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Gliotoxin and Bis-methyl-gliotoxin production by *Trichoderma spp.* as biocontrol agents

Running Title: Human risks potential by using *Trichoderma spp.* metabolites

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Abstract

Trichoderma-based products are used worldwide as biological control agents, due to its properties and benefits regarding to synthetic pesticides. Some of the *Trichoderma* species can produce secondary metabolites, which can be toxins, medicines or even both. The properties of those metabolites seem to be responsible for the extensive use of these species against plant-pathogens, being the biotechnological potential of *Trichoderma* and its metabolites also extended to Industry and Human Health. Gliotoxin belongs to the epipolythiodioxopiperazine class and is a *Trichoderma spp.* metabolite. It is toxic for humans and animals, but it has effects on human tumor cells and against human pathogens. The aim of this study was to evaluate the effect of plant-pathogens, such as *Alternaria tomatophila* and *Rhizoctonia solani*, in the production of gliotoxin and bis-methyl-gliotoxin by *Trichoderma spp*. in order to explore the potential use of these metabolites as biocontrol agents. The results show that the production of secondary metabolites by *T. virens* and by *T. afroharzianum* may affect the growth of plant-pathogen *A. tomatophila*. It was also pointed that probably *T. reesei* is not able to inhibit the growth of *A. tomatophila*. The lack of information of the secondary metabolites studied is of major importance, due to their wide presence in the environment. Despite the recognized environmental benefits, the exposure of workers to gliotoxin and bis-methyl-gliotoxin can put in risk their health, since a chronic exposure can lead to occupational diseases. Further studies should be made, to better understand the extension and the health consequences of occupational-human exposure to *Trichoderma spp.* and its metabolites. The risks and benefits of the use of *Trichoderma spp.* as a biocontrol agent should be better measured, in order to develop regulations to control the use of *Trichoderma spp.*, and subsequently its metabolites.

Keywords: *Trichoderma spp.;* Gliotoxin; Bis-methyl-gliotoxin; Biocontrol agents; Agronomy; Fungi interactions; Human health; Occupational exposure.

Resumo

Os produtos derivados do *Trichoderma* são utilizados a nível mundialcomo agentes de controlo biológico, pelas suas propriedades e benefícios, em relação aos pesticidas sintéticos. Algumas das espécies de *Trichoderma* podem produzir metabolitos secundários, que podem ser toxinas, medicamentos, ou ambos. As propriedades desses metabolitos parecem ser responsáveis pelo uso extensivo dessas espécies contra fitopatógenos, sendo o potencial biotecnológico de *Trichoderma* e dos seus metabolitos também estendido à Indústria e à Saúde Humana. A gliotoxina pertence à classe epipolitiodioxopiperazina e é um metabolito de *Trichoderma spp.*. É tóxico para humanos e animais, mas tem efeitos sobre células tumorais humanas e contra patógenos humanos. O objetivo deste estudo foi avaliar o efeito de fitopatógenos, como *Alternaria tomatophila* e *Rhizoctonia solani*, na produção de gliotoxina e bis-metil-gliotoxina por *Trichoderma spp*. Com o objetivo de explorar o potencial uso desses metabolitos como agentes de biocontrolo, os resultados mostraram que a produção de metabolitos secundários por *T. virens* e por *T. afroharzianum* pode afetar o desenvolvimento do fitopatógeno *A. tomatophila*. Também foi realçado que *T. reesei* não é capaz de inibir o crescimento de *A. tomatophila*. As lacunas de informação sobre o estudo dos metabolitos secundários é de grande importância, devido à sua ampla presença no meio ambiente. Apesar dos reconhecidos benefícios a nível ambiental, a exposição dos trabalhadores à gliotoxina e bis-metil-gliotoxina poderá ter efeitos na saúde, pela exposição crónica, pelo que sugere-se o seu estudo, no sentido de compreender a extensão e consequências na saúde resultantes da exposição humana ocupacional a *Trichoderma spp.* e seus metabolitos. Os riscos e benefícios do uso de *Trichoderma spp.* como um agente de biocontrolo devem ser criteriosamente medidos, no sentido de desenvolver regulamentação.

Palavras-chave: *Trichoderma spp*.; Gliotoxina; Bis-metil-gliotoxina; Agentes de Biocontrolo; Agronomia; Interação fúngica; Saúde Humana; Exposição Ocupacional.

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List of Abbreviations

AT – *Alternaria tomatophila*

BCA's – Biological Control Agents

EU – European Union

GV 29-8 – *Trichoderma virens* (strain GV 29-8)

IPM – Integrated Pest Management

LUT - Luteolin

MBCA'S – Microbial Biologial Control Agents

MS + MES – Murashige & Skoog +MES

ROS – Reactive Oxygen Species

RP-HPLC-UV – Reversed phase High-Performance Liquid Chormatography with Ultra-Violet detector

RS – *Rizhoctonia solani*

RUT C30 – *Trichoderma reesei* (strain RUT C30)

SD – Standard Deviation

T6776 – *Trichoderma afroharzianum* (strain T6776)

TFA – Trifluoroacetic Acid

USB – Università degli studi di Brescia

WDL – Weindling Medium

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1. Introduction

About 40 percent of the reduction of the world crop yield is caused by the large number and diversity of crop pest species (Chandler et al., 2011). In order to protect crops and to control plant diseases and pests, the use of chemical pesticides (e.g., insecticides, fungicides, herbicides, acaricides and plant growth regulators) has been carried out. This practice can have several negative effects on ecosystems, such as inhibiting the growth of beneficial microorganisms (Woo et al., 2014).

In the last decades, the use of synthetic pesticides has increased considerably (Fiorini, 2016). Around 250 000 tones of synthetic pesticides (mostly fungicides) are estimated to have been used per year, in Europe (Woo et al., 2014). However, this practice is not currently considered sustainable, once, in addition to have negative impacts on the income of farmers, the excessive use of these products has an impact on public health and on environment (Fiorini, 2016).

