and more towards developing biological control agents and integrated pest management techniques, with the aim of reducing the use of chemical pesticides. Several microorganisms are antagonists of important plant pathogens and they include bacteria (*Bacillus* spp., *Pseudomonas* spp. and *Enterobacter* spp.), numerous yeasts (*Pichia* spp., *Candida* spp.) and fungi (*Trichoderma* spp., and *Gliocladium* spp.). These agents (BCAs) have been largely used to control disease, alone or in combination.

Many *Trichoderma* strains utilize highly effective antagonistic mechanisms to survive and colonize the competitive environment of the rhizosphere, phyllosphere and spermosphere. The biocontrol activity of effective *Trichoderma* is due also to the production of a variety of secondary metabolites that have a toxic or inhibitory effect on the phytopathogens as well as induce disease resistance mechanisms in plants.

The above BCAs are used worldwide as alternative or in combination of conventional method of disease management. In most of the cases, the available products are made of propagules of the living microbes formulated in a variety of manners. However, the use of these biopesticides/biofertilizers has suffered from a few constrains as listed below:

- Loss of efficacy following varieties of environmental conditions (pH, temperature, water, light, soil types etc.)
- Variable effects on different plant cultivar
- Inconsistent dose-effect response
- Loss of efficacy upon long storage
- Susceptibility to chemicals used in agriculture, natural toxins and other microbes.

Biologically active secondary metabolites are considered a valid alternative to the use of living BCA because they are able to produce the same beneficial effect on crops while overcoming the problems described above.

The aim of this work was to investigate the possibility of improving the effectiveness and the usefulness of microbial biocontrol agents by using selected secondary metabolites able to:

- inhibit the pathogen,
- promote BCA antagonistic activity
- induce systemic resistance in the plant
- stimulate growth and development of different cultures

To achieve this goal, we combined biochemical characterization of SMs and fungal strains with agronomic tests of BCAs, and used agriculturally important plants (*Solanum lycopersicum*, *Brassica rapa*, *Vitis vinifera*) as well as *Arabidopsis thaliana*.

3. Material and Methods

3.1. Fungal strains

All the fungal strains were maintained on potato dextrose agar (PDA) slants at room temperature and subculture bimonthly. *T. harzianum* (M10), *T. atroviride* (P1) and *T. longibrachiatum* (MK1) have been deposited in the culture collection of Agriculture Department – Section of Plant Pathology - University of Naples.

3.2. Liquid culture and metabolite production.

Ten 7 mm diameter plugs of *T. harzianum* (M10) and *T. atroviride* (P1), obtained from actively growing margins of potato dextrose agar (PDA, SIGMA, St Louis, Mo., USA) cultures, were inoculated into 5 L conical flasks containing 2 L of sterile potato dextrose broth (PDB, SIGMA). The stationary cultures were incubated for 21 days at 25 °C. The cultures were filtered under vacuum through filter paper (Whatman No. 4, Brentford, UK).

3.3. Extraction and Isolation of 6-pentyl-α-pyrone (6PP)

The filtered culture broth of *T. atroviride* P1 (3 L) was acidified to pH 4 with 5 M HCl and extracted exhaustively with ethyl acetate. The combined organic fraction was dried (Na_2SO_4) and evaporated in vacuum at 35 °C. The red residue recovered (500 mg) was fractionated by flash column chromatography (Si gel; 200 g Merck, Kiesegel 60, 0.063-0.2 µm), eluting with a gradient of EtOAc/petroleum ether (1:1 to 10:0). Fractions showing similar TLC (Si gel, Kieselgel 60, GF_{254} di 0,25 mm, Merck, Darmstadt; Germany) profiles were combined.

Twelve fractions were collected, and of these, fraction 3 consisted of oleaginous material that showed the same mass spectra and 1H and 13C parameters as those of 6-pentyl-α-pyrone (6PP).

The compounds were detected on TLC plates using UV light (254 or 366 nm) and/or by spraying the plates with 5% (v/v) H_2SO_4 in EtOH followed by heating at 110 °C for 10 min.

^{1}H and ^{13}C NMR spectra were recorded with a Varian 400 instrument operating at 400 (^{1}H) and 125 (^{13}C) MHz, using residual and deuterated solvent peaks as reference standards. High resolution spectra were recorded using a Waters Alliance e2695 HPLC connected to a Waters LCT Premier XE mass spectrometer with an electrospray ionisation source (ESI).

3.4. Extraction and Isolation of 2-hydroxy-2-[4-(1-hydroxy-octa-2,4-dienylidene)-1-methyl-3,5-dioxo-pyrrolidin-2-ylmethyl]-3-methyl-butyric acid (Harzianic acid HA)

The filtered culture broth (2 L) was acidified to pH 4 with 5 M HCl and extracted exhaustively with ethyl acetate (EtOAc). The combined organic fraction was dried (Na_2SO_4) and evaporated in vacuo at 35 °C. The red residue recovered was dissolved in $CHCl_3$ and extracted three times with 2 M NaOH. Harzianic acid was then precipitated with 2 M HCl. The solid was recovered (135 mg), solubilised and subjected to RP-18 vacuum chromatography (20 g), eluting with a gradient of methanol (MeOH):H_2O:CH_3CN (1:8:1 to 10:0:0). After the separation, 45 mg of pure HA were collected.

The homogeneity of pure pooled products was verified by analytical reverse-phase TLC (glass pre-coated Silica gel 60 RP-18 plates - Merck Kieselgel 60 TLC Silica gel 60 RP-18 F254s, 0.25 mm) using 3:4:3 CH_3CN - MeOH -H_2O as eluent (Rf of HA: 0.3). The compounds were detected on TLC plates using UV light (254 or 366 nm) and/or by spraying the plates with 5% (v/v) H_2SO_4 in EtOH followed by heating at 110 °C for 10 min.

HA (**1**). UV, IR, ^{1}H NMR, ^{13}C NMR and HR-FABMS were identical to those reported by Sawa et al. (1994). ESMS (+) *m/z* 753.3 $[M_2 + Na]^+$, 404.2 $[M + K]^+$, 388.2 $[M + Na]^+$,366.2 $[M + H]^+$; 264.2 $[M +H – C_7H_{18}]^+$.

^{1}H and ^{13}C NMR spectra were recorded with a Varian 400 instrument operating at 400 (^{1}H) and 125 (^{13}C) MHz, using residual and deuterated solvent peaks as reference standards. High resolution spectra were recorded using a Waters Alliance e2695 HPLC connected to a Waters LCT Premier XE mass spectrometer with an electrospray ionisation source (ESI).

3.5. CAS agar plates assays

The method to detect siderophore production was previously described by Schwyn and Neilands (1987). Orange halos around the colonies, growth on Chrome Azurol S (CAS) plates, are indicative of siderophore activity. CAS solution was also used for detection of siderophore production in culture filtrate (50 µl of culture was added to 950 µl of CAS solution, after reaching equilibrium the absorbance was measured at 630 nm). The CAS assay was also used to test the chelating properties of a solution 10^{-3} M of HA in methanol.

The CAS assay (Schwyn and Neilands 1987) was modified to test the ability of strain M10 to produce iron-binding compounds eventually avoiding the growth inhibition caused by the toxicity of the CAS-blue agar medium (Milagres et al. 1999). Petri dishes (10 cm in diameter) were prepared with the Malt Extract Agar (MEA) medium. After have solidified, the medium was cut into halves, one of which was replaced by CAS-blue agar. The halves containing culture medium (MEA) were inoculated with M10 plugs. The plates were incubated at 25°C for 6 days.

3.6. Iron binding affinity of HA

In order to measure the iron binding affinity of HA, the method of Kaufmann et al. (2005) was used with some modifications. Stock solutions of ferric chloride (10 mM)

and HA (10 mM) were prepared with 4:1 MeOH / 0.1 M NaOAc buffer solution (pH 7.4). Aliquots of both stock solutions were diluted and the absorbances of the formed complexes were measured at 290 nm in triplicate in the presence and absence of EDTA (10 mM and saturated solution).

3.7. LC/MS of HA–Fe(III) complex

The Fe(III)-binding properties of the HA were investigated by adding 100 µl of a Fe(III) chloride solution (10 mM) to 100 µL of 10 mM HA in MeOH. The solution turned red and was directly injected using a syringe pump into the LC/MS system. Full-scans in the range *m/z* 100–1,200 were performed on a Bruker 6340 ion trap mass spectrometer equipped with an electrospray ionization source and operating in the positive ion mode. High resolution spectra were recorded using a Waters Alliance e2695 HPLC connected to a Waters LCT Premier XE mass spectrometer with an electrospray ionisation source (ESI). Samples were injected using the onboard injector in 10 µL injection volumes and eluted with 20% acetonitrile/water at a flow rate of 0.3 mL/min to the time-of-flight mass spectrometer. For the HA-Fe(III) complex, positive ESI-HRMS found *m/z* 491.0574 ($[C_{19}H_{27}NO_6FeCl_2]^+$ requires 491.0565)

3.8. Hytra1 purification from culture filtrate

One hundred microliter of a *T. longibrachiatum* Mk1 spore suspension 10^8/ml was used to inoculate Erlenmeyer flasks containing 100 ml of Murashige e Skoog (M&S base medium) added with 1% of tomato plant tissue. After 7 days of growth at 25 °C and 150 rpm (revolution per minute) the culture filtrate (CF) was separated from the biomass by filtration with Miracloth paper (Calbiochem La Jolla, CA, USA) and subsequently centrifugation at 20.000g rpm for 20 min. The obtained clear CF was poured in a separator funnel, vigorously shaken for 5 minutes and decanted for 5 more minutes. At this point, two different phases appeared into the funnel, a clear liquid with a consistent foam on the surface which was recovered and dissolved in 70% ethanol. Protein

concentration was determined by a Bradford Dc protein assay (Bio-Rad, Richmond, CA, USA) and samples were stored at -20 °C until use.

3.9. Tomato plant growth promotion

Tomato (*Lycopersicum esculentum cv. Roma*) seeds were surface sterilized using 70% EtOH for 2 min, followed by 2% NaClO for 2 min, thoroughly washed with sterile distilled water and used for the following experiments.

3.9.1. *In vitro* assay

Seed germination

Sterile tomato seeds were placed on magenta box containing half-strength Murashige and Skoog salt (MS) medium (ICN Biomedicals) containing 1 % agar and 1.5% sucrose, adjusted to pH 5.7, and vernalized for 2 days at 4°C in the absence of light. Sterile solutions of HA, 6PP and Hytra1 were added to the substrate before the solidification of agar using these concentrations:

1. Control (only water)
2. HA 10µM
3. HA 1µM
4. HA 0.1µM
5. 6PP 10µM
6. 6PP 1µM
7. 6PP 0.1µM
8. Hytra1 0.01µM
9. HA 10µM/ 6PP 1µM
10. HA 1µM/6PP 10µM
11. HA 10µM/6PP 10µM

12. HA 1μM/ 6PP 1μM
13. HA 1μM/ 6PP 1μM/ Hytra1 0.01μM

Each treatment consisted of five replicates and the experiment was repeated four times. Data from the experiments were combined since statistical analysis determined homogeneity of variance ($P \leq 0.05$).

Rooting assays

Sterile tomato seeds were allowed to germinate in the dark in sterile plastic boxes containing a salt medium SM Agar plus sucrose 1.5 % for 10 days. The small seedlings were cut into small pieces that were transferred to new sterile boxes containing different solution listed below:

Exp. N.	Substrates
1	SM+1.5% sucrose (negative control)
2	SM +1.5% sucrose + HA 0.1μM
3	SM +1.5% sucrose + HYTRA1 0.01μM
4	Germon E (GE) (L. Gobbi, Italy) +HYTRA1 0.01μM
5	GE +HA 0.1μM
6	GE +HA 0.1μM + HYTRA1 0.01μM
7	SM +1.5% sucrose + HA 0.1μM + HYTRA1 0.01μM
8	GE (positive control)

The composition of Salt Medium in one liter of water was as follows: KH_2PO_4 680 mg L^{-1}, K_2HPO_4 870 mg L^{-1}, KCl 200 mg L^{-1}, NH_4NO_3 1 g L^{-1}, $CaCl_2$ 200 mg L^{-1}, $MgSO_4$. 7H2O 200 mg L^{-1}, $FeSO_4$ 2 mg L^{-1}, $MnSO_4$ 2 mg L-1, $ZnSO_4$ 2 mg L^{-1}, Sucrose 10 g L^{-1}, agar 10 g L^{-1} (all purchased from SIGMA). Each treatment consisted of five replicates and the experiment was repeated four times. Data from the experiments were combined since statistical analysis determined homogeneity of variance ($P \leq 0.05$).

3.9.2. In vivo assay

Pot experiments

Tomato seedlings were placed on plastic pots and grown in a phytotron (16h photoperiod); the temperature was maintained at 25 ±1°C with a relative humidity of 65–75 %. Sterile solutions of HA and 6PP were added (drenched – 50 ml) every two days at concentrations of 10µM, 1µM, 0.1µM for tomato plant. Untreated plants were used as controls.

Plant development was measured daily. Each treatment consisted of five replicates and the experiment was repeated four times. At the end of each experiment, the whole plants were dried and weighed. Data from the experiments were combined since statistical analysis determined homogeneity of variance ($P \leq 0.05$).

Glass plate experiments

Tomato (*Lycopersicum esculentum cv. Roma*) seeds were surface sterilized using 70% EtOH for 2 min, followed by 2% NaClO for 2 min, thoroughly washed with sterile distilled water then placed on sterile glass plates separated by spacers of 2 mm containing 20 g of 50% peat and 50% normal soil. The plants were drenched with 5 ml of the following metabolite solutions:

HA 1µM

6PP 1µM

Hytra 1 0.01µM

HA 1µM/ 6PP 1µM

HA 1µM/ 6PP 1µM/ Hytra 1 0.01µM

Root length was measured daily. Each treatment consisted of five replicates and the experiment was repeated four times. Data from the experiments were combined since statistical analysis determined homogeneity of variance ($P \leq 0.05$).

3.10. Broccoli plant growth promotion and glucosinaltes analysis

Brassica rapa (subsp. sylvestris var. esculenta ecotype "Sessantino") seeds were surface sterilized with the same protocol used for tomato. Furthermore, 900-1000 broccoli seeds were coated with 8 mL of *Trichoderma* spp. (*atroviride* strain P1 or *harzianum* strain M10) spore solutions (10^8 sp/mL) and dried overnight under laminar flow. Then the treated and untreated seeds were placed on plastic pots containing sterile 50% peat and 50% normal soil. The untreated seedlings (no coated) were drenched with sterile solutions of HA and 6PP (1 µM) every two days. Untreated (no living fungi and no metabolites applications) plants were used as controls.

Seedlings were grown in a phytotron (16h photoperiod); the temperature was maintained at 25 ±1°C with a relative humidity of 65–75 %.

Stem length was measured daily. Each treatment consisted of five replicates and the experiment was repeated four times. At the end of each experiment, the whole plants were dried and weighed. Data from the experiments were combined since statistical analysis determined homogeneity of variance ($P \leq 0.05$).

3.10.1. Glucosinaltes analysis

Broccoli plants were frozen, lyophilized and the glucosinolates were extracted with MAE extraction (Microwave-assisted-extraction). An Ethos-1 laboratory microwave system (*Ethos 1* labstation, Milestone, USA) equipped with a 12-vessel carrousel operated in the closed-vessel mode was used for analytical tests. For the extraction, carried out in duplicate in the vessel, 0.180 g of dry-tissue were weighed, to which were added 10 mL of an aqueous solution of 70% methanol (v / v). In each vessel were also added 20 µL of 2-propenylglucosinolate (sinigrin - 60 mM) as internal standard. Both temperature and pressure were monitored in a single vessel during operation through an ATC-400 FO automatic control system.

Glucosinolates were analysed after extraction by HPLC (Shimadzu LC 10, Shimadzu, Japan) at a flow rate of 1 ml/min, using a Prodigy column 5 l ODS3 100A, 250 - 4.60

mm (Phenomenex, USA). The mobile phase was a mixture of ultra-pure water (A) and acetonitrile (B). Compounds elution was achieved using the following linear gradient: starting condition 0-15% B (10 min), 15-40% B (5 min), 40-50% B (5 min), 50-0 % B (5 min). Flow: 0.8 mL/min. Chromatograms were recorded at 227 nm.

LC-MS-MS analyses were performed by a LC/MS/MS System (API 3000, MDS SCIEX). The mass spectrometer is equipped with a Model 11 syringe pump (Harvard, Apparatus, Holliston, MA, USA) and with an APCI interface. The mass spectrometer was used exclusively in the triple quadrupole mode. Detection of the compounds was performed using IDA (information dependent acquisition), an artificial intelligence-based product ion scan mode, generating a survey scan, single MS spectra with molecular mass information, product ion spectra, and extracted ion fragmentograms (XIC). The APCI source was used in negative mode at temperature set at 400 °C. All solvents were of HPLC grade. Sinigrin (allyl glucosinolate) was obtained from Sigma-Aldrich (USA).

3.11. *Vitis vinifera* plant growth promotion and qualitative analysis

3.11.1. *In vivo* assay

One year old plants of *V. vinifera* cv. Sangiovese were planted in pots (12 cm of diameter) containing sterile peat and soil (1:1 v:v). Plants were grown for 2 months, from April to June, in greenhouse at 25°C with a natural photoperiod. Plants were treated with purified secondary metabolites (6PP and HA) solution applied at a concentration of 10µM and 1µM, or with spore suspensions of P1 and M10 (applied at 10^8 sp/mL).

Stem length was measured daily. Each treatment consisted of five replicates and the experiment was repeated four times. At the end of each experiment, the whole plants were dried and weighed. Data from the experiments were combined since statistical analysis determined homogeneity of variance ($P \leq 0.05$).

3.11.2. Field experiment

The purified secondary metabolite 6PP and a commercial *T.harzianum* strain T22 were applied in a field of *Vitis vinifera*. The experimental field consisted of 9 rows each containing 12 plants. The 6PP solution was applied at 1µM (3 rows) and spore suspension of T22 was applied at 10^8 sp/l (3 rows) and compared to the untreated plant (3 rows). Treatments (every 14 days) begun one month after plants sprouted and finished with harvest.

3.11.3. Analysis of polyphenols

Polyphenols were extracted from fruits. 5 g of fruit tissues were homogenised for 1min in 20 ml of extraction solution containing methanol/water/formic acid (60:37:3 v/v/v) and centrifuged for 5 min at 5000 rpm. Aliquots (4 ml) of supernatant were evaporated to dryness using a SpeedVac concentrator (ThermoSavant, Holbrook, NY, USA) with no radiant heat and resuspended in 1 ml of extraction solution.

The amount of total polyphenols in the extracts was determined according to the Folin–Ciocalteau method and using HPLC methods (LC-10Ai - Shimadzu), UV/VIS SCL-10AVP (Shimadzu) detector and a Prodigy column ODS3 100 Å, 250x4.6 mm, 5 µm (Phenomenex, CA, USA). The mobile phase was a mixture of ultra-pure water/ 0.2% formic acid (A) and acetonitrile/methanol (60/40 v/v) (B). Compounds elution was achieved using the following linear gradient: starting condition 20-30% B (6 min), 30-40% B (10 min), 40-50% B (5 min), 50-90% B (11 min). Flow: 0.8 mL/min. Wavelengths misured : 280 nm, 360 nm e 510 nm.

Gallic acid was employed as a standard and results were expressed as gallic acid equivalents (GAE) (mg GAE/100 g of seeds or skin dry matter (DM)). The absorbance was measured using a UV–vis spectrophotometer (Lambda 25, PerkinElmer, Italy) at the wavelength of 750 nm.

3.11.4. Antioxidant activity

The antioxidant activity was measured using ABTS/HRP decoloration methods. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. Because ABTS and potassium persulfate react stoichiometrically at a ratio of 1:0.5, this will result in incomplete oxidation of the ABTS. Oxidation of the ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. The radical was stable in this form for more than two days when stored in the dark at room temperature. For the study of phenolic compounds and food extracts, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 (60.02) at 734 nm and equilibrated at 30°C. Stock solutions of phenols in ethanol were diluted such that, after introduction of a 10- ml aliquot of each dilution into the assay, they produced between 20%–80% inhibition of the blank absorbance. After addition of 1.0 ml of diluted $ABTS^{\bullet+}$ solution (A734nm 5 0.700 6 0.020) to 10 ml of antioxidant compounds or Trolox standards (final concentration 0–15 mM) in ethanol. Solvent blank was run in assay. All determinations were carried out at least three times, and in triplicate, on each occasion and at each separate concentration of the standard and samples. The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of concentration of antioxidants and of Trolox for the standard reference data. The concentration - response curve for 5 sequentially and separately prepared stock standards of Trolox is illustrated in Fig. 3.1.

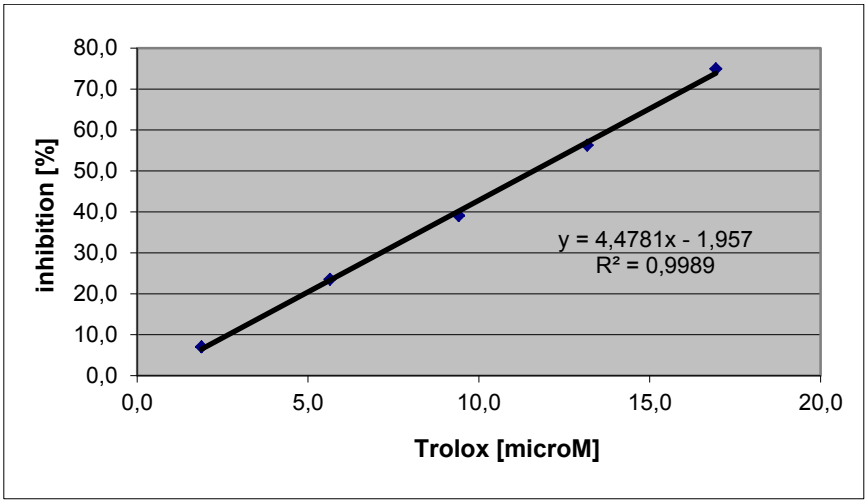

Figure 3.1: calibration curve using Trolox (standard) in the ABTS method. The assessment of the antioxidant activity is calculated as a percentage of decrease in absorbance, also known as "percentage of inhibition".

3.12. *Arabidopsis thaliana* plant growth promotion

3.12.1. *In vitro* assay

Arabidopsis Thaliana (Columbia-0: Col-0) seeds were surface sterilized using using 70% EtOH for 2 min, followed by 5% bleach/ 1% SDS solution for 15 min, thoroughly washed with sterile distilled water three times then placed on squared plastic plates containing MS agar (for 2L: 4.8 g/L pH=5.7, 6,4g/L of Agar then autoclaved). Roots length was measured daily. treatment consisted of three replicates and the experiment was repeated three times. Data from the experiments were combined since statistical analysis determined homogeneity of variance ($P \leq 0.05$).

3.12.2. *In vivo* assay

Arabidopsis Thaliana (Columbia-0: Col-0) seeds were sown in Levington's F2 compost plus sand (JFC Munro, Devon; http://www.jfcmonro.com) and chilled for 2 days at 4 °C. Plants were grown under short-day conditions (10 h light) in a controlled-

environment chamber, at 22 °C during the day and 18 °C at night with 60% relative humidity for 5–6 weeks before transplanting. Sterile solutions of HA, 6PP and Hytra 1 were drenched every two days at concentrations of 0.1 µM, 0.1 µM, 0.01 µM respectively for one month. Untreated plants were used as controls.

At the end of each experiment, fresh and dry weight of the whole rosette was detected. Data from the experiments were combined since statistical analysis determined homogeneity of variance ($P \leq 0.05$).

3.13. *Arabidopsis thaliana* metabolome

Wild type *Arabidopsis* (Col-0) plants were treated with *Trichoderma* metabolites (HA, 0.1mM; 6PP, 0.1mM; Hytra 1, 0.01mM) for one month (five biological replicates for each treatment). Then freeze dried leaf powder (10 mg) was extracted in 0.8 ml 20% methanol containing an internal standard (36 µg ml^{-1} umbelliferone). After centrifugation (10 min at 16,100* g, 4°C), the samples were filtered through a 0.2 µm polyvinylidine fluoride (PVDF) syringe filter (Chromacol, Welwyn Garden City, UK). Metabolite profiling was performed using a QToF 6520 mass spectrometer (Agilent Technologies, Palo Alto, USA) in MS mode coupled to a 1200 series Rapid Resolution HPLC system. 5 µL of sample extract was loaded onto a Zorbax StableBond C18 1.8 µm, 2.1 9 100 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA). Mobile phase A comprised 5% acetonitrile with 0.1% formic acid in water and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. The following gradient was used: 0 min—0% B; 1 min—0% B; 5 min—20% B; 20 min—100% B; 25 min—100% B; 26 min—0% B; 9 min post time. The flow rate was 0.25 ml min^{-1} and the column temperature was held at 35°C for the duration. The source conditions for electrospray ionisation were as follows: gas temperature was 350°C with a drying gas flow rate of 11 l min^{-1} and a nebuliser pressure of 55 psig. The capillary voltage was 3.5 kV and the data shown here were collected in negative ion mode. The fragmentor voltage was 115 V and skimmer voltage 70 V. Scanning was performed at three scans sec^{-1}. Features (i.e. predicted compounds with neutral mass and retention time) were

extracted from each sample using the molecular feature extraction facility in Mass Hunter (Aligent Technologies, Palo Alto, USA). This method extracts ions (of charge 1 or 2) which have defined chromatographic features above a set peak height (in this case, just above the noise level 100 counts peak height). A peak is generated from a deconvolution process in mass spectra. Count peak height is the output from Mass Hunter and is directly related to the abundance of a feature. The presence of co-eluting ions differing by the appropriate m/z values allows the identification of commonly - occurring adducts. Adducts are then collapsed into a single feature with a predicted neutral mass and retention time. Where a feature is detected in the absence of multiple adducts its neutral mass is calculated on the assumption that it is deprotonated or protonated. The list of features from each sample is subjected to alignment and PCA (Principal component analysis) as reported by Venura and coworkers (2012).

4. Results

4.1. Characterization of harzianic acid (HA) and *Trichoderma harzianum* M10.

In the first part of this chapter we purified and characterized the main metabolites (in terms of concentration) produced by selected strains of *Trichoderma* fungi (M10 and T22)

4.1.1. Isolation and chemical characterization of HA

T. harzianum M10 was grown in PDB for 21 days, and the culture filtrate was extracted with ethyl acetate, from which HA (98 mg) was isolated as described in the materials and methods section.

The high resolution mass spectrum (figure 4.1) of HA showed a molecular peak $[M+H]^+$ at 366.1892 m/z (calcd for $C_{19}H_{27}NO_6$ + H, 366.1872), and its pattern corresponded to that of 2-hydroxy-2-[4-(1-hydroxy-octa-2,4-dienylidene)-1-methyl-3,5-dioxo-pyrrolidin-2-ylmethyl]-3-methyl-butyric acid described by Sawa et al. (1994) and Vinale et al. (2009).

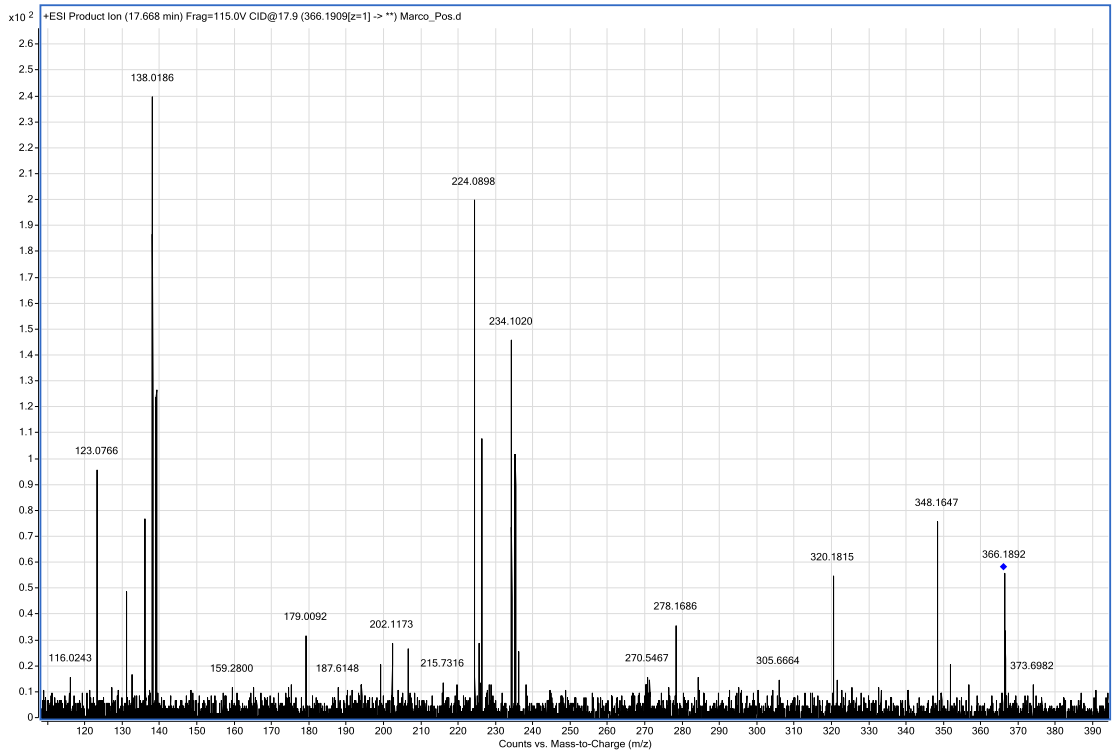

Figure 4.4 HR ESI-MS spectrum of harzianic acid

The HA structure was confirmed by NMR experiments (^{1}H NMR; ^{13}C NMR, COSY; TOCSY; DEPT 135; HMBC; HSQC). In figure 4.2 is reported the ^{1}H NMR of HA.

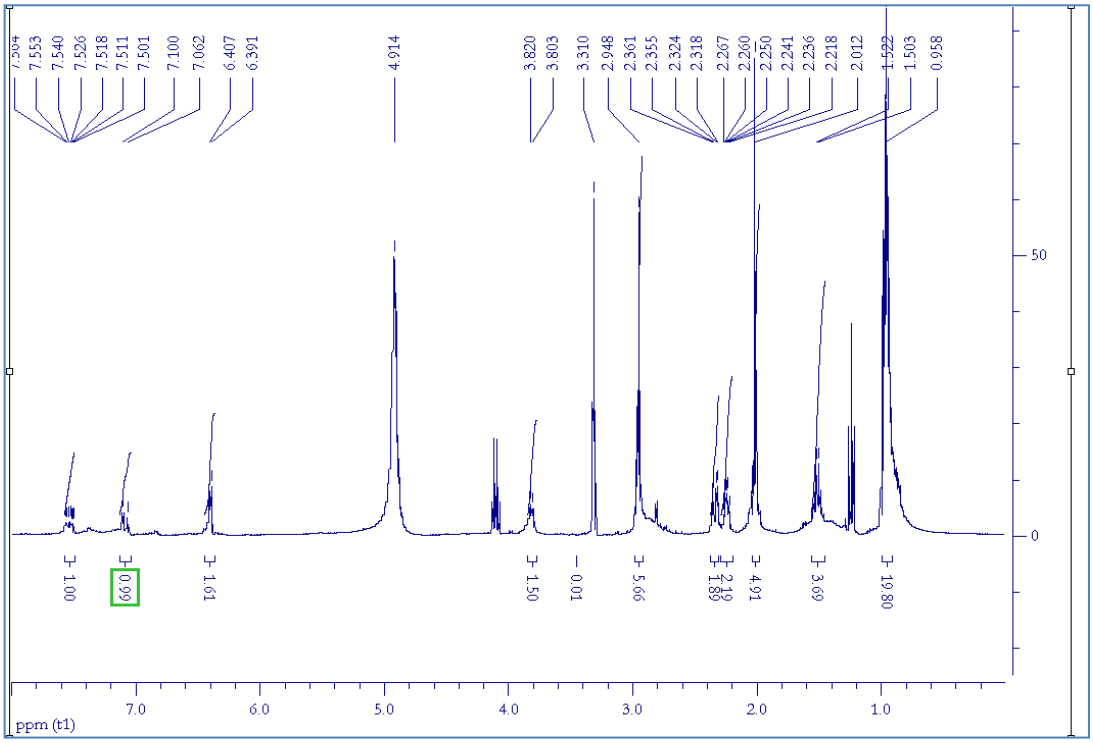

Figure 4.5 ^{1}H NMR spectrum of harzianic acid

4.1.2. Iron (III) binding activity of *Trichoderma harzianum* M10 and HA and characterization of HA-Fe (III) complex

Iron (III) binding activity of M10 and of its secondary metabolite HA was evaluated with chrome azurol S (CAS)-blue agar assay. The fungus grew on CAS blue agar and the iron(III) chelating compounds, secreted by the microorganism, diffused throughout the medium producing a color change from blue to orange.

Purified HA decolorized CAS blue agar, indicating that it could form a complex with Fe(III) (figure 4.3). In fact, the compound in aqueous solution was pale orange while the addition of Fe(III) resulted in the appearance of a red color, indicating that an iron complex was formed.

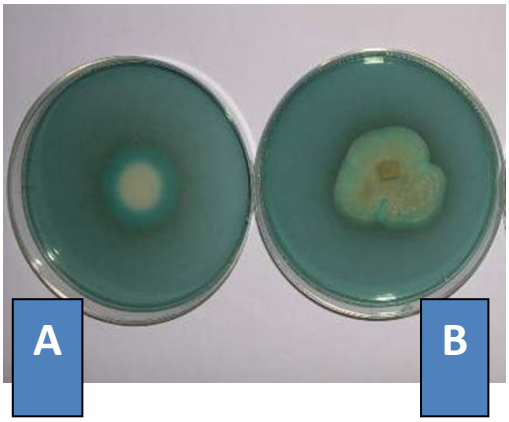

Figure 4.6 Agar plate containing chrome azurol S blue agar (CAS Agar) media inoculated with: Harzianic acid (10 ml of a 100µM solution) (A), Trichoderma harzianum M10 (B).

The interaction of the fungal metabolite HA with iron (III) was further investigated. When Fe(III) was added as $FeCl_3$, the mass spectra showed additional signals at 455.1 m/z and 491.1 m/z (figure 4.4) corresponding to a 1:1 chloride containing complex (figure 3.5), $[M-H+Fe(III)+Cl2+H]^+$ (m/z 491.1) or $[M-2H+Fe(III)+Cl+H]^+$ (m/z 455.1), as determined by isotopic distribution.

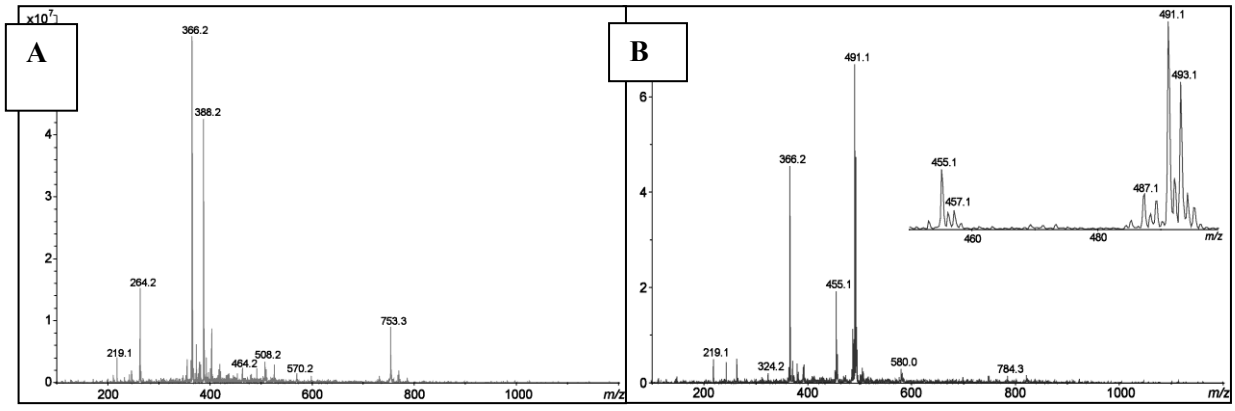

Figure 4.4: ESI-MS of HA (A) and HA-Fe(III) complex (B).