To control the use of pesticides, recently, the European Union (EU) stipulates a framework to obtain a sustainable use of these products (Woo et al., 2014). Thus, for a pesticide being commercialized and used in the EU, it has to be scientifically tested that that it is effective against its target pests as well as it is not deleterious for human health and environment (Woo et al., 2014). Actually, some governments in Europe are implementating a regulation of the pre-market assessment of safety. These regulations and also of the community sensitivity to a sustainable and safe agriculture compel farmers to find alternatives to synthetic pesticides (Fiorini, 2016).

In order to increase the security for consumers and to decrease the risks to human health and environment, an integrated pest management (IPM) has been implemented, in the European Union (EU 2009/128/EC, 2009). This practice comprised the use of biocontrol agents as alternative to synthetic pesticides (Woo et al., 2014). IPM uses a group of plant protection called the Biopesticdes. They are derived from natural products, such as fungi, pheromones, bacteria, among others (Woo et al., 2014), which can be parasites, predatory insects and microbial antagonists of plant pathogens (Fiorini, 2016). Therefore, IPM is a sustainable and effective approach of managing crop pests and natural competitors, increasing the food availability and reducing the use of chemical pesticides (EU 2009/128/EC, 2009; EPA, 2017; Fiorini, 2016).

Furthermore, most of these biopesticides are developed inexpensively and the waste produced is little or no toxic, making them more appealing than chemical pesticides (Fiorini, 2016). *Trichoderma*-based products are used more commonly than other microbial biopesticides (Fiorini, 2016).

Some of the fungi used as biological control agents produce secondary metabolites, which have antimicrobial properties, used as mechanisms of biocontrol. These properties, in the case of *Trichoderma spp*., can be related to the increased production of some secondary metabolites, when the fungi are in interaction with pathogens (Bezuidenhout et al., 2012; Corsi, 2016; Scharf, Heinekamp & Brakhage., 2014). Therefore, it can be considered that, depending on the interactions of the fungi, the presence of pathogens in the environment can affect the production of secondary metabolites, as a form of biocontrol.

The biological control has several advantages, such as: (1) the low phytotoxicity and pathogenicity to humans and to other biocontrol agents, (2) the induction of better crop quality and (3) a reliable pest control. However, for the implementation of biological control it is required to have substantial knowledge on the subject (Fiorini, 2016) and considering all risks and benefits, including take in to account the potential consequences of occupational human exposure.

The aim of this thesis was to evaluate the effect of well-known plant-pathogens in the production of secondary metabolites by *Trichoderma spp*., namely gliotoxin and bis-methyl-gliotoxin (bmGT).

2. Literature Review: The genus *Trichoderma*

Trichoderma is a genus of asexually reproducing filamentous fungi (Gajera, Domadiya, Patel, Kappora & Golakiya, 2013) that belongs to the phylum *Ascomycota*, class of *Sordariomycetes*, order of *Hypocreales* and family of *Hypocreaceae* (D´Acunti, 2017; Gajera et al., 2013). It is an ubiquitous genus that occurs worldwide, being distributed and developed in soil, decaying material, wood, plant material, particularly in its roots and leaves, colonizing fruits and forest tree crops (Contreras-Cornejo, Macías-Rodríguez, del-Val & Larsen, 2016; Druzhinina, Kopchinskiy & Kubicek, 2006; Fiorini, 2016; Gajera et al., 2013).

This genus of fungi is known as a biocontrol agent against some of the most important plant diseases and pests since 1930s (Bezuidenhout, Rensburg & Rensburg, 2012; Corsi, 2016; Gajera et al., 2013; Howell, 2003; Phupiewkham , Sirithorn, Saksirirat & Thammasirirak, 2015). It is also known for stimulating the growth of the plant with which they interact (D'Acunti, 2017). *Trichoderma* produces numerous spores (conidia), secondary enzymes and metabolites with antibiotic properties (Bezuidenhout, 2012; D'Acunti, 2017).

The *Trichoderma*-based products are distributed and used worldwide, especially in the Asian countries, where there is the largest distribution of these products, followed by Europe, South-Central America and, lastly, North America (Woo et al., 2014). The *Trichoderma* products have a wide variety of uses in the environment, health and even industry (Woo et al., 2014).

According to Fiorini et al. (2016), more than 60 percent of all registered biopesticides are *Trichoderma*-based. This is because of the properties of these fungi to improve plant defenses, to antagonize, to parasitize and to even kill other fungi (Atanasova et al., 2013; Bezuidenhout, 2012; Firoirni et al., 2016; Howell, 2003; Mukherjee et al., 2013).

A comparative study between different strains of Trichoderma performed by Atanasova et al. (2013), "revealed that mycoparasitism is the innate 'property of this genus". Mycoparasitism is the parasitism of one fungus by another. It "describes the interactions in which some organisms benefit at the expense of the fungi" (Atanasova et al., 2013). Mycoparasitic *Trichoderma* strains can recognize the host fungi, coil around its hyphae and use cell wall degrading enzymes to penetrate the host cell and use its contents as a nutrient source (Harcz, 2004).

There are more than 75 species of *Trichoderma*, resulting in more than 1100 different strains already isolated (Contreras-Cornejo et al., 2016). Most of *Trichoderma* strains produce asexual spores, which indicate that most of the strains do not have a sexual stage in the reproduction process. However, the strains that have this sexual stage do not have, commonly, biocontrol properties (Harman, 2000).

The main reasons for the application of *Trichoderma spp.* in the rhizosphere are shown in the section "*Trichoderma* in the environment". The characterization and description of the *Trichoderma* strains used in this thesis, *Trichoderma virens*, *Trichoderma reesei* and *Trichoderma afroharzianum*, are presented below.

2.1. *Trichoderma spp.*

2.1.1. *Trichoderma virens*

Trichoderma virens (strain GV 29-8) is a naturally occurring saprophytic fungus and it is considered one of the main strains for conducing biocontrol studies and for the promotion of plant growth (Anitha & Murugesan, 2005; Atanasova et al., 2013; Contreras-Cornejo et al., 2016).

T. virens is able to produce secondary metabolites, among them gliotoxin – this species owns 8 of the 12 genes of *Aspergillus fumigatus* gliotoxin cluster (Corsi, 2016; Zeilinger, Gruber, Bansal & Mukherjee, 2016), gliovirin and viridian (Howell and Stipanovic, 1983; Jones and Hancock, 1987), with antifungal, antibacterial, antibiotic and herbicidal properties (Howell, 1991). The kinds and amounts of these produced metabolites are influenced by the substrate on which they grow (Howell, 1991).

Therefore, this fungus is widely used against several phytopathogenic fungi, such as *Rhizoctonia solani*, and against many soilborne root and seedling diseases (Anitha & Murugesan, 2005; Howell, 1991; Vargas et al., 2014; Wilhite & Straney, 1996). This species also play an important role in the stimulation of plant defense responses (Mukherjee et al., 2012).

2.1.2. *Trichoderma reesei*

Trichoderma reseei (strain RUT C30) was generated through the wild-type *T. reseei* QM6a, in 1970s, originated by a mutagenic process with UV light. This strain of *T. reseei*, RUT C30, is able to produce high quantities of cellulase (Corsi, 2016; D'Acunti, 2017) and because of that is commonly found in decaying wood (Mukherjee et al., 2012). There is no indication of the use of this fungus as a plant growth promoter or as a biocontrol agent (Contreras-Cornejo et al., 2016; Corsi, 2016; D'Acunti, 2017).

It is known that *T. reseei* has 6 genes of the 12 genes of *A. fumigatus* gliotoxin cluster (Zeilinger et al., 2016). Despite that, the production of gliotoxin has not been observed (Corsi, 2016; D'Acunti, 2017; Zeilinger et al., 2016). According to Mukherjee et al. (2012) and to Atanasova et al. (2013), the small genome is probably the cause of the loss of mycoparasitism specific genes.

In a test assay performed with *Rhizoctonia solani*, *T. reesei* was not able to stop the growth of the pathogen, but, after 10 days, a barrage zone was created between the two fungi and afterward *T. reseei* overgrown *R. solani* (Atanasova et al., 2013).

2.1.3. *Trichoderma afroharzianum*

Trichoderma harzianum (strain T6776) (University of Pisa fungal collection) recently reclassified by Fiorini et al. (2015), was isolated from the soil of a pine forest in Pisa, Italy. This species is known as a fungicide and as a biocontrol agent, being a mycoparasite of pathogenic fungi in plants (Atanasova et al., 2013; Mukherjee et al., 2012; Phupiewkham et al., 2015). This species is considered the model of biocontrol of pathogens and of the mechanisms involved in this process (Bezuidenhout, 2012; Fiorini, 2016). In a study performed by Fiorini et al. (2015), this specific strain, in interaction with Micro-tom plants, was able to produce hormones to improve the chlorophyll photosynthesis, the growth rate and the biomass production (D'Acunti, 2017; Corsi, 2016; Fiorini, 2016; Fiorini et al., 2016; Sarrocco et al., 2013), and produce many secondary metabolites, such as antibiotic substance and cell-wall degrading enzyme (Gajera et al., 2013; Phupiewkham et al., 2015).

According to our knowledge in the literature, there is no reference to the production of gliotoxin by this fungus. In addition and thanks to the genome sequencing by Baroncelli

et al. (2015), it was possible to find a cluster in this strain similar to the one of *A. fumigatus* (Crotti et al., 2016).

This species of *Trichoderma* was used for controlling plant diseases, such as Brown spot in rice and disease caused by *Phytophthora erystroseptica*, *Fusarium sp.* and *Rhizoctonia solani* in tomato and potato plants (Fiorini, 2016; Phupiewkham et al., 2015; Sarrocco et al., 2013). Nevertheless, despite the benefic properties that this species has to plants, some of its isolates were found to be opportunistic human pathogens (Fiorini, 2016).

2.2. Biological Control

Biological control relies to the use of parasites, predators or pathogens in order to control the activity of other pathogenic organisms. Basically, it consists in the ability of an agent to maintain a density of population of other organisms lower than it would occur in the absence of that agent (Bezuidenhout, 2012; D'Acunti, 2017).

The use of biological control agents (BCA's) was necessary as an alternative for the extense use of chemical pesticides. Futhermore, the use of these agents instead of the chemical pesticides has other advantages especially to a sustainable environmental management and to public health (D'Acunti, 2017).

Since BCA's consist of biological products, they are not a threat for the environment. Instead, they do not contaminate groundwater and they help in the soil enrichment (D'Acunti, 2017). The lower amounts used, the ability to grow and to easily reproduce, as well as the resistance in the environment of the BCA's are another advantage of this practice. Another fact is that, usually, these agents live in symbiosis with their host and, since they are part of the ecosystem, their introduction on the environment will not have such a negative impact as the insertion of chemical pesticides (Konstantinovas et al., 2017). Interestingly, besides to protect the plants and control the development of a plant, the BCA's can also decrease the effects that the pathogen and the chemical pesticides would have on the farm workers, supporting the existent control strategies.

However, in contrast, some studies report that the use of BCA's can increase the exposure of workers to microbial agents and the resistance of the spores in the environment can be a risk for the consumers. The prolonged survival of these agents can make them an overall risk to the human health and environment (Konstantinovas et al., 2017). It was also

reported that the exposure to these agents could lead to diseases, such as allergies and asthma, compromising the respiratory tracts of mammals, as well as general infections (Konstantinovas et al., 2017).

According to Konstantinovas et al. (2017), "there are around 135 products commercialized as BCA's". The BCA's can be viruses, bacteria, fungi and protists, and they can be classified as microbial or biochemical pesticides. Biochemical agents are non-living parts of microorganism, such as hormones, enzymes and metabolites (Konstantinovas et al., 2017). The microbial biological control agents (MBCA's) go through genetic modifications, in order to optimize their biocontrol activity (Konstantinovas et al., 2017).

The mechanisms that the BCA's use vary depending on the agent (Gajera et al., 2013; Woo et al., 2014). There are around 13 species of fungi that have biocontrol properties, such as *T. virens* and *Beauveria bassiana*. *Trichoderma* was recognized as a potential BCA in 1930 and, over time, the number of diseases controlled by these fungi has increased (Bezuidenhout, 2012; Howell, 2003). The mechanisms used by *Trichoderma* against plant pathogens were described by Gajera et al. (2013) which include: biocontrol by competitions; antibiosis; mycoparasitism; and stimulation of plant resistance and plant defenses (D'Acunti, 2017; Woo et al., 2014).