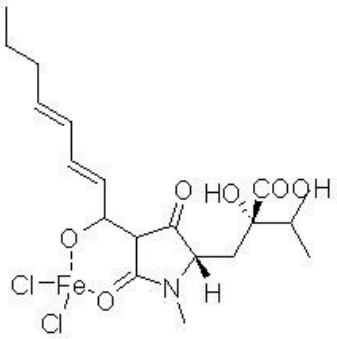

Figure 4.5: HA-Fe(III) complex

High resolution mass spectrum of the HA-Fe(III) complex showed signals at m/z 491.0574 ($[C_{19}H_{27}NO_6FeCl_2]^+$ requires 491.0565), confirming the 1:1 HA–Fe complex. The resulting mass spectrum is shown in figure 4.6.

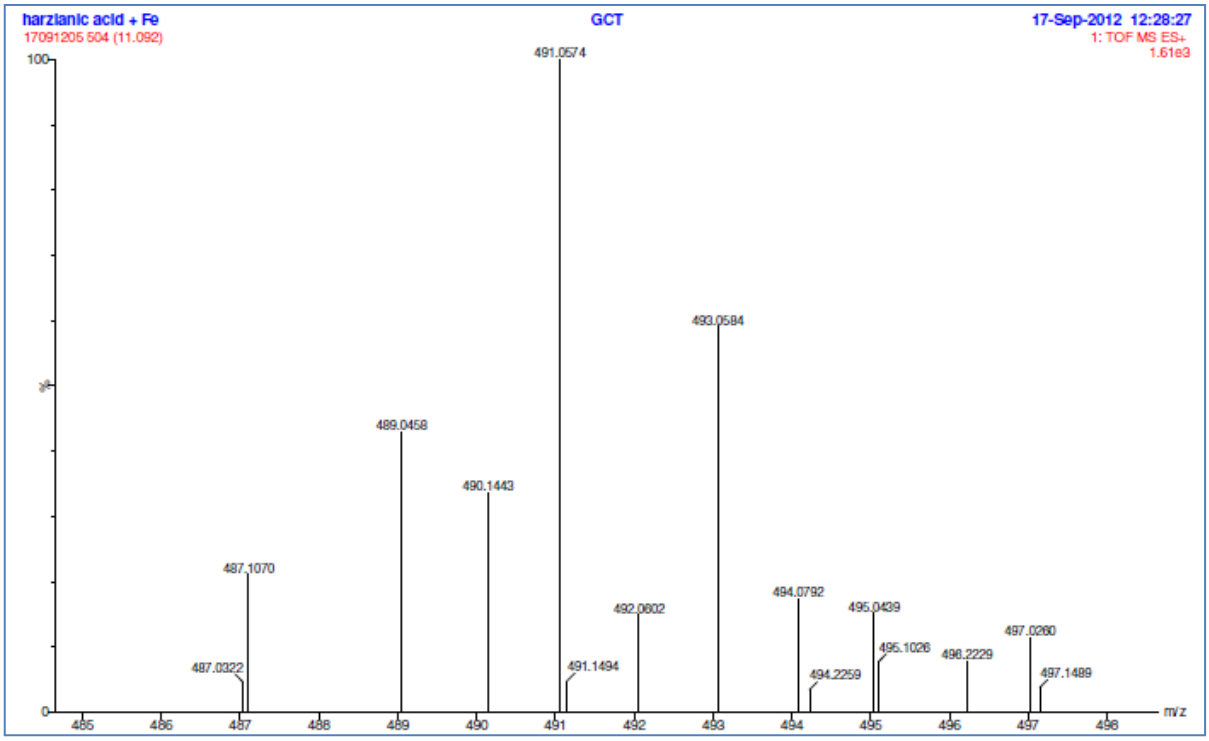

Figure 4.6: HR ESI-MS of HA-Fe(III) complex

In addition, we investigated the effect of adding different concentrations of ferric chloride and tetramic acid and observed the formation of a HA-Fe(III) complex by spectrophotometric analysis. These experiments were performed by using a previously described protocol based on the competition between HA and EDTA for iron and the

detection of the complexes was determined by measuring the characteristic absorption (Wang et al., 2002). The loss of HA-Fe (III) absorbance at 340 nm upon addition of EDTA was used to calculate the equilibrium constant, presuming the formation of a 1:1 HA–Fe complex, according to the following equation:

$$\text{EDTA-Fe} + \text{HA} = \text{EDTA} + \text{Fe-HA}$$

$$K_{eq} = [\text{EDTA}][\text{Fe-HA}] / [\text{HA}][\text{EDTA-Fe}] = K_{d;EDTA} / K_{d;HA}$$

By using the known affinity of EDTA for Fe(III) (5.00×10^{-23} M), we were able to determine the relative affinity ($K_{d,app}$) of HA for Fe^{3+}:1.79×10^{-25} M.

4.2. Isolation and chemical characterization of 6-penthyl-α-pyrone (6PP)

The filtered culture broth of *T. atroviride* strain P1 (3 L) was acidified to pH 4 with 5 M HCl and extracted exhaustively with ethyl acetate. The combined organic fraction was dried (Na_2SO_4) and evaporated in vacuum at 35° C. The red residue recovered (500 mg) was fractionated by flash column chromatography (silica gel; 200 g), by eluting with a gradient of EtOAc/petroleum ether (1:1 to 10:0). Fractions showing similar TLC profiles were combined.

Of the twelve fractions collected, fraction 3 (92 mg) consisted of an oleaginous material that showed the same mass spectra and ^{1}H and ^{13}C parameters as those of 6PP (Moss et al., 1975).

The high resolution mass spectrum of 6PP, showing a molecular peak $[M+H]^{+}$ at 167.1061 m/z (calcd for $C_{10}H_{14}O_2$ + H, 167.1027), is reported in Figure 4.7, while the ^{1}H NMR is reported in Figure 4.8.

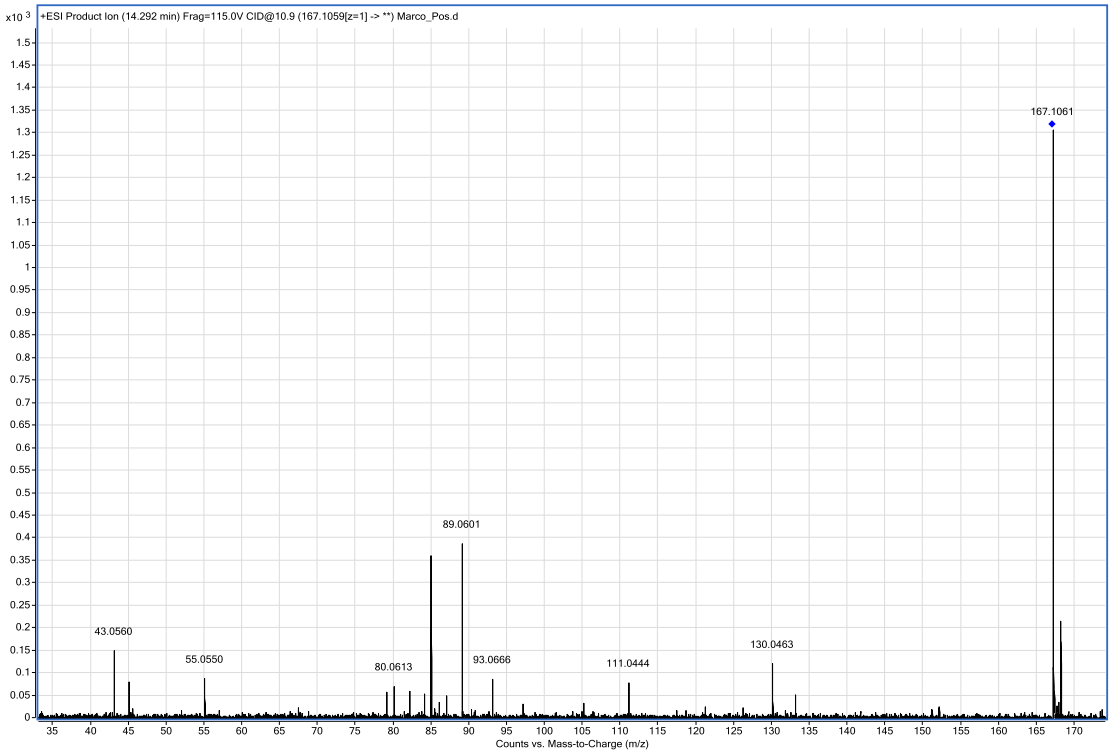

Figure 4.7: HR ESI-MS spectrum 6-pentil-α-pyrone

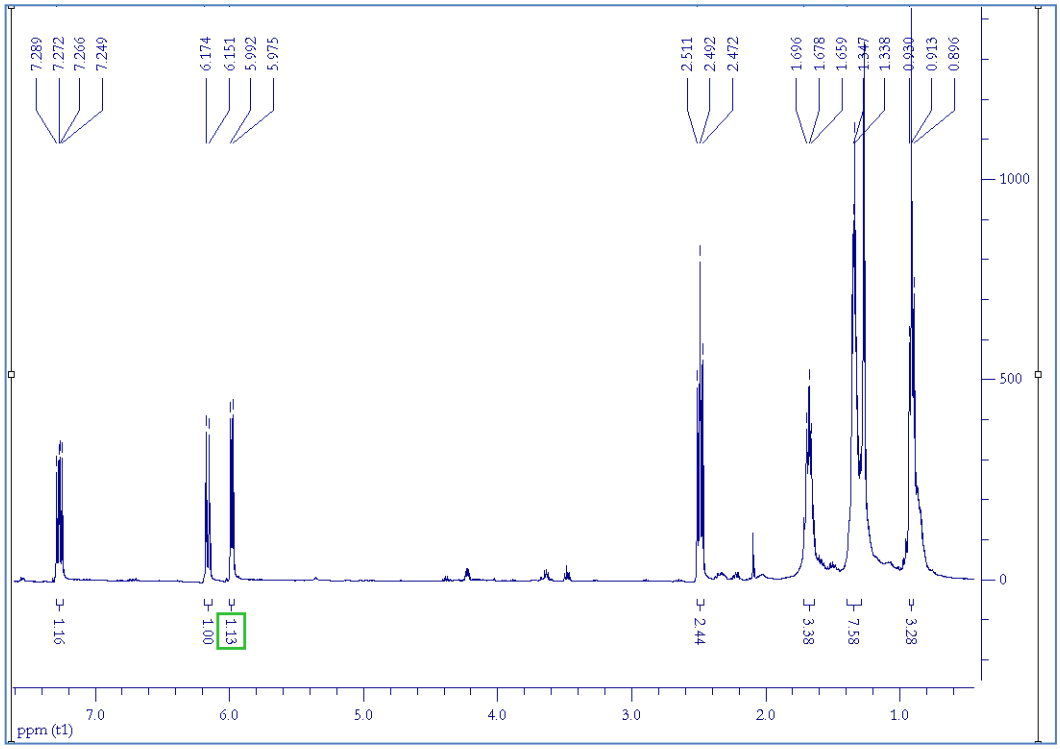

Figure 4.8: ^{1}H NMR spectrum of 6PP.

4.3. Effect of purified metabolites, 6PP and HA, on *Solanum lycopersicum* cv. San Marzano

Purified *Trichoderma* metabolites are applied on tomato seedlings to observe the effect on the seed germination and growth. We study also the effect of application with fungal metabolites, singly or their combination, on *Solanum lycopersicum* growth in terms of seed germination and root development.

4.3.1. *In vivo* assays: seed germination and plant growth promotion

To assess the effects of 6PP and HA at different concentrations (10 µM, 1 µM, 0.1 µM) on seed germination, shoot growth and fresh-dry weight of tomato *in vivo* experiments were carried out in growth chamber at temperature of 25° C.

Both secondary metabolites promoted seed germination. HA had a strong effect at 10µM and 1µM while, 6PP incresed seed germination only at 10 µM. None of the treatments significantly reduced tomato seeds germination (Table 4.1).

Table 4.1 Effect of HA and 6PP at different concentration (10 µM to 0.1 µM per pot) on tomato seed germination (percent of the tested seeds).

Treatment	day 1	SD	day 2	SD	day 3	SD	day 4	SD
Control	0 [a]	0	16,70 [a]	3,90	72,20 [a]	7,90	100,00 [a]	0
HA 10 µM	0 [a]	0	88,90 [b]	3,90	94,40 [b]	3,90	100,00 [a]	0
HA 1 µM	0 [a]	0	72,20 [c]	7,90	100,00 [c]	0,00	100,00 [a]	0
HA 0.1 µM	0 [a]	0	27,80 [d]	11,80	72,20 [d]	11,80	100,00 [a]	0
6PP 10 µM	0 [a]	0	44,40 [e]	7,90	88,90 [e]	7,90	100,00 [a]	0
6PP 1 µM	0 [a]	0	25,00 [f]	15,70	50,00 [f]	3,90	100,00 [a]	0
6PP 0.1 µM	0 [a]	0	11,10 [g]	0,00	61,10 [g]	11,80	100,00 [a]	0

Values are means of 3 replicates (20 seeds per pot). SD: standard deviation. Values with the same letter do not differ significantly (P < 0.05).

Seven days after, the plants, resulting from treated seeds, showed, in some cases, a growth promotion effect in terms of stem length (figure 4.9). The application of HA at any tested concentration enhanced the shoots development, while 6PP gave the same effect only if applied at 10 µM.

Furthermore, an increase of fresh and dry weight of the plant was observed for the HA treatments (10 µM to 0.1 µM), while 6PP produced only a significant increase of fresh weight at 10 µM (figure 4.10 and 4.11).

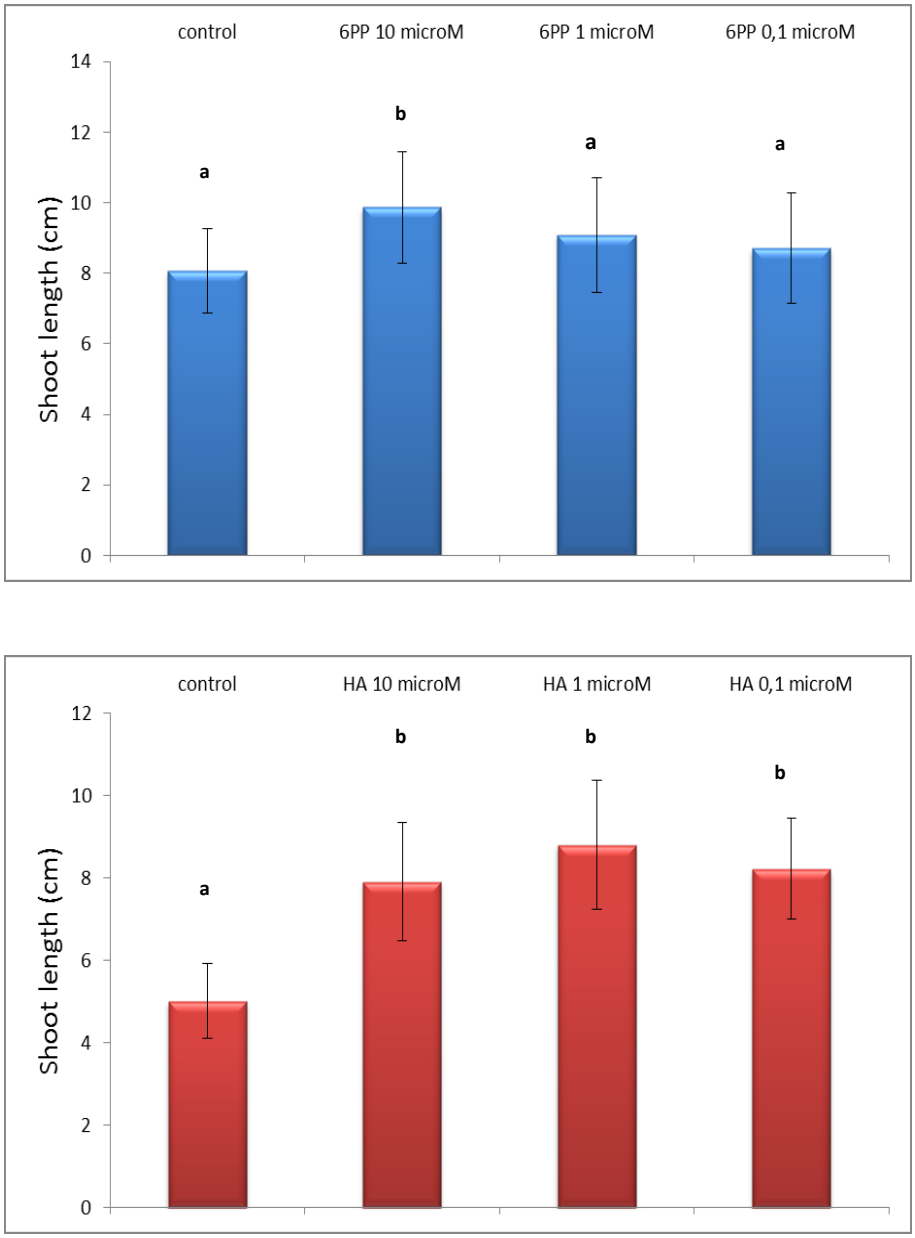

Figure 4.9: Effect of 6PP and HA at different concentration on tomato shoot growth. Concentration ranged from 10 to 0.1 µM. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