The activity of biocontrol by competitions can be made by to different processes, the fungistasis and the competition for nutrients. The first one, fungistasis is the inhibition of the growth and reproduction of fungi without killing them. *Trichoderma* strains are able to do that to some phytopathogens, such as *R. solani* (Gajera et al., 2013). The mechanism of competition for nutrients is based on the use of the nutrients available in the soil, in order to limit their availability to the pathogens. Since *Trichoderma* strains grow faster, they use the nutrients more efficiently than the pathogens and this result in the starvation of the phytopathogen, causing its death (Gajera et al., 2013; Mukherjee et al., 2012).

The antibiosis process involves the production or release of compounds (antibiotics or hydrolytic enzymes) that will kill or inhibit the growth of pathogens. *Trichoderma* strains are known to produce volatile and non-volatile toxic metabolites, which have this property against pathogens (Contreras-Cornejo et al., 2016; Gajera et al., 2013; Mukherjee et al., 2012).

Mycoparasitism is a process of direct attack of one fungus on another. It involves the recognition, attack, penetration and killing of the pathogen (Bezuidenhout, 2012; Gajera et al., 2013; Mukherjee et al., 2012). Basically, *Trichoderma* can recognize the host and attach to it and, after, coil its hyphae. It also involves secretion of antibiotic metabolites that will disarm and kill the pathogen (Contreras-Cornejo et al., 2016; Corsi, 2016).

The stimulation of plant resistance and plant defense mechanism consists in the production of a metabolite that is able to protect the plant from the pathogen. It was shown that *Trichoderma* could transfer these products to the plant, stimulating the plant resistance against the pathogen (Contreras-Cornejo et al., 2016; Gajera et al., 2013; Konstantinovas et al., 2017; Woo et al., 2014).

Some of BCA's use only one of these mechanisms to control pathogens. However, others BCA's use more than one combined. The latter are considered the most successful biocontrol agents, such as *Trichoderma* (D'Acunti, 2017; Gajera et al., 2013).

The several uses of these BCA's involve the application in the field, nurseries, greenhouses, golf courses and home gardens. In the agriculture, they can be used in a variety of crops: vegetables, cereals, oilseeds, vineyards, fruits orchards, as well as in ornamental trees and plants. The *Trichoderma* BCA's can be applied in other areas than agriculture, such as medicine, dentistry and environmental ecology (Woo et al., 2014).

2.3. Secondary metabolites production

According to Scharf et al., (2014), "secondary metabolites are small organic molecules produced by various microorganisms through the action of large enzymes". Usually, these metabolites are not absolutely necessary for the growth of the producer, but they can benefit for the producing organisms, depending on the conditions and habitats that they are produced (Scharf et al., 2014).

Usually, secondary metabolites, especially the epipolythiodioxopiperazines (ETP's), are produced by species of fungi that are distributed worldwide, such as *Trichoderma*, *Aspergillus* and *Penicillium* (Kwon-Chung & Sugui, 2009). However, the production of secondary metabolites is strain dependent (Bezuidenhout et al., 2012). The biosynthesis genes responsible for the production of secondary metabolites by fungi are, normally, disposed of in clusters (Mukherjee et al., 2012; Scharf et al., 2014).

Trichoderma spp., the main fungi of the scope of this thesis, is known for producing some secondary metabolites that contribute for the fungi pathogenicity and also, in the case of fungi-plant interaction, they have a role in the activation of plant defenses and growth regulation (Bezuidenhout et al., 2012; Corsi, 2016; Scharf et al., 2014). Other properties of these molecules are the ability to disrupt lipid membranes and to induct the resistance of the plant (Bezuidenhout, 2012; Phupiewkhamet al., 2015). It is also known that some secondary metabolites have antibiotic and anti-microbial properties (Phupiewkham et al., 2015; Woo et al., 2014).

Zeilinger et al. (2016) published a review presenting data of the secondary metabolism of *Trichoderma spp*., which can be non-ribossomal peptides, polyketides, terpenoids and 6-Pentyl pyrone. The first include the peptaibiotics, epipolythiodioxopiperazines, siderophores and the metabolites produced by "NRPS-like" gene clusters.

2.4. The applications of *Trichoderma* in the environment

2.4.1. Agriculture

The alternative for the extensive use of synthetic pesticides, led to the wide study of *Trichoderma spp.* fungi and for their use as MBCA's in agriculture against some plant pathogenic fungi (Bezuidenhout et al., 2012; Gajera et al., 2013; Mukherjee et al., 2012; Woo et al., 2014). The implications of using chemical pesticides, including the effect on public health, the safety of agrifood products and the impact on the environment were reported by Woo et al., 2014.

Trichoderma species are used worldwide as biopesticides, bioprotectants, bio-inoculants, biodecomposers, plant growth enhancers, biofertilizers and stimulants of natural resistance (Bezuidenhout et al., 2012; Contreras-Cornejo, Macías-Rodrígues, Alfaro-Cuevas & López-Bucio, 2014; Fiorini et al., 2016; Howell, 2003; Mukherjee et al., 2012; Woo et al., 2014). Considering that BCA's are a sustainable alternative to chemical pesticides (Fiorini, 2016; Woo et al., 2014), it was reported a survey by Woo et al. (2014), showing that more than 250 *Trichoderma*-based products are available in the world and this number has been growing (Fiorini et al., 2016; Woo et al., 2014).

The major reasons why *Trichoderma* species are used in rhizosphere are supported by: a) their high reproductive capacity; b) ability to survive under very unfavorable conditions;

c) efficiency in the utilization of nutrients; d) capacity to modify the rhizosphere and, e) strong aggressiveness against plant pathogens" (Bezuidenhout, 2012; Corsi, 2016).

Some of the metabolites produced by *Trichoderma* species have distinct properties, involving the interaction between plants, plant-pathogens and *Trichoderma* (Corsi, 2016; Phupiewkham et al., 2015), thus being considered BCA's (Bezuidenhout, 2012; D'Acunti, 2017; Howell, 2003; Scharf et al., 2016). Therefore, these compounds are of great importance for human health and for the environment (D'Acunti, 2017; Woo et al., 2014).

As reported before, *Trichoderma*-based products have several properties that can protect plants (Contreras-Cornejo, Macías-Rodrígues, Cortés-Penagos & López-Bucio, 2009; Contreras-Cornejo et al., 2016; Woo et al., 2014), however, there seem to be differences in the mechanisms used by *Trichoderma* to respond to a pathogenic fungi attack (Atanasova et al., 2013).

Trichoderma spp. can improve plant defences against biotic and abiotic stress and act as plant growth promoters (Contreras-Cornejo et al., 2016; Fiorini et al., 2016; Woo et al., 2014), These fungi can be soil inoculants to improve nutrient ability (Woo et al., 2014), decomposers and biodegradators (Woo et al., 2014) and, as mycoparasitic, can protect the plant from pathogenic fungi (Bezuidenhout, 2012; Corsi, 2016; Gajera et al., 2013; Mukherjee et al., 2012; Phupiewkham et al., 2015; Woo et al., 2014).

Some *Trichoderma* species are able to sense the presence of a pathogen from distance, a process called priming (Atanasova et al., 2013; Woo et al., 2014). Its growth and colonization can be done in association with plant roots, being the reasons for the extensive use of *Trichoderma* in agriculture (Corsi, 2016; Gajera et al., 2013; Mukherjee et al., 2012). It is described by several authors that some *Trichoderma* strains can colonize and penetrate the roots of plants and lead to changes in the physiology and in the defense plant systems (Fiorini et al., 2016; Mukherjee et al., 2012).

The beneficial effects that *Trichoderma* species provide to the plants are the protection against pathogens, which can involve the mechnism of biocontrol as the nutrient competition, the fungistasis, the mycoparasitism, the antibiosis and the stimulation of plant resistance and plant defense mechanism, that are described in "Biological Control" (Atanasova et al., 2013; Bezuidenhout et al., 2012; Contreras-Cornejo et al., 2016; Corsi,

2016; Gajera et al., 2013; Konstantinovas et al., 2017; Mukherjee et al., 2012; Woo et al., 2014).

Another beneficial effect of *Trichoderma* to plants is the plant growth promotion. This advantage can be expressed by plant growth regulators, which encompass the production of plant hormones like auxin (Bezuidenhout, 2012; Contreras-Cornejo et al., 2016; Mukherjee et al., 2012; Woo et al., 2014), or it is expressed by nutrient solubilization that is characterized by the solubilization of some mineral nutrients that are limited to plants in the rhizosphere (Contreras-Cornejo et al., 2016; Woo et al., 2014).

Finally, other beneficial effect of *Trichoderma* is the abiotic stress adaptation. It is described that some *Trichoderma* strains allow the plant to grow or the germination of the seeds under abiotic stress (Contreras-Cornejo et al., 2016; Mukherjee et al., 2012; Woo et al., 2014).

2.4.2. Industry

Trichoderma species are known to produce several enzymes and chemicals (Kredics et al., 2014; Mukherjee et al., 2013; Schuster and Schmoll, 2010). This was first discovered by the US Army in the Second World War and, nowadays, has innumerous applications, being used in various types of industry (Mukherjee et al., 2013; Schuster and Schmoll, 2010), such as in the biofuels industry, as a producer of enzymes and chemicals, in the food industry, as aroma compounds and food additives (Mukherjee et al., 2013; Schuster and Schmoll, 2010), in the pulp and paper industry, in the textile industry (Schuster and Schmoll, 2010) and in the biotechnology industry, as producer of nanoparticles (Mukherjee et al., 2013).

The principal cellulases producer of the genus *Trichoderma* is the *Trichoderma viride*, known now as *Trichoderma reesei*. Other important metabolites produced by this species of *Trichoderma* (*T. reesei*) are plant cell wall-degrading enzymes (Mukherjee et al., 2013). Currently, *T. reesei* is considered to be a model system for plant cell wall degradation (Mukherjee et al., 2013).

There have been some ongoing studies to deepen the knowledge of the applications of these fungi and their metabolites (Mukherjee et al., 2013). The investigation is based on the improvement of the potential of *Trichoderma spp.* to degrade plant cell wall, to

produce heterologous protein and to the sexual crossing (Mukherjee et al., 2013; Schuster and Schmoll, 2010), in order to increase the uses of this genus of fungi in the industry and other areas.

The use of microorganisms has become a major interest in industries. The research developed is increasing the applications of these fungi. The concern of the population and, in the industry world, of stakeholders, led industries to seek for environment-friendly techniques, compounds and processes.

2.4.3. Human Health and other Environments

Trichoderma species are fungi that are distributed worldwide and, it can have influence in the human health and in the health of other organisms present in the environment (other microorganisms, plants and animals) (Kredics et al., 2014; Mukherjee et al., 2013; Schuster and Schmoll, 2010).

The fungi that belong to this genus can have both positive and negative effects in humans, depending on the species. *Trichoderma spp.* can display an important role in the biodegradation and recycling of complex polymers, can remediate heavy metals, toxins and xenobiotics and are great produders of secondary metabolites. Some of those metabolites can be medicines, toxins or both (Kredics et al., 2014; Mukherjee et al., 2013). For example, gliotoxin, the first discovered *Trichoderma* antibiotic is also considered a mycotoxin.

According to Mukherjee et al. (2013), *Trichoderma longibrachiatum* is known to be a human pathogen to immunosuppressed individuals, but other species can also cause infection in this type of individuals, such as *T. citrinoviride* and *T. harzianum* (Schuster and Schmoll, 2010). More recently, it was reported that some *Trichoderma* species are causing infections in healthy individuals (Mukherjee et al., 2013).

The list of infections that could be caused by *Trichoderma spp.* is extensive, including "peritonitis and intra-abdominal abscess in patients undergoing continuous ambulatory peritoneal dialysis, liver infection, acute invasive sinusitis and disseminated infections of transplant recipients, brain abscess, skin infection, necrotizing stomatitis and pulmonary infections of patients with hematological malignancies, fungemia by contaminated saline, rhinosinusitis, pulmonary myeloma and fibrosis, hypersensitivity pneumonosis,

endocarditis, otitis externa, cerebrospinal fluid infection and allergic reactions" (Kredics et al., 2014, as referred in Kredics et al., 2011b),

As referred above, *Trichoderma* species are able to remediate heavy metals, toxins and xenobiotics, thereat becoming a major interest for the environment itself (Mukherjee et al., 2013). The importance of these properties in several environments can be a propellent for its use, as well as its properties as MBCA's (Gajera et al., 2013; Howell, 2003; Konstantinovas et al., 2017; Woo et al., 2014).