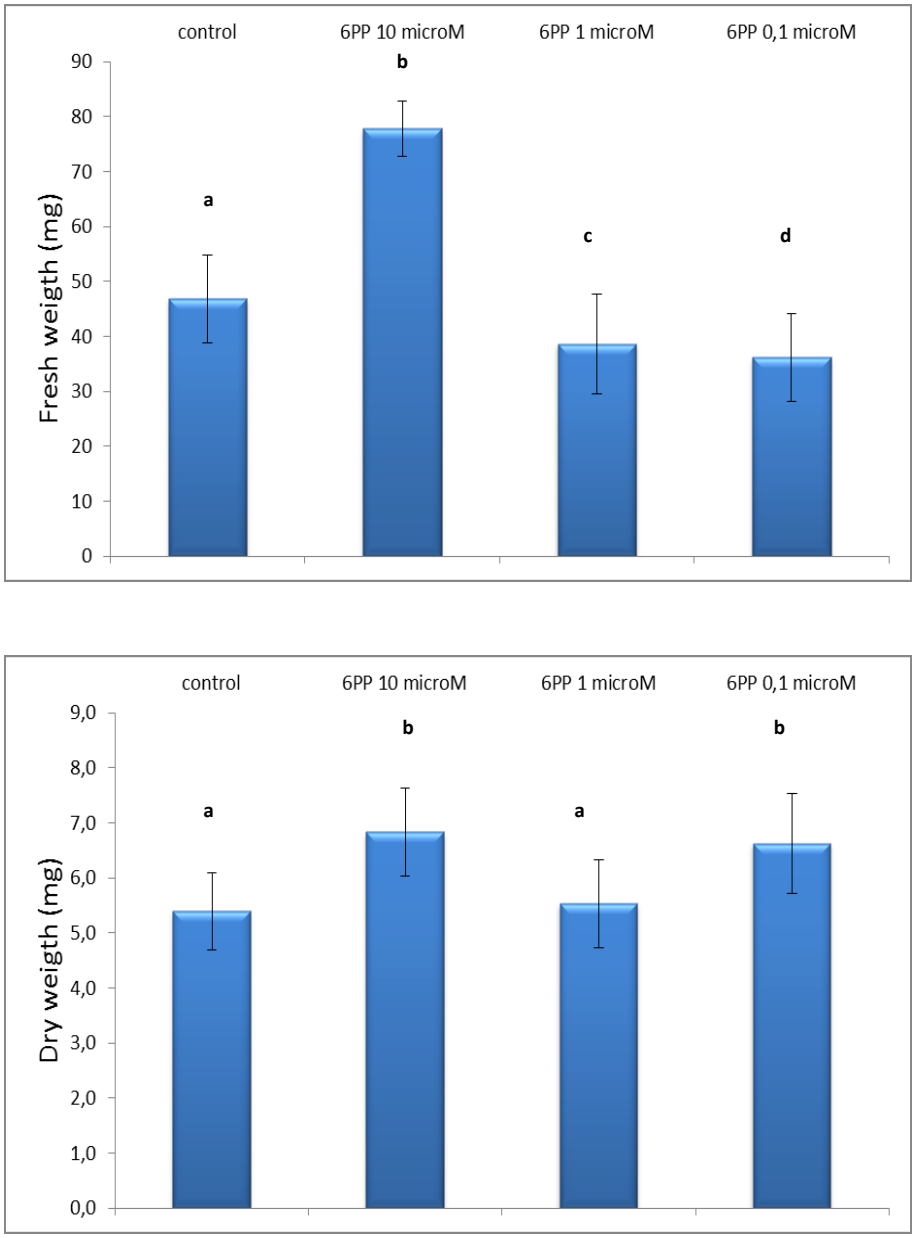

Figure 4.10: Effect of 6PP at different concentration on tomato fresh/dry weight. Concentration ranged from 10 to 0.1 µM. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

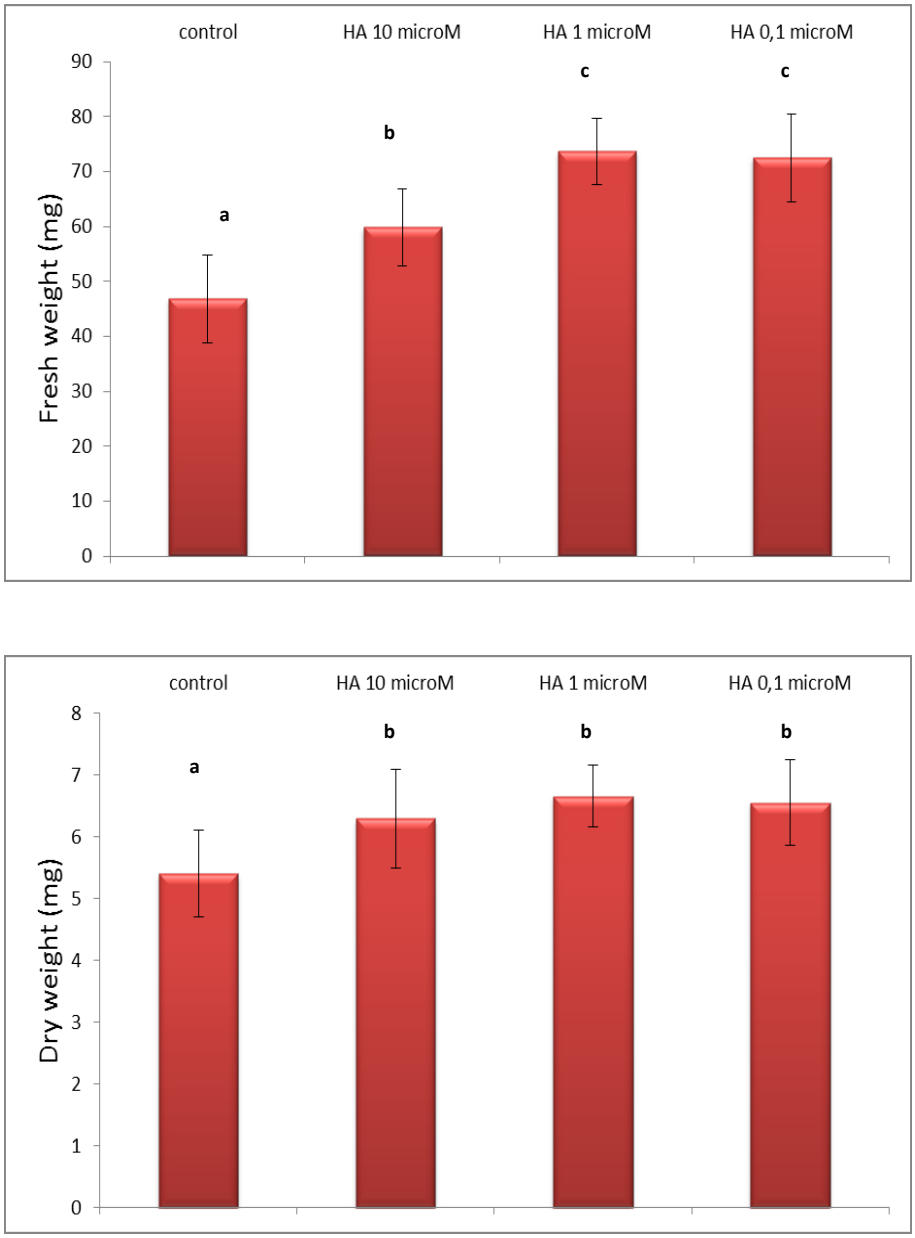

Figure 4.11: Effect of HA at different concentration on tomato fresh/dry weight. Concentration ranged from 10 to 0.1 µM. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly ($P < 0.05$).

4.3.2. Effect of purified metabolites 6PP and HA on *Solanum lycopersicum* cv. San Marzano: seed germination assay

In vitro assays (Figure 4.12) were performed to determine the effect on tomato seed germination of different combinations of HA, 6PP and the hydrophobin Hytra1,

obtained from *T. longibrachiatum* strain MK1 (Ruocco et al. 2008). As reported in the figure 3.13 seed germination was increased by 44% using a mixture of HA 1µM and 6PP 1µM, while by 42 % using HA 10 µM plus 6PP 10 µM. Application of the metabolites singly (HA, 6PP and Hytra 1) also stimulated germination at different concentration. In particular the percentage values were, respectively, by 39% and 42% for HA 10µM and 1µM, while, by 41% and 27% for 6PP 10µM and 1µM.

Unexpectedly, the combination of Hytra 1 (0.01µM), HA (1µM) and 6PP (1µM) stimulated seed germination (33%) less than the treatment with the protein alone (49%) (Figure 4.13).

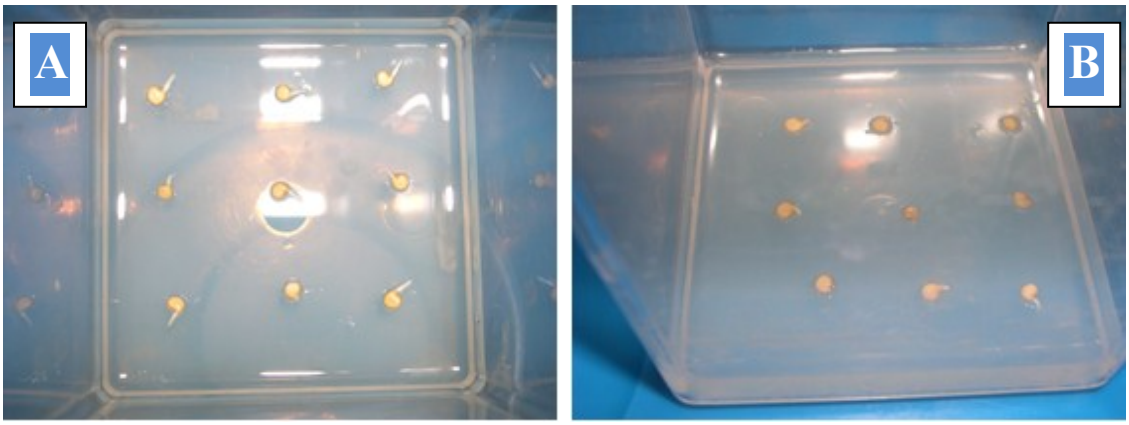

Figure 4.12: Effect of HA 10µM (A) on tomato seed germination compared with the control (B)

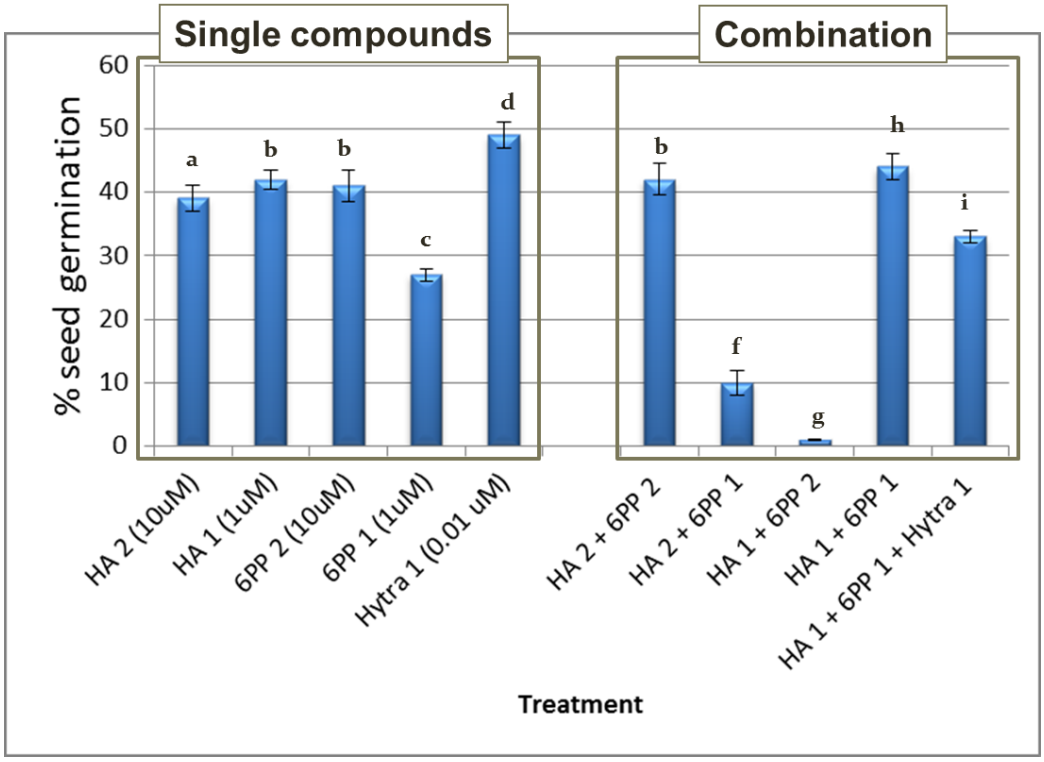

Figure 4.13: Effect of *Trichoderma* metabolites or their combinations on tomato seed germination. Concentration ranged from 10 to 0.01 µM. Percentages of seed germination relatively to the untreated control are indicated. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

4.3.3. Effect of *Trichoderma* metabolites and their combination on *Solanum lycopersicum* cv. San Marzano cuttings: root growth promotion assay.

The effect of *Trichoderma* metabolites on root development of tomato cuttings was also evaluated. Tomato seeds were allowed to germinate in the dark in sterile plastic boxes containing a salt medium SM (see Material and Methods section) plus sucrose 1.5 % for 10 days. The small seedlings were cut into small pieces that were transferred to new sterile boxes containing different solutions of the *Trichoderma* metabolites and the commercial formulation GE (Germon E). The figures 4.14, 4.15 and 4.16 show the results of this experiment. The effects of the fungal metabolites and of the commercial rooting hormone formulation were morphologically different: while the purified compounds stimulated the formation of true roots, the hormone preparation induced the formation of calli from which an array of new roots was then generated (not shown).

Generally, only the treatments containing Hytra 1 at 0.01µM and HA alone at 0.1µM consistently stimulated root length, dry and fresh weight (Figures 4.14, 4.15 and 4.16), while the use of the metabolite combinations, also of rooting hormone, inhibited the root development, in terms of length.

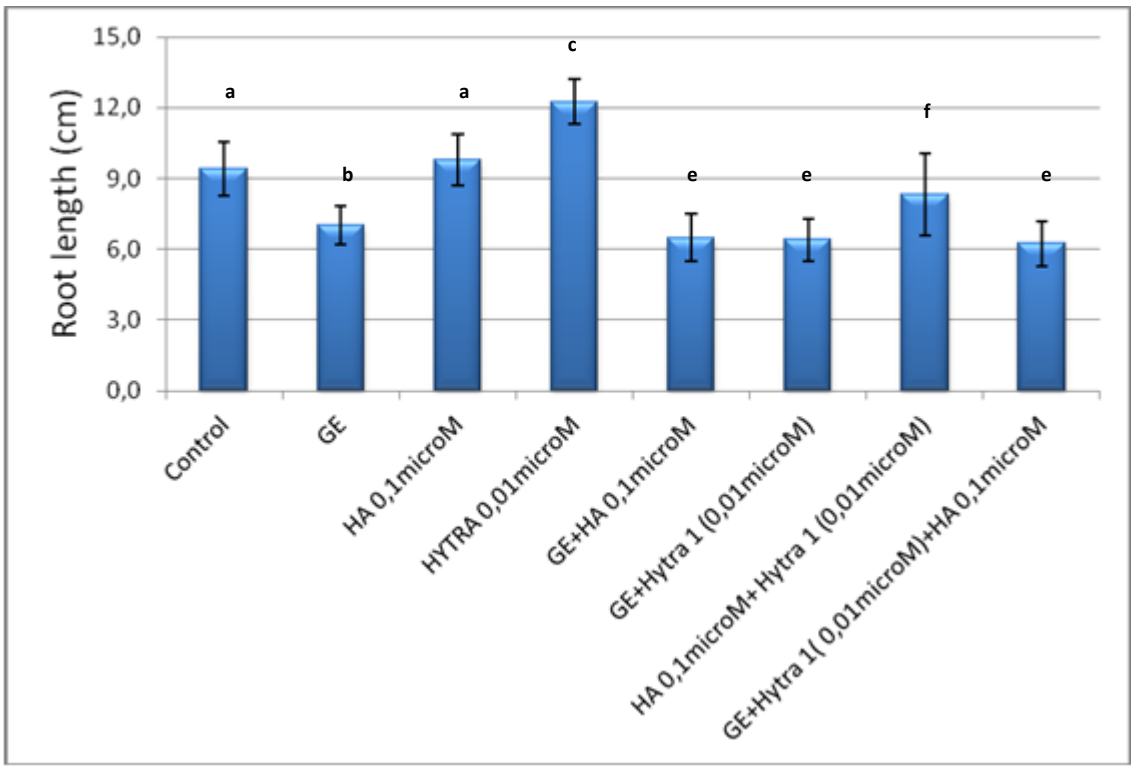

Figure 4.14: Effect of *Trichoderma* metabolites or their combination, also the product GE (Germon E), on tomato root development (length). Concentration ranged from 0.1 to 0.01 µM. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

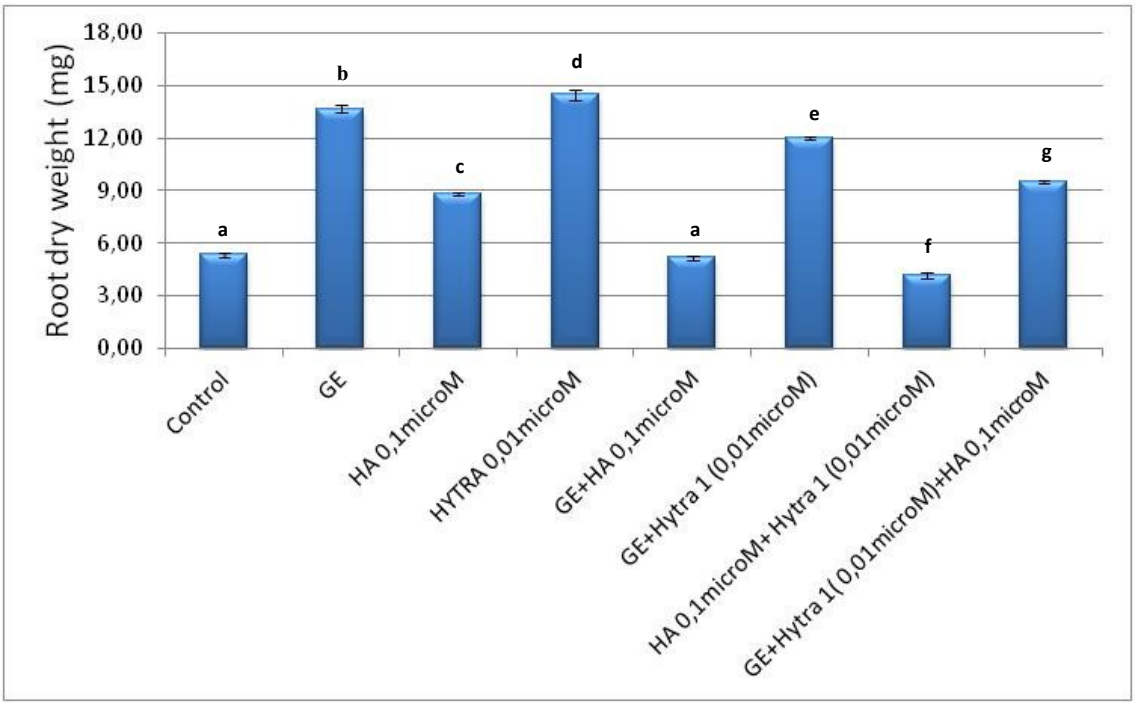

Figure 4.15: Effect of *Trichoderma* metabolites or their combination, also the product GE (Germon E), on tomato root development (dry weight). Concentration ranged from 0.1 to 0.01 µM. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