It is also known that *Trichoderma* species are abundant in marine, particularly in marine sponge species, and freshwater environments, with special interest in the samples found in bottled water. In the marine water environments, studies reported the production of metabolites with a wide range of potential activities, such as antibacterial, cytotoxicity against human cancer cells, between others (Kredics et al., 2014). Related to the freshwater environments, the concern is about the bottled water samples contaminated with *Trichoderma* fungi and other human pathogenic fungi (for example, *Aspergillus* and *Penicillium*) (Kredics et al., 2014). This contamination can be a major problem for public health, making it more crucial to control these situations.

Another high representative environment where *Trichoderma* species were found is in the air and settled dust, such as places like homes, flats, hospitals, air ducts and air conditioners, libraries and archives (Kredics et al., 2014). As well as the contamination of bottled water, the contamination of air is also a concern for public health, even more in places like hospitals, where can be immunosuppressed people, in which, as reported before, *Trichoderma* species can play a role as human pathogens. Despite the fact that *Trichoderma spp*. and its conidia can be in the air, there is no information about the species found in specific occupational environments (Kredics et al., 2014). The knowledge about the species in occupational environments is important, in order to reduce the exposure and the risks associated to that species. Measures should be taken to prevent occupational exposure and diseases.

It was suggested in the literature that potential virulence factors of *Trichoderma* species could be the ability to grow at elevated temperatures and neutral pH, the production of extracellular proteases and the ability to use amino acids as carbon and nitrogen sources (Kredics et al., 2014). Therefore, the species that manifest these properties should not be used in crop fields, as BCA's, in order to ensure the safety of humans (Mukherjee et al.,

2013). According to what was described before, a deeper control and regulation of the *Trichoderma spp.* in the environment should be carried out, in order to guarantee the safety of workers, of buildings occupants and of the general population.

2.5. Gliotoxin

2.5.1. What is Gliotoxin?

Gliotoxin was first discovered in 1934 as a compound produced by *Trichoderma virens* (Brian and Hemming, 1945). This non-ribossomal peptide was described as an antibiotic against the plant pathogen *Rhizoctonia solani* (Anitha & Murugesan, 2005; Bezuidenhout et al., 2012; Grovel, Kerzaon, Petit, Du Pont & Pouchus, 2006; Konstantinovas et al., 2017; Scharf et al., 2016; Vargas et al., 2014). It was known to be toxic to other plant pathogens, such as *Sclerotinia americana* (Bezuidenhout et al., 2012; Howell et al., 2003; Konstantinovas et al., 2017; Scharf et al., 2016; Vargas et al., 2014). This compound has high persistence in the water, soil and air, a major factor to be consider a risk to the human health (Santa Cruz Biotechnology, Inc., 2016).

Gliotoxin is a toxin that belongs to epipolythiodioxopiperazine class (Dolan, O'Keeffe, Jone, Doyle, 2015; Kwon-Chung & Sugui, 2009; Scharf et al., 2016). Several fungi, such as *A. fumigatus*, a human pathogen, and *T. virens*, produce glitoxin (Anitha & Murugesan, 2005; Bok et al., 2006; Bugli et al., 2014; Kwon-Chung & Sugui, 2009; Sugui et al., 2017).

According to Waring and Beaver (1996), this toxin is toxic for both animals and humans, being lethal (Grovel et al., 2006) in relatively low concentrations. This potent immunosuppressive mycotoxin has a particularity in its structure, elucidated by Bell and

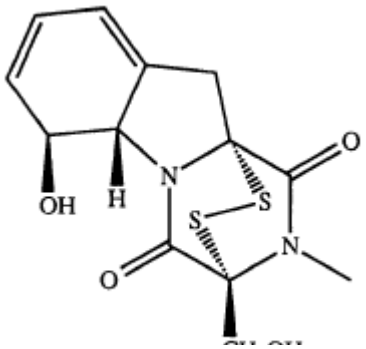

Figure 1- Gliotoxin chemical structure, with the disulfide bridge.

Adapted from Waring and Beaver, 1996

colleagues in 1958. Gliotoxin, $C_{13}H_{14}O_4N_2S_2$ (Elvidge and Spring, 1949) (Figure 1), contains a disulphide bridge, which is responsible for the inactivation of proteins, when in reaction with thiol groups and it is also what ensures the toxicity of gliotoxin (Bugli et al., 2014; Scharf et al., 2012; Sugui et al., 2017).

The stability of gliotoxin in rhizosphere is influenced by pH. Although Weidling (1934) reported that gliotoxin is stable at a low pH, Wright (1954) affirms that the low pH value is not a requisite for gliotoxin production (Anitha & Murugesan, 2005; D'Acunti, 2017; Scharf et al., 2016). The light is not a factor that influences the toxin production (Anitha & Murugesan, 2005).

Trichoderma spp. is used worldwide as BCA, in part because of the properties that gliotoxin has against plant-pathogens (Scharf et al., 2016). Gliotoxin has a wide range of properties, such as cytotoxic (Grovel et al., 2006; Vargas et al., 2014), phytotoxic (Scharf et al., 2016; Vargas et al., 2014), antimicrobial (Vargas et al., 2014), immunosuppressive (Grovel et al., 2006; Konstantinovas et al., 2017; Kwon-Chung & Sugui, 2009; Scharf et al., 2016), antiviral (McDougall, 1969), fungistatic (Elvidge and Spring, 1949) that affects humans, animals, plants and microorganisms.

The mechanisms of gliotoxin toxicity are: 1) generation of reactive oxygen species (ROS) (Kwon-Chung & Sugui, 2009; Gardiner & Howlett, 2005; Gardiner, Waring & Howlett, 2005; Waring & Beaver, 1996); 2) mixed disulfide formation with thiol groups (Gardiner & Howlett, 2005; Gardiner et al., 2005; Waring & Beaver, 1996).