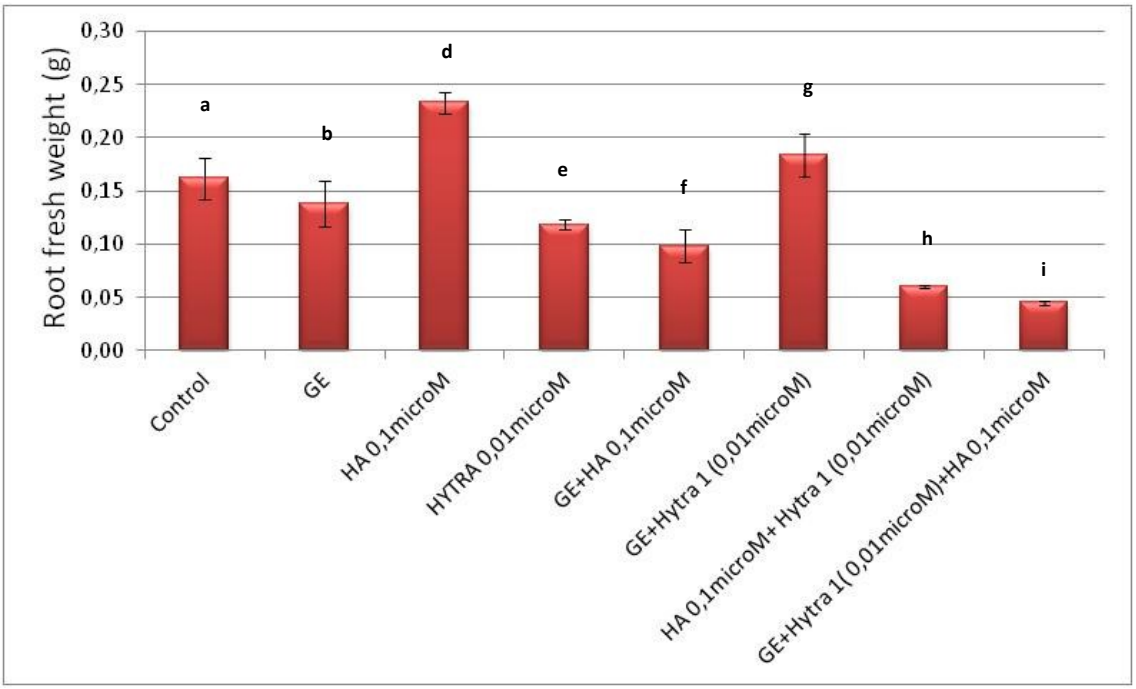

Figure 4.16: Effect of *Trichoderma* metabolites or their combination, also the product GE (Germon E), on tomato root development (fresh weight). Concentration ranged from 0.1 to 0.01 µM. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

4.3.4. Effect of *Trichoderma* metabolites and their combination on *Solanum lycopersicum* cv. San Marzano: root growth promotion assay plate experiments.

In vivo experiments were carried out to assess the root growth promotion activity of Hytra1, HA and 6PP and their combinations. Tomato seeds were placed between two glass plates separated by 2mm of soil as shown in figure 4.17, and the thin layer was watered with the metabolite solution (HA 1µM, 6PP 1µM, Hytra 1 0.01µM, HA 1µM/ 6PP 1µM, HA 1µM/ 6PP 1µM/ Hytra 1 0.01µM).

Root length was measured 7 days after the treatment.

Table 4.2 Effect of HA and 6PP at different concentration (10 µM to 0.1 µM per pot) on tomato root length (percentage increase compared to control).

Treatment	root length	root fresh weight	root dry weight
HA 1µM	+26%	+50%	+41%
HA 1µM + 6PP 1µM	+37%	+40%	+19%
HYTRA 1 0.01µM M	+26%	+55%	+41%
HA 1µM + HYTRA 1 0.01µM	+25%	+31%	+45%

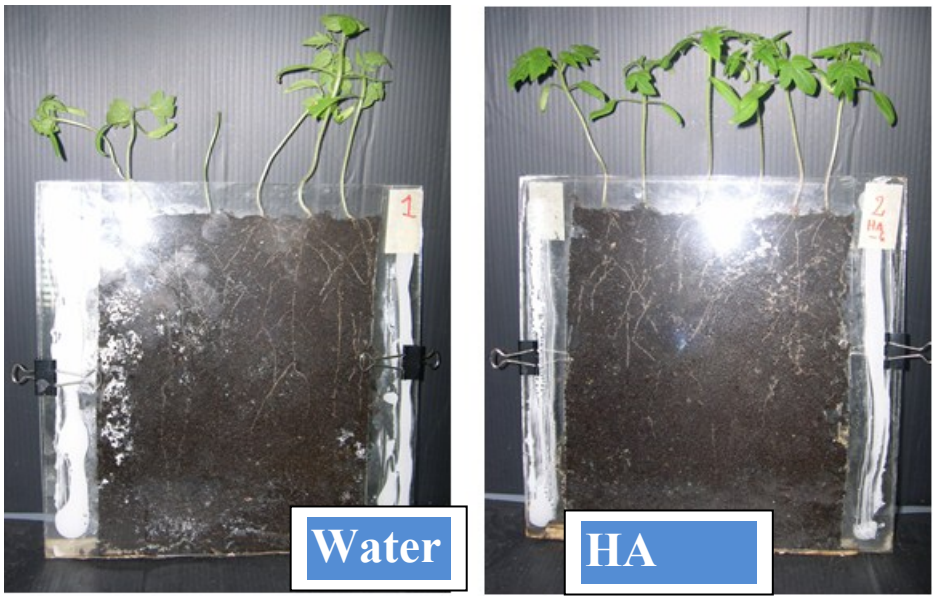

Figure 4.17: Effect of HA on tomato seedling placed in soil on the glass plate experiment. HA treatment was at 1µM.

Either HA, Hytra 1 or their combination promoted tomato development in terms of root length (table 4.2). 6PP showed significant positive results only in combination with HA (data not shown). Moreover, the stems of tomato seedlings treated with the metabolite solution, particularly with HA solution or its combination, grew more uniform compared to the control.

4.3.5. Effect of *Trichoderma* metabolites and their combination on *Solanum lycopersicum* cv. San Marzano: plant growth promotion assay in pot experiment.

The application of the *Trichoderma* metabolites (HA, 6PP, Hytra 1 and their combinations) in pot experiments affected the shoot and root length of tomato plants (Figure 4.18 and 4.19).

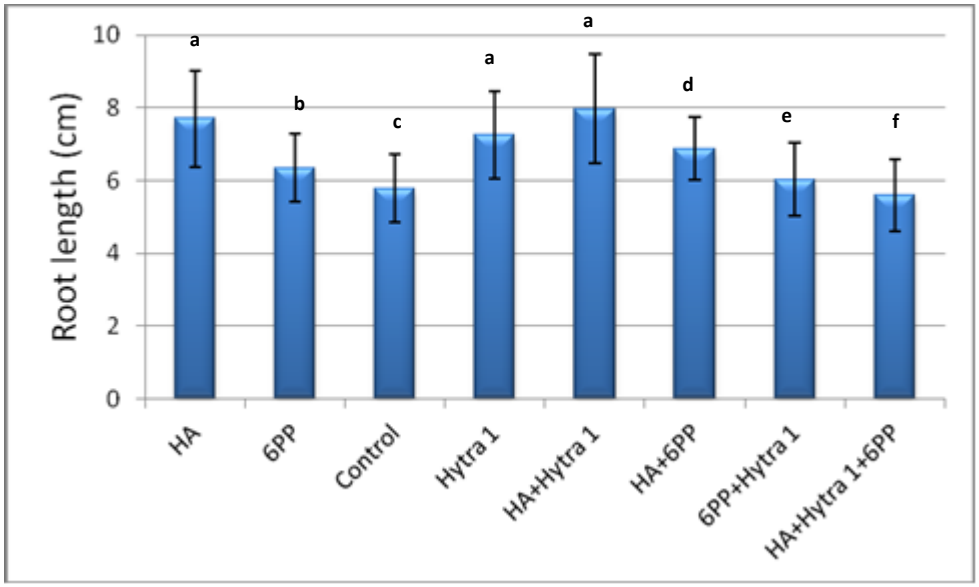

Figure 4.18: Effect of *Trichoderma* metabolites or their combination on tomato root growth. Compound concentration: HA 0.1µM, 6PP 0.1µM and Hytra 1 0.01µM. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

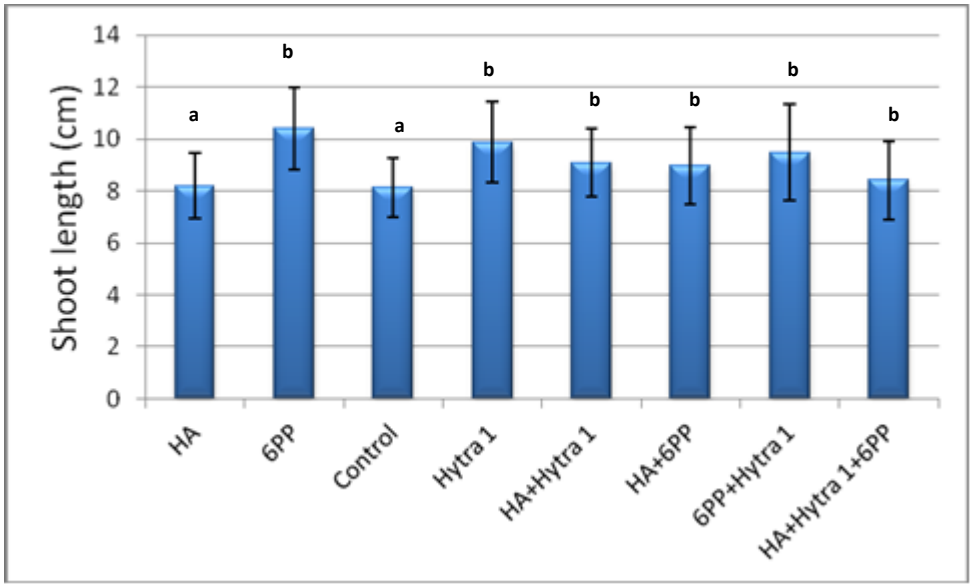

Figure 4.19: Effect of *Trichoderma* metabolites and their combination on tomato shoot growth. Compound concentration: HA 0.1µM, 6PP 0.1µM and Hytra 1 0.01µM. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

HA (0.1µM), Hytra 1 (0.01µM) and 6PP (0.1µM) increased the root length by 33%, 25% and 9% respectively (Figure 4.18). The highest level of promotion (37%) was obtained by combining HA (0.1µM) and Hytra 1 (0.01µM). No significant differences were detected with other metabolite combinations except for HA (0.1mM) plus 6PP (0.1mM), which increased the root length by 19%. The shoot length was not affected by HA application. On the contrary, it was improved by 27% with 6PP (0.1mM) and 21% with Hytra 1 (0.01mM) treatment (Figure 4.19). Combinations of metabolites did not produce significant differences in comparison with the single compound application.

4.4. Effect of *T atroviride* P1, *T. harzianum* M10, 6PP and HA on *Brassica rapa* subsp. sylvestris var. esculenta ecotype "*Sessantino*" (Broccoli)

In this part we have observed the effect of treatment with two *Trichoderma* strains (*T. harzianum* M10 and *T. atroviride* P1) and two secondary metabolites (HA and 6PP) on *Brassica rapa* growth. Moreover we measured the effect of application on the

glucosinolate production. These are compounds very important in plant defense as well as in growth.

4.4.1. Plant growth promotion *in vivo*

The effect of *T atroviride* P1, *T. harzianum* M10, 6PP and HA on *Brassica rapa* subsp. *sylvestris* var. *esculenta* ecotype *"Sessantino"* growth was evaluated by measuring shoots length and fresh/dry weight.

Shoot growth was enhanced by the application of M10, P1 (50 ml at concentration of 10^9 spore/ml for pot), as well as purified HA and 6PP (50 ml at concentration of 1 µM for pot). M10, P1, HA and 6PP increased shoot length by 60%, 63%, 79% and 30% (Figure 4.20).

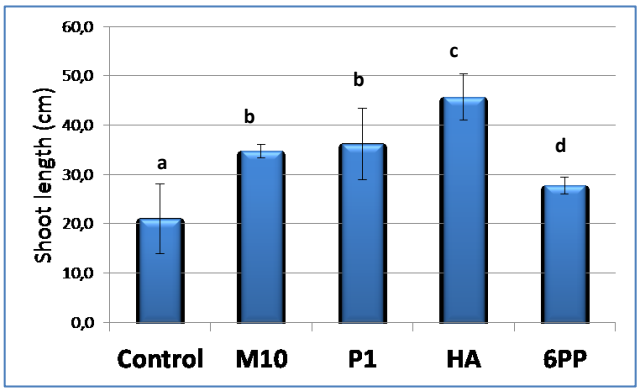

Figure 4.20: Effect of M10, P1 (10^9spore/ml), purified HA (1 µM) and purified 6PP (1 µM) on *B. rapa* growth. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

A promoting effect of the living fungi (M10 and P1) and the purified metabolites was also observed on both fresh and dry weight of the whole plant (Figure 4.21). *Trichoderma* M10 and 6PP increased fresh and dry weight by around 50% if applied at concentrations of 10^9 spore/ml and 1 µM, respectively; while, *Trichodema* P1 and HA, used at the same rate, increased fresh and dry weight by around 30%.

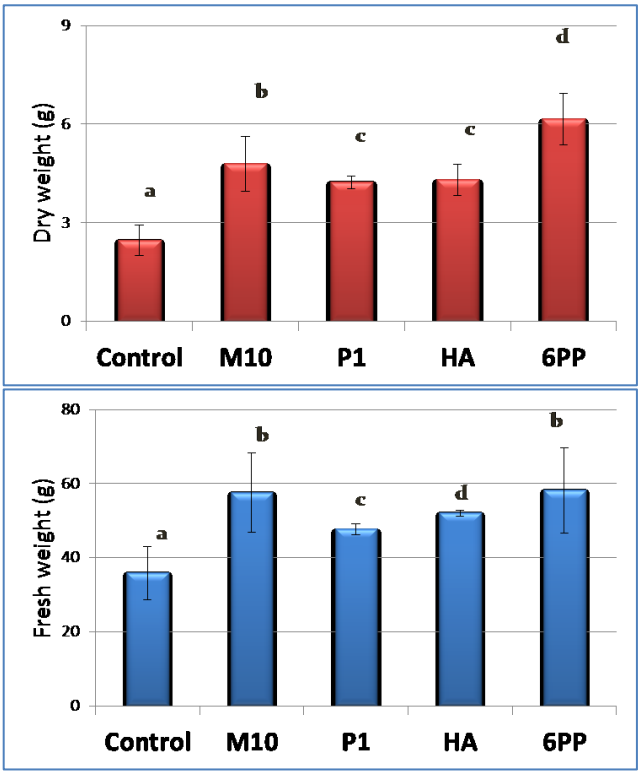

Figure 4.21: Effect of M10, P1 (10^9spore/mL), HA (1 µM) and 6PP (1 µM) on *B. rapa* fresh/dry weigth Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

4.4.2. Effect on the plant: production of glucosinolates

The effect of treatment with two *Trichoderma* strains (M10 and P1 used at concentration of 10^9 spore/ml), and two purified metabolites 6PP and HA (1 µM) on accumulation of 4 different glucosinoletes was determined at different time points (12, 24, 36 and 72 hours) after the application. The molecular weights and retention times, obtained by LC/MS/MS analysis, of the main glucosinolates are shown in the table 4.3.

Table 4.3: Molecular weight and retention time (RT) of the main glucosinolates extracted from *Brassica rapa*

GLUCOSINOLATES	MOLECULAR WEIGHT (g/mol)	RT (min)
neoglucobrassicin	477	19.03
glucoiberin	422	18.39
glucobrassicin	447	17.08
gluconapin	372	11.48

Interestingly the LC-MS/MS analysis of the plant extracts showed a decreased level for all the glucosinolates tested at 72 h (end of the experiment) for any of the treatments (Figure 4.22 to 4.25). Among the metabolites, only 6PP increased the accumulation of neoglucobrassicin, glucobrassicin and gluconapin, which was evident already at 36h (Figure 4.24 and 4.25).

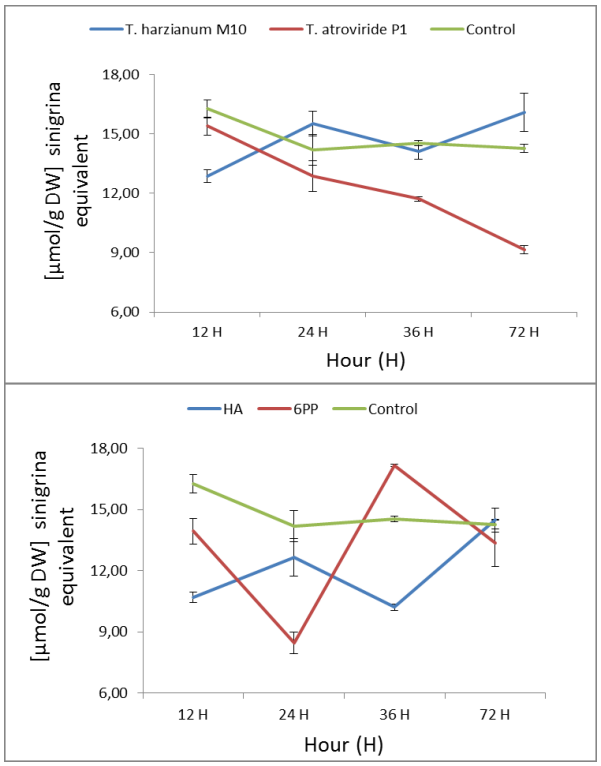

Figure 4.22 Effect of M10/P1 fungi (10^9spore/mL) and HA/6PP metabolites (1 µM) on *B. rapa* neoglucobrassicin production at different time after treatment

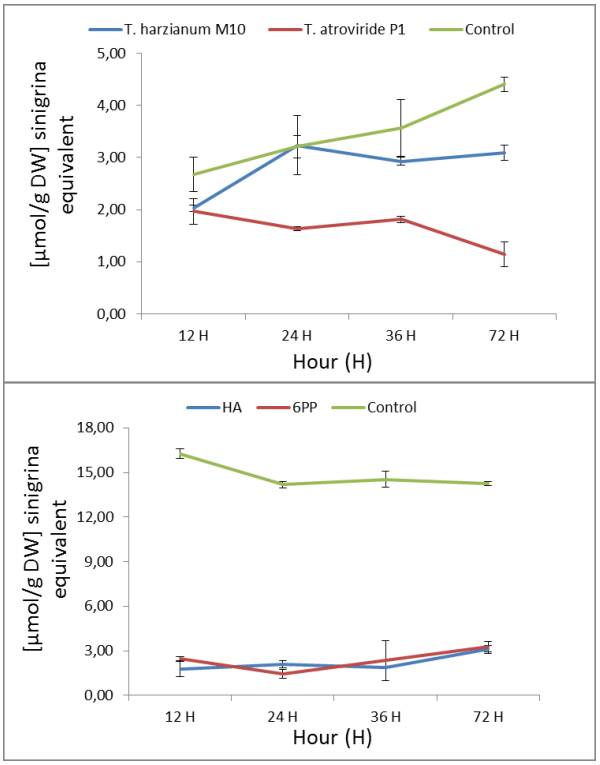

Figure 4.23 Effect of M10/P1 fungi (10^9spore/mL) and HA/6PP metabolites (1 µM) on *B. rapa* glucoiberin production at different time after treatment

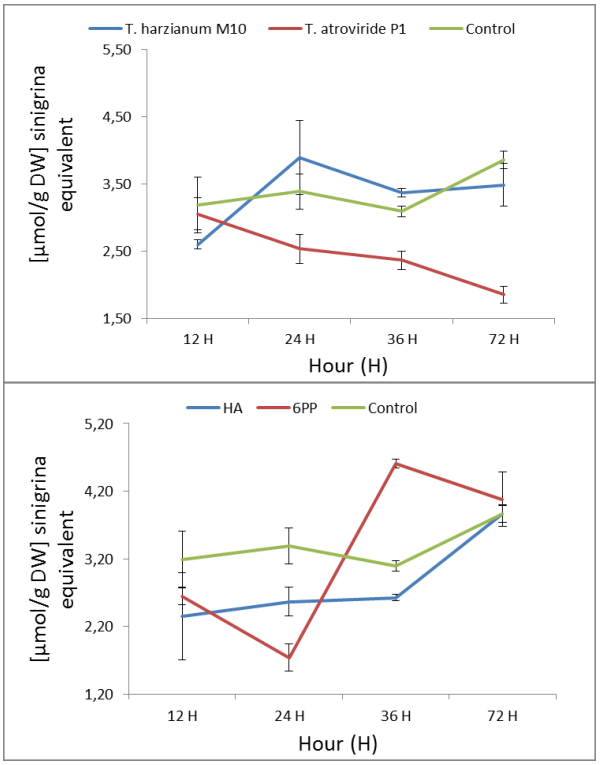

Figure 4.24 Effect of M10/P1 fungi (10^9spore/mL) and HA/6PP metabolites (1 µM) on *B. rapa* glucobrassicin production at different time after treatment

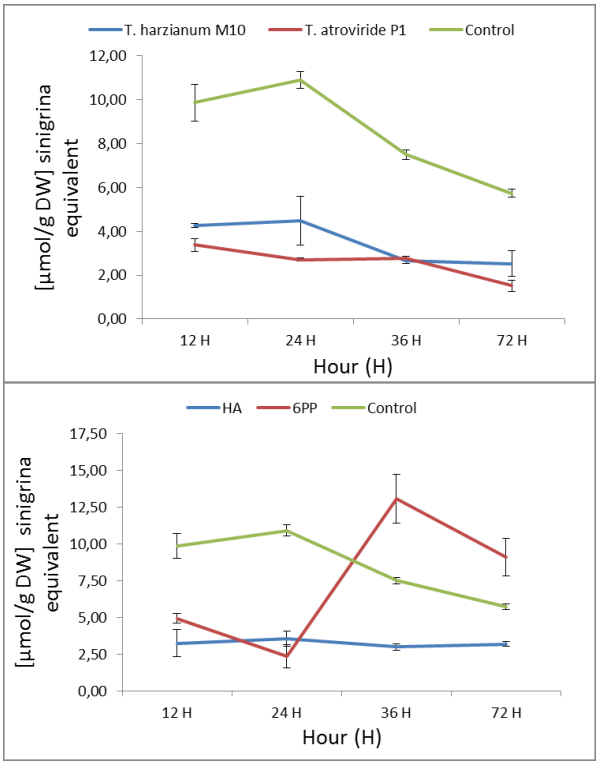

Figure 4.25 Effect of M10/P1 fungi (10^9spore/mL) and HA/6PP metabolites (1 µM) on *B. rapa* gluconapin production at different time after treatment

If the experiment was prolonged until the end of the plant vegetative cycle, different glucosinolate levels were found. An increased level of glucosinoletes was found for *T. atroviride* P1 treatment (up to 50%) (neoglucobrassicin, glucoiberin, glucobrassicin and gluconapin), *T. harzianum* M10 treatment (neoglucobrassicin and glucobrassicin), HA (neoglucobrassicin, glucoiberin, glucobrassicin and gluconapin) and 6PP (glucoiberin and gluconapin). In this case the effect was lower in comparison with living-fungus application (Figure 4.26 and 4.27).

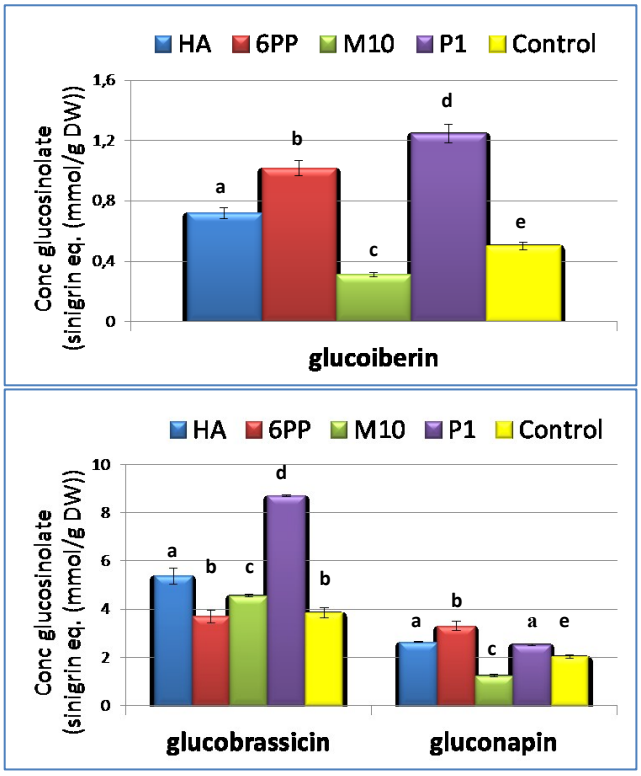

Figure 4.26: Effect of treatments on glucoiberin, glucobrassicin and gluconapin concentration at the end of vegetative cycle.Values are means of 5 replicates. Bars indicate standard deviation.

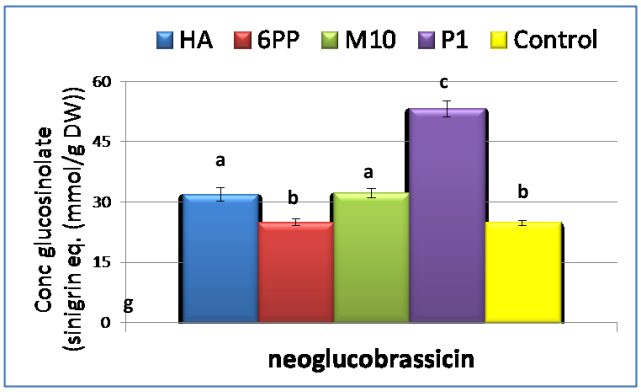

Figure 4.27: Effect of treatments on neoglucobrassicin concentration at the end of vegetative cycle. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly ($P < 0.05$).

4.5. Effects of *T atroviride* P1, *T. harzianum* M10, 6PP and HA on *Vitis vinifera* cv. Sangiovese

In this part we investigated if the application of living BCA, belonging to *Trichoderma* genus (used as alternative to synthetic pesticides) applied on *V. vinifera* (cv. *Sangiovese*) plants, could be improved or substituted by treatments with selected bioactive secondary metabolites (obtained from beneficial microbes) able to: i) inhibit the pathogen; ii) promote BCA antagonistic activity; iii) induce systemic resistance in the plant; iv) stimulate growth and development of different cultures.

4.5.1. Plant growth promotion in growth chamber.

In vivo experiments on *V. vinifera* were performed in growth chamber at temperature of 25 °C. The plants were drenched or sprayed with 50ml of a *Trichoderma* solution (*T. atroviride* P1 and *T. harzianum* M10 strains at 10^9 spores/mL) or with 50 ml of a secondary metabolite solution (HA and 6PP) at concentration of 10 and 1 µM. The effects on the plant appearance were determined 30 days after. The plants treated with M10 and P1 were tell, more developed and carried leaves apparently greener as compared with untreated controls (Figure 4.28). No significant differences were found among treatments applied as spray or drenching. Application of fungi or 6PP (not in the case of HA) by spraying or drenching did not produce significant differences between same treatments.

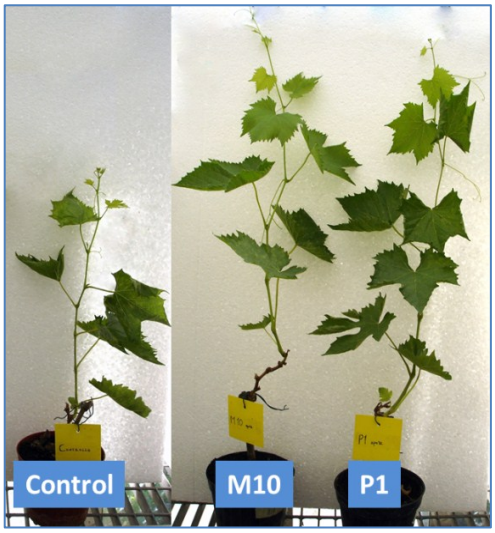

Figure 4.28: Effects of M10 or P1 (10^8 spores/ml) on grapevine growth.

6PP improved plant development and increased leaves size compared to controls, with a dose-dependent effect (10µM > 1µM) (Figure 4.29).

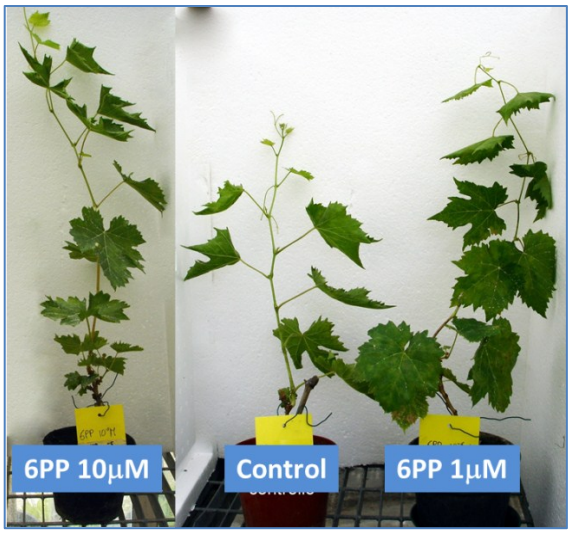

Figure 4.29: Growth promotion effect on grapevine plants watered with 6PP solutions at concentrations of 10 and 1 µM.

The grapevine growth, in terms of shoot-length and leaves size, was enhanced by drenching the soil with HA (50 ml of a HA solution at 10 and 1µM) but a phytotoxic effect (i.e. chlorosis, irregular development of leaves) was detected when HA was sprayed at the concentration of 10 µM directly on the leaves. Application of HA at the

lower concentration (1µM) produced no significant effects neither positive nor negative (Figure 4.30).

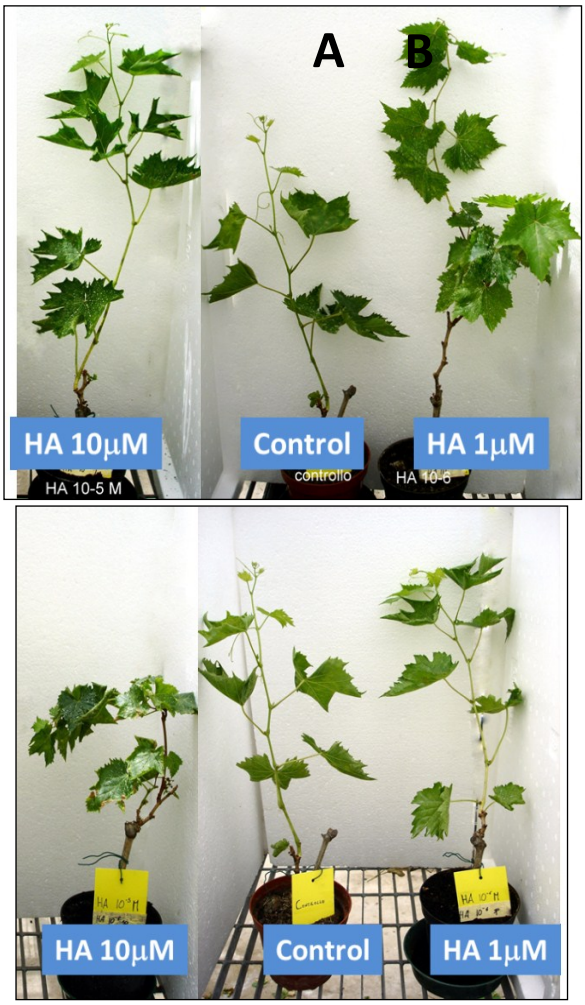

Figure 4.30: Differences between grapevine plants watered (A) and sprayed (B) with HA solutions at different concentrations (10 and 1 µM.)

4.5.2. Plant growth promotion and other effects in field experiment.

Field experiments (Figure 4.31) were carried out in order to evaluate the effects of treatment on *V. vinifera* of the *Trichoderma* metabolite, 6PP (5 L applied at a concentration of 1 µM for 3 rows of plants), in comparison to a commercial formulation based on the highly-effective strain T22 of *T. harzianum* (5 L applied at a concentration

of 10^8 sp/ml for 3 rows of plants). Data were collected only at the end of the experiments (90 days after) by evaluating:

i) Average of grape cluster;

ii) antioxidant activity in the grape;

iii) total amount of polyphenols in the grape;

iv) HPLC profiles of anthocyanins and polyphenols in the grape.

Figure 4.31: Field experiment performed at ARBOPAVE department of the University of Naples "Federico II"

6PP and T22 treatments increased average of grape cluster (in terms of Kg) by 63% and 97%, respectively, in comparison to untreated plants (Figures 4.32 and 4.33).

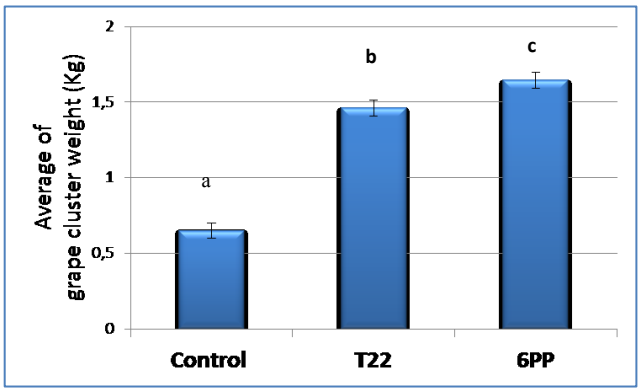

Figure 4.32: Effects of 6PP and T22 on grape production. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

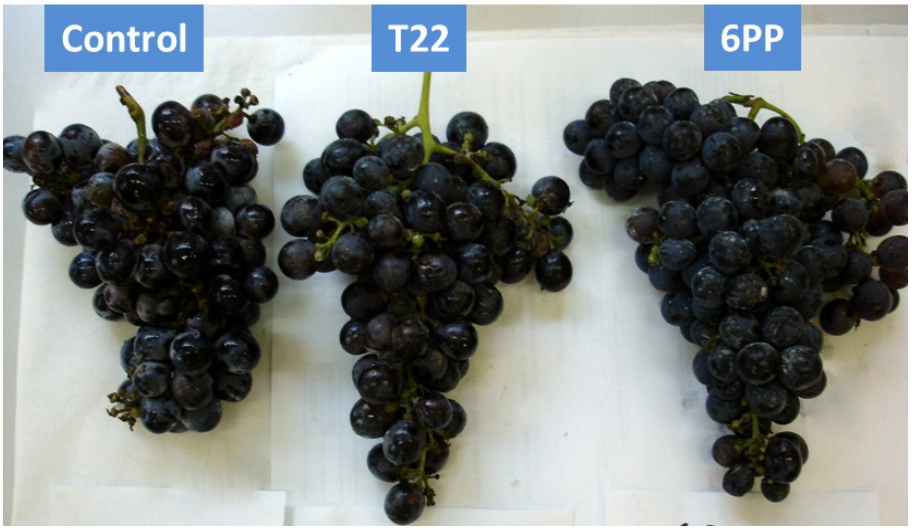

Figure 4.33: Effects of 6PP and T22 on bunch size

In order to evaluate if 6PP and T22 can affect the quality of the fruit, total antioxidant activity in the grapes was measured using the ABTS assay. In this method the antioxidants present in the sample reduce the absorbance of the pre-formed radical cation ABTS depending on the antioxidant activity level, the concentration of the antioxidant and the duration of the reaction. Thus, the extent of decolorization as "percentage inhibition" of the $ABTS^{\bullet+}$ radical cation is determined as a function of concentration and time and calculated relatively to the reaction of Trolox, used as a standard, under the same conditions.