2.5.2. Gliotoxin producers

Gliotoxin was first discovered as a compound produced by *T. virens* (Bezuidenhout et al., 2012; Brian and Hemming, 1945; Konstantinovas et al., 2017; Scharf et al., 2016; Vargas et al., 2014). Nowadays, it is known that gliotoxin is produced by several fungi species, such as *A. fumigatus*, *T. virens*, *Trichoderma lignorum*, *T. viride*, *Gliocadium fimbriatum*, *Eurotium chevalieri*, *Neosartorya pseudofischeri*, some *Penicillium spp*. and some *Acremonium spp.* (Harcz, 2004; Scharf et al., 2016). Some researchers claim that *Candida spp.* produces gliotoxin (Bezuidenhout, 2012) and others do not detect the production of gliotoxin by this genus (Scharf et al., 2016).

A. fumigatus can produce gliotoxin, which has already been detected on sprouce wood, chipboard surfaces and, particularly, in warm and humid areas, becoming a risk for occupational (farmers) and public health (Konstantinovas et al., 2017).

T. virens can produce gliotoxin in soil/rhizosphere, as well as in liquid culture, on agar and in other natural substrates (Scharf et al., 2016). Despite the substrate, some *T. virens* strains cannot produce gliotoxin. Howell and colleagues, in 1993, classified *T. virens* into two groups: the P strains and the Q strains. The P strains do not produce gliotoxin and the Q strains produce gliotoxin (Scharf et al., 2016; Vargas et al., 2014).

2.5.3. Gliotoxin biosynthesis pathway within *T. virens*

According to literature, the gene biosynthetic cluster of gliotoxin in *T. virens* has only 8 of the 12 genes identified in the cluster of *A. fumigatus* (Dolan et al, 2005; Kwon-Chung & Sugui, 2009; Mukherjeet et al., 2012; Scharf et al., 2016). The biosynthetic pathway of gliotoxin production (gene clusters) was recently discovered for *T. harzianum* and *T. reesei* (Scharf et al., 2016).

The cluster in *T. virens* is composed by genes that encode: an aminocyclopropane carboxilacetase synthetase (gliL), a dipeptidase (gliJ), a non-ribossomal peptide synthetase (gliP), two cytochrome P450 monooxygenase (gliC and gliF), a 0-methyltransferase (gliM), a glutathione S-transferase (gliG), a γ-glutamyl ciclotransferase-like protein (gliK), a transporter (gliA) and a methyltransferase (gliN) (Gardiner & Howlett, 2005; Kwon-Chung & Sugui, 2009; Mukherjee et al., 2012; Scharf et al., 2016; Scharf et al., 2010; Vargas et al., 2014; Zeilinger et al., 2016).

The difference between the gene cluster from *A. fumigatus* and *T. virens* is the absence of a thioredoxin reductase (gliT) and of a transcriptor factor zincfinger (gliZ) (Gardiner & Howlett, 2005; Kwon-Chung & Sugui, 2009; Mukherjee et al., 2012; Scharf et al., 2016; Vargas et al., 2014; Zeilinger et al., 2016). It is known that gliP is the key enzyme for the gliotoxin biosynthesis, thus without this gene there is no gliotoxin production (Kupfahl et al., 2006).

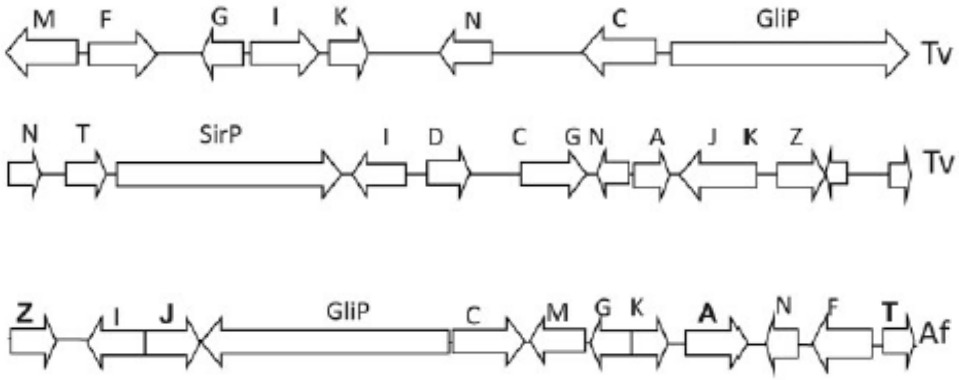

Figure 2 - Putative biosynthesis gene cluster of gliotoxin in *Trichoderma virens* (Tv) and in *Aspergillus fumigatus (*Af).

Adapted from Mukherjee et al., 2012.

2.5.4. Effects of Gliotoxin

Gliotoxin is a mycotoxin that has severe effects in humans as well as in other organisms. It can affect innumerable cell mechanisms, leading to the death of the cells (Konstantinovas et al., 2017). The effects of gliotoxin on cells seem to be related not only with the concentration, but also with the cell type (Scharf et al., 2016).

It is known that this molecule is lethal in low concentrations, being considered as toxic for humans and animals, if ingested (Grove et al., 2006; Scharf et al., 2016). Gliotoxin belongs to the group of immunosuppressives agents, making it important to take into consideration regarding its effects on humans. In Table 1, are presented some of the main effects of gliotoxin on humans and the respective route of exposure.

Table 1- Acute and chronic effects of gliotoxin on human health, considering the route of exposure.

Route of Exposure	Acute Health Effects	Chronic Health Effects
Ingested	May be fatal or produce serious damage to the health	May produce immunosuppression in individuals occupationally exposed May cause cancer and mutations
Eye Contact	May cause tearing or conjunctival redness and eye windburn	
Skin contact	May cause abrasive damage (can result in absorption)	
Intravenous	May produce systemic injury	

Inhaled	May be harmful and produce respiratory discomfort

Adapted from Santa Cruz Biotechnology, Inc., 2016.

Despite that and according to Scharf et al. (2016), gliotoxin has effects on different types of tumor cells and against some important human pathogens (for example, *Plasmodium falciparum*), suggesting that gliotoxin has potential therapeutic applications.