This activity increased after the treatments with *T. harzianum* T22 and 6PP by 48.7% and 60.3%, respectively (Figure 4.34).

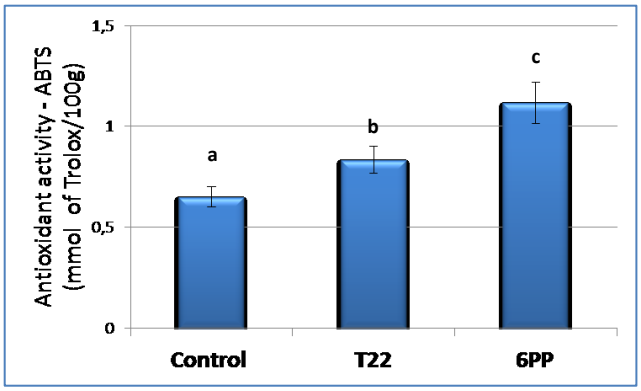

Figure 4.34: Effect of 6PP and T22 on antioxidant activity of the grape. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

Polyphenol concentration was measured in triplicate for each sample by using the Folin-Ciocalteu reagent. Gallic acid was used as standard (absorbance measured at 765 nm). Results are reported in figure 4.35 as mg equivalents of gallic acid.

In the control the polyphenol concentration was lower than in the samples obtained by the treated plant, with no significant differences between the two treatments.

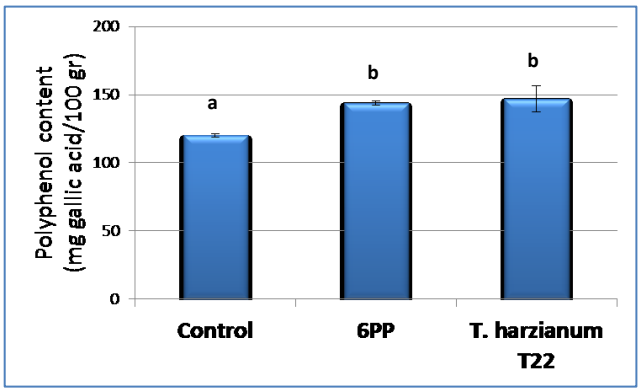

Figure 4.35: Effect of 6PP and T22 on polyphenol production of the grape. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

The HPLC analysis showed different chromatographic profiles among the treatments in terms of polyphenol concentrations (peaks area). The polyphenols were detected by using three different wavelengths:

- anthocyanins at λ 510 nm (two peaks) ;
- flavonols (quercetin and rutin) at λ 360 nm (two peaks);
- stilbene (resveratrol) and flavan-3-ols (catechin and epicatechin) at λ 280 nm (two peaks).

Both treatments increased all peak areas as compared to the control, with the exception of two peak areas detected at 360 and 280 nm for the T22 treatment (table 4.4).

Table 4.4: Effect of T22 and HA treatment of grapevine determined with HPLC analysis. In the last two raw indicate indicate the percentage of peak area compared to untreated control.

Wavelength λ (nm)	510		360		280	
n. peaks	1	2	1	2	1	2
RT (min)	16,2	18,5	17,2	18,1	3,4	23,6
6PP	56	77	46	46	26	73
T22	49	70	26	-14	8	-18

4.6. Effects of 6PP, HA and Hytra 1 on *Arabidopsis thaliana* ecotype Columbia (col-0)

In this part we examined the effect of *Trichoderma* metabolite treatments on *A. thaliana* (col-0) growth. Moreover, we investigated the plant metabolic changes and measured the alterations in the level of hormones related to growth and development, as well as to defense response.

4.6.1. Plant growth promotion

Experiments *in vitro* were performed by applying on *A. thaliana* (col-0) the secondary metabolites HA and 6PP at concentration of 0.1µM, and the *Trichoderma* protein Hytra 1 at concentration of 1nM.

The root length was improved by treatment with the metabolites and the protein indicating with HA and Hytra 1 the highest effect (Figure 4.36 and 4.37 A).

Furthermore, all metabolites stimulated secondary roots production (Figure 4.37 B): 110% of increment was observed with HA, while 56.4% and 62.5% with 6PP and Hytra 1, respectively.

Figure 4.36: *In vitro* effect of *Trichoderma* metabolites HA or 6PP (0.1µM) and Hytra 1 (1 nM) on *A. thaliana*.

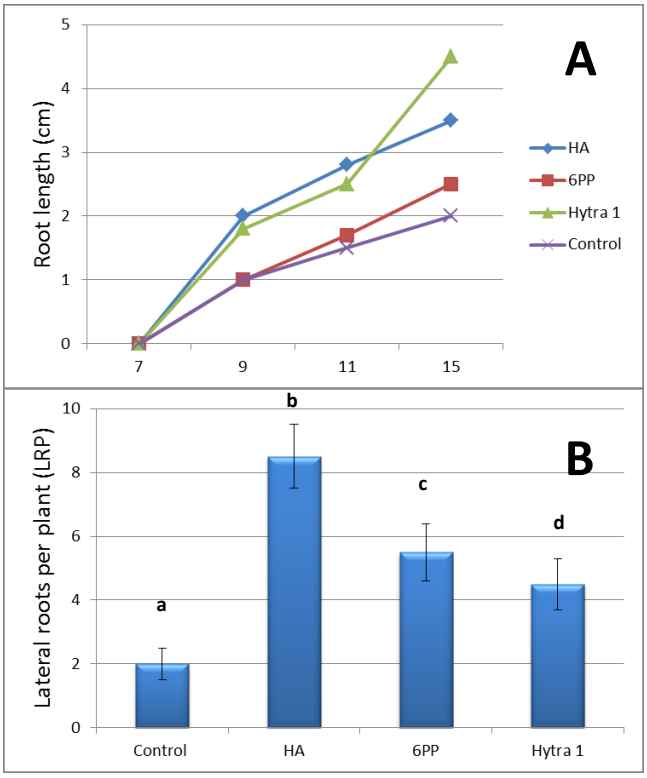

Figure 4.37: Effect of *Trichoderma* metabolites HA, 6PP (0.1µM) and Hytra 1 (1 nM) on the root length (A) and secondary roots production(B) of *A. thaliana*, the latest measured as number of later roots per plant (LRP). Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

In vivo tests confirmed the plant growth promotion activity of purified *Trichoderma* metabolites (Figures 3.38 and 3.39).

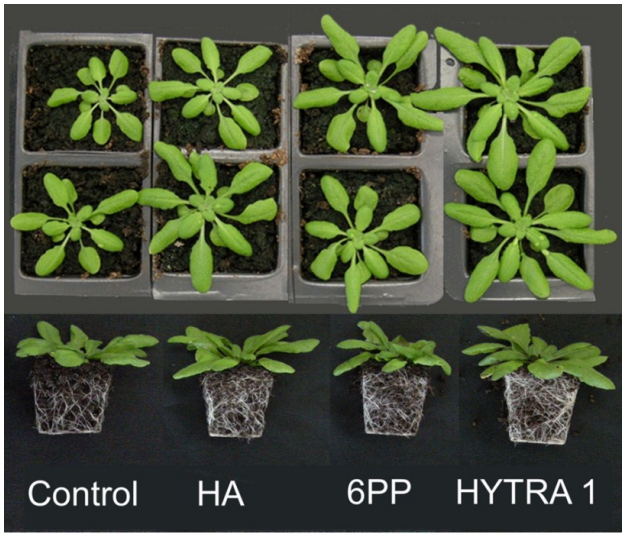

Figure 4.38: *in vivo* effect of purified *Trichoderma* metabolites HA, 6PP (0.1µM) and Hytra 1 (1 nM) on *A. thaliana* (col-0)

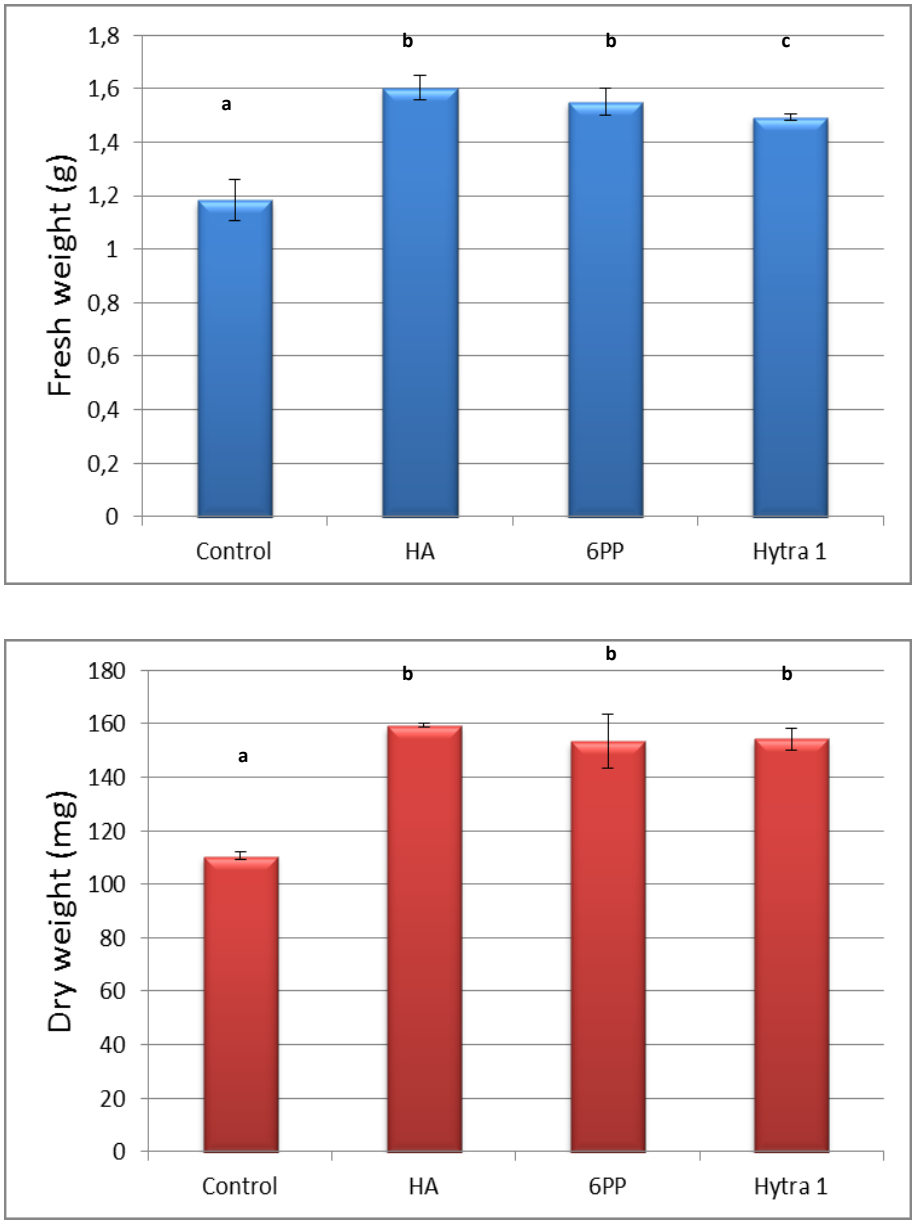

Figure 4.39 Effect of *Trichoderma* metabolites on *A. thalina* plant fresh and dry weight. Values are means of 5 replicates. Bars indicate standard deviation. Values with the same letter do not differ significantly (P < 0.05).

4.6.2. Metabolic changes in *A. thaliana*.

Leaf tissue from the treated and untreated plants was extracted with methanol/water (80/20 v:v). The extract was subjected to LC-MS-Qtof analysis in order to analyze the plant metabolome. The multiple output data were processed using the principal

components analysis (PCA). PCA of full unfiltered data at 95% confidence intervals was used to evaluate the major changes in the metabolome (figure 3.40).

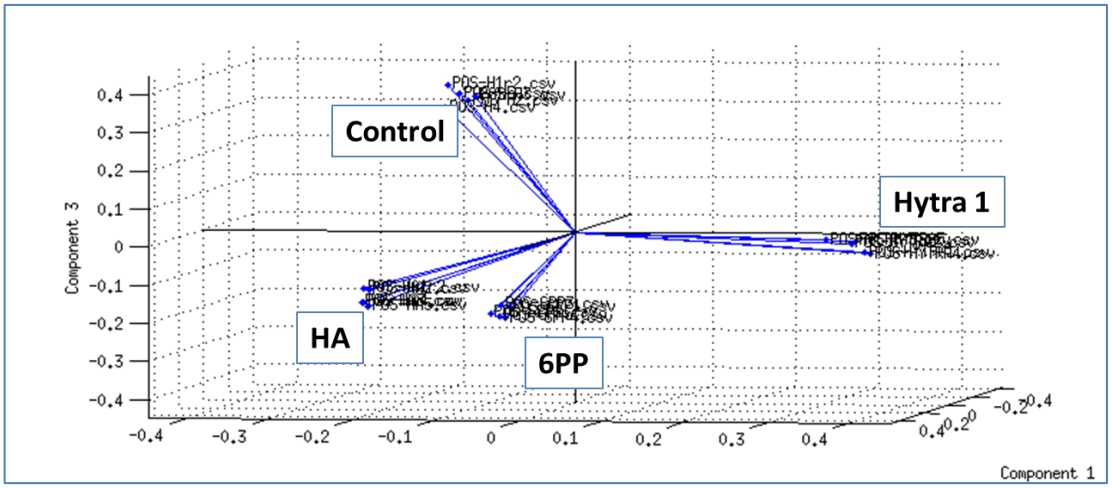

Figure 4.40 Differences between the effect of *Trichoderma* metabolites on *A. thaliana* also versus the untreated control determined by PCA (principal component analysis) performend with LC-MS-Qtof assay 80% methanol extracts. Data points represent biological replicates (five replicates in each experiment).

This analysis showed that the treatments were clearly separated and the samples clustered along different trajectories. The metabolome was affected by *Trichoderma* metabolites, with HA and 6PP causing a similar global change of the metabolic profile, while a strongly different response was obtained for Hytra1.

The metabolome of *A. thaliana* (col-0) grown alone was used as a control for comparison with the three treatments (HA, 6PP, Hytra 1). More than 224 differential plant metabolites were significantly changed (produced *ex novo*, increased or decreased) when *A. thaliana* was exposed to the *Trichoderma* metabolites (Figure 4.41). In particular, when the HA treatment was compared to the control, 28 compounds appeared to be produced *ex novo*, 45 were up-regulated and 2 down-regulated, indicating that the presence of HA induces major changes in the metabolome of treated plant. Application of 6PP also determined a differential accumulation of several metabolites: 20 new, 15 metabolites were up-regulated and 3 down-regulated compounds.

The unique metabolic response of *A. thaliana* to Hytra 1, as determined by PCA, produced 10 novels, 5 increased and 2 decreased metabolites in the treated plant.

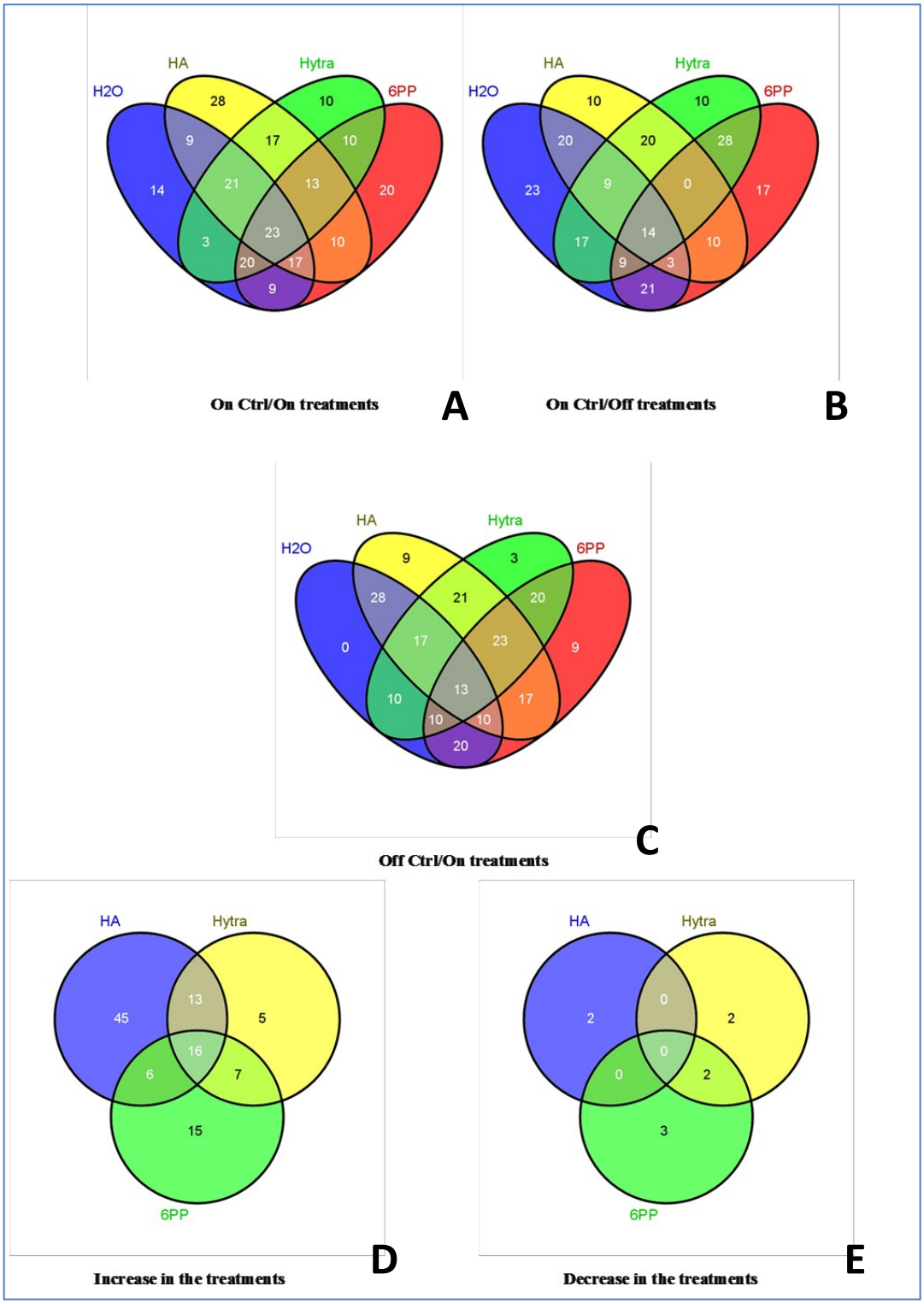

Figure 4.41 Changes occurring in the metabolome of *A. Thaliana* (Col-0) grown alone (H2O = control), with HA (HA), with 6PP (6PP) and Hytra 1 (Hytra). The numbers of plant metabolites in common between the different treatment (A) are indicated. The numbers of plant metabolites newly found (C) or unfound (B) are also indicated. In addition, the numbers of plant metabolites increased (in terms of concentration)(D) or decreased (E) compared to the control are indicated.

Finally the accumulation of plant hormones related to growth and development, as well as to defense response, was determined in the analyzed extracts.

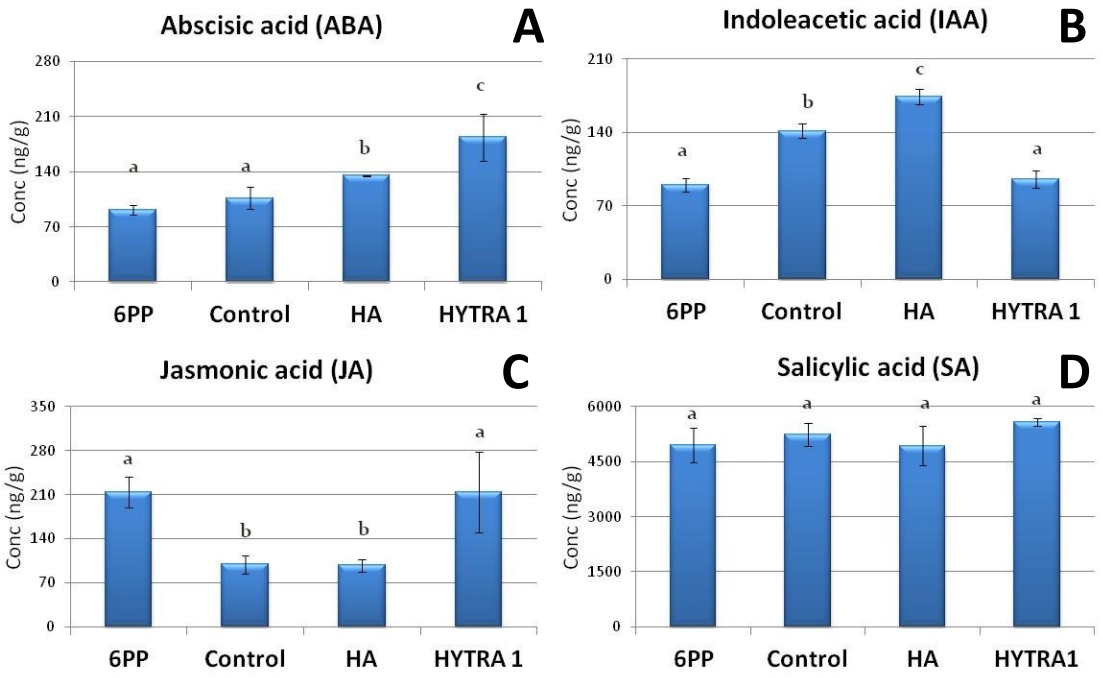

Figure 3.42: Effect of *Trichoderma* metabolites treatments (HA, 6PP (0.1µM) and Hytra 1 (1 nM)) on hormones accumulation*:* Abscisic acid (ABA) (a), Indolacetic acid (IAA) (b), Jasmonic acid (JA) (c).and Salicylic acid (SA) (d)

5. Discussion

The interaction of *Trichoderma* spp. with plants confers several benefits to the associated host including: i) the suppression of phytopathogens by using direct antagonistic mechanisms (i.e. antibiosis, mycoparasitism, competition for nutrient and space); ii) plant growth promotion; iii) enhanced nutrient availability and uptake, and iv) induction of plant resistance mechanisms (Howell, 2003; Harman et al., 2004; Vinale et al., 2008). In addition, some *Trichoderma* strains produce compounds that can cause substantial changes in the metabolism of the host and enhance the ability of *Trichoderma* spp. to activate defense response and/or regulate plant growth (Vinale et al., 2008).

Harzianic acid (HA), 6-pentyl-α-pyrone (6PP) and the protein Hytra1 are *Trichoderma* metabolites that showed plant growth promotion activity. In the present study we investigated the chemical and biological properties of HA, 6PP and Hytra1 comparing the effect of their application on the plants with that of their producing fungi.

The metabolite, isolated by RP-18 vacuum chromatography of NaOH 2 M extract, shows the ^{1}H and ^{13}C parameters of HA (2-hydroxy-2-[4-(1-hydroxy-octa-2,4-dienylidene)-1-methyl-3,5-dioxo-pyrrolidin-2-ylmethyl]-3-methyl-butyric acid), a compound belonging to the chemical class of tetramic acids.

The naturally occurring tetramic acid derivatives have attracted significant attention because of their wide distribution and remarkable diversity of biological activities, including the chelation of Fe(III) (important for ion transport across cell membranes). It has been found that in some cases the metal complexes formed have a higher biological activity than their ligands taken singly (Royles, 1995; Ghisalberti, 2003; Schobert and Schlenk, 2008; Athanasellis et al., 2010).

Both the living microorganism and the purified HA, when tested in the CAS blue agar plates, caused a colour change of the substrate from blue to orange, suggesting that the the tetramic acid derivative is involved in the iron(III) binding properties of the fungus. Moreover, the LC-MS analysis of a HA – Fe^{+3} solution showed additional signals at 455.1 m/z and 491.1 m/z corresponding to a 1:1 chloride containing complex, [M-

$H+Fe(III)+Cl2+H]^+$ (m/z 491.1) or $[M-2H+Fe(III)+Cl+H]^+$ (m/z 455.1). Since chloride is a coordinating ligand for iron, it is possible that the chloride anion is directly bound to the metal (Caudel et al., 1994).

The value of K_d of HA–Fe(III) complex (1.79×10^{-25} M) may be directly compared with that of other chelators showing a 1:1 Fe:ligand stoichiometry, such as desferrioxamine (DFO), EDTA, 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione (HPD), pyoverdin and pyochelin (Kaufmann et al., 2005). As shown in Table 1, HA has lower affinity to Fe(III) than DFO, pyoverdin and HPD, while it has a stronger affinity for iron(III) than EDTA or pyochelin. These data suggest that HA could compete for available iron in solution supporting its solubilization by the fungus.

Table 1. Affinity constants of chelators for Fe(III).

Compound	K_d
EDTA	5.00×10^{-23} M
DFO	2.51×10^{-26} M
Pyoverdin	10^{-32} M
Pyochelin	10^{-5} M
HPD	1.6×10^{-29} M
HA	1.79×10^{-25} M

Siderophores produced by beneficial agents may have important effects on both microbial and plant nutrition. Fe3+ - siderophores complexes can be recognized and taken up by several plant species, and this activity is considered crucial for root iron uptake, particularly in calcareous soils (Weyens et al., 2009; Sharma et al., 2003). Our data suggest a role of HA in the competition for iron of *Trichoderma* with other microbes and in iron solubilisation for plant nutrition. Microbial siderophores are iron chelating agents that can regulate the availability of iron in the rhizosphere. It has been assumed that competition for iron depends on: i) the affinity of the siderophore for the metal; ii) the type and the concentration of the siderophores; iii) the kinetics of ion exchange; iv) and availability of Fe-complexes to microbes as well as plants.

The pyrone 6PP has been isolated from culture filtrate of *T. atroviride* strain P1 by flash chromatography. The chemical structure has been confirmed by NMR and mass spectra. 6PP was characterized by Collins and Halim (1971), while Moss et al. (1975) isolated the pyrone from *T. viride* and demonstrated its antibiotic activity against *Phytophthora cinnamomi*. Vinale et al. (2008) studied the plant growth promotion effect of this compound, highlighting its role in the *Trichoderma*-plant interaction.

The hydrophobin Hytra1 has been isolated from *T. longibrachiatum* strain MK1 culture filtrates. It is a protein of 71 aa, with a molecular weight of 7218 Da and 8 cysteine residues arranged in the strictly conserved motif X_n-C-X_{5-10}-C-C-X_{11-44}-C-X_{8-23}-C-X_{5-9}-C-C-X_{6-18}-C-X_m typical of class II hydrophobins. *Trichoderma* genus is considered to have the largest number of class II hydrophobins among ascomycetes (Seidl-Seiboth et al., 2011). Hydrophobins are involved in many processes including the formation of aerial hyphae, spores and fruiting bodies (Wösten, 2001). Hytra1 shows an antimicrobial activity against *B. cinerea* and *R. solani* both *in vitro* and *in vivo* tests (Ruocco et al., 2007). Ruocco et al. (2008) demonstrated that Hytra1 induces a hypersensitive reaction (HR) and systemic resistance in tomato plants.. Physiological analyses of tomato leaves treated with Hytra1 showed that this hydrophobin can induce an oxidative burst in plant cells. Low Hytra1 concentrations also triggered activation of the antioxidant system that controls the accumulation of reactive oxygen species (superoxide anions and peroxides). Process that leads to the accumulation of lipoperoxides and defense-related molecules such as riscitin and PR proteins.

In vivo assays of tomato plants (*Solanum lycopersicum* cv. Roma) have shown that HA and 6PP application stimulate seed germination and improve development in terms of root/stem length and fresh/dry weight. In particular, HA enhanced the plant growth when used at three different concentration (10, 1 and 0.1 μM), while 6PP showed the same effect only when applied at 10 μM. This result is in agreement with Cutler et al. (1986 and 1989) and Parker et al. (1995 and 1997) that reported the isolation, identification and biological activity of some secondary metabolites produced by *T. koningii* (koninginins A-C, E, G) and *T. harzianum* (6-pentyl-α-pyrone; 6PP), that affect plant growth. These metabolites had a concentration-dependent effect on wheat coleoptiles (phytotoxic activity detected at 10^{-3} M, but not at 10^{-4} M).

Effect of *Trichoderma* metabolite combinations on tomato growth has also been observed *in vitro* and *in vivo*. It has been determined the percentage of seed germination by using different metabolite mixtures. The results indicate that all the compounds used, singly or in combination, stimulate seed germination. However, only for the combination of HA 1μM with 6PP 1μM a strong synergic effect has been noted, while in some cases the other combinations produced an inhibitory effect.

A rooting-effect on tomato cuttings of HA, 6PP and Hytra1 or their combinations has been observed also in comparison with a commercial hormone formulation, the Germon E. The effects were different: while the fungal compounds stimulated the formation of true roots, the commercial preparation induced the formation of calli from which an array of new roots was then generated. However, among the combinations only those with Hytra1 stimulated the root development in terms of length. Ruocco et al. (2009) demonstrated that Hytra1 induces plant root growth in a dose-dependent manner. The protein can affect the auxin pathway because Hytra1 at 0.3 μM stimulate root development in terms of length, tomato cuttings immerse in a solution containing Hytra1 form *de novo* roots and cuttings from Hytra1-expressing plants immerse in water are stimulated in terms of root formation.

Application of two *Trichoderma* strains (P1 and M10) or their metabolite (6PP and HA) affected *Vitis vinifera* growth. In particular, an effect on the plant was observed when the fungi or purified compounds were drenched on the soil or sprayed on the leaves. The treatments improved the plant development, but a phytotoxic effect was detected when HA was applied directly on the leaves at 10 μM. *Trichoderma* secondary metabolites may have an auxin-like action, which is typically expressed at low concentrations (10^{-5} and 10^{-6} M) while producing an inhibitory effect at higher doses (Brenner, 1981 and Cleland, 1972). Moreover, an auxin-like activity was observed on etiolated pea (*Pisum sativum*) stems treated with 6PP, which also affected positively the growth of tomato (*Solanum lycopersicum*) and canola (*Brassica napus*) seedlings (Vinale et al., 2008).

In field experiments, the application of 6PP (1 μM) and *T. harzianum* T22 (as commercial formulation) on *V. vinifera* increased crop yield as measured in terms of Kg of grapes/ plant and bunch size. Similarly, Di Marco and Osti (2007) reported that the

treatments with a commercial product based on *T. harzianum* T22 on grapevine improved the quantitative and qualitative characteristics of the root system, and increased the grape production.

Treatments with 6PP and T22 increased the polyphenol contents and the total antioxidant activity (in particular with 6PP) in the fruits. These increments are associated with plant defense response to an abiotic or biotic stress (Cho et al., 2004; Ames et al., 1993). Calderon et al. (1993) studied the ability of an enzymatic elicitor obtained from *T. viride* (used as commercial preparation) to induce HR in a grapevine cultivar susceptible to *B. cinerea*. Together with the beneficial effect on the plant physiology and metabolism, the application of the microbial compound increase the synthesis and accumulation of resveratrol, a phytoalexin of grapevines belonging to the chemical class of polyphenols (Langcake & Pryce, 1977). As reported by Harman et al. (2004), Woo et al. (2006 and 2007) and Vinale et al. (2008), different strains of *Trichoderma* may enhance the plant defense in the interaction with the host through the production of bioactive molecules (BAMs). These BAMs include: i) proteins with enzymatic activity, such as xylanase; ii) avirulence-like gene products able to induce defense reactions in plants; iii) some secondary metabolites (i.e. 6PP and peptaibols); and iii) low molecular- weight compounds released from either fungal or plant cell walls by the activity of *Trichoderma* enzymes. Our data indicate that the effect of the purified 6PP is comparable or, in some cases, better of that observed by using the commercial formulation based on the highly-effective strain T22 of *T. harzianum.* The results suggest that the application of metabolites isolated from *Trichoderma* strains may be used in alternative to the living BCAs.

Plant growth promotion effect have been observed on *Brassica rapa* treated with two *Trichoderma* strains (*T. harzianum* M10 and *T. atroviride* P1) or their secondary metabolites (HA and 6PP). Particularly, HA increased stem length better than its producing fungus (*T. harzianum* M10), while in the case of 6PP and *T. atroviride* P1 the opposite was true.

Compared to untreated control a reduced accumulation of glucosinolates (neoglucobrassicin, glucobrassicin, glucoiberin and gluconapin) was also detected in the plant 72h after the treatments followed by an increase at the end of vegetative cycle

particularly when M10 and P1 were applied. These effects can be related to the plant defense response to the fungus or the *Trichoderma* metabolites as in the case of grapevine.

The effect of three *Trichoderma* metabolites on *A. thaliana* growth has been assessed. HA, 6PP and Hytra 1, applied at concentration of 0.1, 0.1 and 0.01 μM respectively, promoted the plant growth both *in vivo* and *in vitro*. Moreover, it is interesting to note that the metabolite applications *in vitro* stimulated particularly the production of secondary roots. Harman (2000) and Vinale et al. (2008 and 2012) demonstrated that some *Trichoderma* strains or their metabolites, when applied on the plant, were able to stimulate lateral root growth through an auxin-dependent mechanism.

In order to evaluate the effects of *Trichoderma* metabolite applications on the production of plant hormones related to growth and development, as well as to defence response, an LC-MS analysis was performed. Our data indicated that: i) HA increased the concentration of IAA and ABA; ii) 6PP the level of JA; iii) Hytra1 increased JA and ABA. These results suggest that the hormones can be affected by metabolite applications, although it is not possible to demonstrate that the *Trichoderma* compounds are directly involved in the specific biosynthetic pathway.

The plant metabolism changes induced by the application of *Trichoderma* metabolites have been investigated by LC-MS-Qtof and all data were subjected to a principal component analysis (PCA). The PCA scores plot revealed a clear separation of the four different groups (according to the different treatments: Control, HA, 6PP and Hytra1), with the five replicates of each treatment clustering together. This finding demonstrated the high reproducibility between the biological replicates and the differential effects of the purified compounds on the plant metabolome. These effects are reported in the Venny diagrams (results section – Figure 4.41) that compare the number of plant metabolites found in the untreated control, with that found in the different treatments. HA caused a pronounced increase in the number of plant metabolites, while smaller changes were observed in the case of 6PP and Hytra1 treatments.

Furthermore, as shown in Figure 4.40 HA and 6PP caused a similar global change of the metabolic profile, while a different response was obtained with Hytra1. The results

indicated that different *Trichoderma* metabolites produced different effect/changes on the plant physiology and metabolome.

Brotman et al. (2012) demonstrated that *Trichoderma* root colonization alters the *A. thaliana* metabolic profile, including significant changes of amino acids involved in the biosynthesis of plant hormones and plant defence metabolites. The promotion of plant growth may require an increased energy supply that is directly correlated with the metabolic changes induced by *Trichoderma* spp. (Brotman et al., 2012).

Our data suggest that, as reported for the living beneficial fungus known to ameliorate the physiological state of the plant, also the purified metabolite can substantially alter the metabolic profile by directly modulating several biosynthetic pathways.

6. Conclusion

The data indicate comparable beneficial effect on the plant between treatment with *Trichoderma* metabolites and treatment with the living microbes. These natural compounds are involved in regulation of plant growth and development and elicit also defence responses against pathogens.

The isolation and application of bioactive compounds, produced by beneficial microbes responsible for the desired positive effects on plants, is a promising alternative to the use of living antagonists. These formulations could also include mixtures of enzymes and secondary metabolites mixed with microbial propagules. Conditions can be selected for the production of substances with high biological activity, and these compounds can be made in diverse commercial formulations (i.e. powder, granules, dip, drench), and applied directly to vegetation in the field or greenhouse.

Clearly there is a prospective for the application of *Trichoderma* metabolites to induce host resistance and/or promote plant growth, as they can be i) produced inexpensively in large quantities on industrial scale; ii) easily separated from the fungal biomass; iii) dried and formulated as spray or drench applications.

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