It also is involved in biocontrol of plant pathogens, through its antibiotic activity (Scharf et al., 2016; Wilhite et al., 1994), such as *Rhizoctonia solani* (Anitha & Murugesan, 2005) and *Phytium ultimum* (Howell, 1991; Scharf et al., 2016), inducing in these pathogens necrosis of cytoplasmatic material, inhibition of spores germination and mycelial growth (Konstantinovas et al., 2017; Lewis, Wiederhold, Lionakis, Prince & Kontoyiannis, 2005). In fungi, this molecule also induces redox stress (Carberry et al., 2012; Dolan et al., 2015; Scharf et al., 2016) and interferes with protein modification (Dolan et al., 2015).

Like other mycotoxins, there is the possibility that gliotoxin can enter the food chain and can reach high amounts of food (Scharf et al., 2016). If that happens, humans and animals can ingest gliotoxin, what will affect negatively their health. The fact that there are no values or control of the gliotoxin content in food makes possible the public health risk (Scharf et al., 2016).

In mice, there is some information about the toxicity (LD_{50}) of gliotoxin (Table 2).

Table 2 - Toxicological information (LD_{50}) of gliotoxin in mice in mg/Kg.

Route of Exposure	**Toxicity – LD_{50} (mg/Kg)**
Oral	67
Intraperitoneal	32
Subcutaneous	25
Intravenous	7.8

Adapted from Santa Cruz Biotechnology, Inc., 2016.

Gliotoxin is toxic for humans, animals and even plants (Bezuidenhout, 2012; Grove et al., 2006; Lewis et al., 2005; Waring & Beaver, 1996), but some studies reported that gliotoxin can be a therapeutic drug against some tumors (Dolan et al., 2005; Scharf et al., 2016), can inhibit bacterial multiplication and the germination of fungal spores (Anitha & Murugesan, 2005; Brian and Hemming, 1945).

In humans and some animals, it is known that gliotoxin inhibits the function of the NADPH oxidase enzyme complex (Dolan et al., 2015; Grovel et al., 2006; Konstantinovas et al., 2017). It can also induce apoptotic cell death of thymocytes, peripheral lymphocytes, macrophages and others (Dolan et al., 2015; Kwon-Chung & Sugui, 2009; Scharf et al., 2016; Waring and Beaver, 1996). It inhibits the activation of NFkB (Dolan et al., 2015; Konstantinovas et al., 2017; Kwon-Chung & Sugui, 2009; Scharf et al., 2016;), the growth of innumerous cell lines (Grovel et al., 2006) and phagocytosis (Konstantinovas et al., 2017; Kwon-Chung & Sugui, 2009).

It is known that gliotoxin inhibits the production of enzymes by some plants (Grovel et al., 2006; Haraguchi, Hamatani, Hamada & Fujii-Tachino, 1996), and affects negatively vegetative growth, by inhibiting growth and seed germination (Haraguchi et al., 1996; Howell, 2006; Scharf et al., 2016; Vargas et al., 2014).

However, the studies made involve different cell types, varied technical procedures and even different concentrations of gliotoxin. These conditions make difficult to compare studies in which are reported the effects of gliotoxin (Bezuidenhout, 2012; Scharf et al., 2016).

2.5.5. Gliotoxin in human health context

As reported before, some gliotoxin-producing *Trichoderma spp.* isolates are used and commercialized as biological control products. Therefore, these isolates are largely used in agriculture (Scharf et al., 2016). This practice and the use of microbial pest control agents, in general, may increase the exposure of farmers to gliotoxin (Konstantinovas et al., 2017). Therefore, not only farmers, but all the workers involved in the production process of biocontrol products with *Trichoderma spp.* can be exposed to gliotoxin.

Despite many different authors reported the presence of gliotoxin in biocontrol *Trichoderma*-based products, there is not much information regarding the presence of this molecule in bioaerosols, commonly used in agriculture. Likewise, the information about the symptoms and the doses that cause the susceptibility are not evident (Konstantinovas et al., 2017). Some safety data sheets available from different laboratories that sell gliotoxin standards provide information about the potential health effects of this molecule, as presented in Table 1.

Considering the high persistence in the environment and despite of the low bioaccumulation rate, gliotoxin can be a hazard to the workers. In this case, the importance of the use of individual protection equipment is high and some of these equipments should be: gloves and protection clothes, to reduce the exposed skin area; chemical goggles, to reduce the possibility of eye contact with the compound; and respiratory protection, especially when the work involves vapor or airborne particles. It is important to emphasize that the toxicity rate of gliotoxin is 3 in a scale of 0 to 4, meaning that the toxicity hazard is high (Santa Cruz Biotechnology, Inc., 2016).

In Table 3, is presented a comparison between the negative effects and the biotechnologic potential of *Trichoderma spp.* and gliotoxin. It is possible to verify that some of *Trichoderma* effects (negative or positive) are similar to the ones reported for gliotoxin. This shows that gliotoxin can be the cause for most of *Trichoderma spp.* properties. However, some species, such as *T. reesei*, are not confirmed to produce gliotoxin and still have some of the desired properties (Contreras-Cornejo et al., 2016). It is important to consider that *Trichoderma* species are great producers of secondary metabolites, not only gliotoxin, being that the porperties reported can also be related to other metabolites (Bezuidenhout, 2012; D'Acunti, 2017).

Table 3 - Comparison of *Trichoderma* spp. and Gliotoxin negative effects and their biotechnologic potential in the environment, industry and health

	Negative Effects		Biotechnologic Potential		References
	Trichoderma spp.	Gliotoxin	*Trichoderma spp.*	Gliotoxin	
Environment	Contamination of bottled water	High persistence in the environment	Commercialized as Biological Control Agent (biopesticides, bioprotectants, bio-inoculants, biodecomposers, plant growth enhancers, biofertilizers and stimulants of natural resistance)	Commercialized as Biological Control Agent	Fiorini, 2016; Woo et al., 2014; Howell, 2003; Mukherjee et al., 2012; Bezuidenhout et al., 2012; Contreras-Cornejo et al., 2016; Kredics et al., 2014; Mukherjee et al., 2013; Carberry et al., 2012; Dolan et al., 2015; Scharf et al., 2016; Wilhite et al., 1994; Anitha & Murugesan, 2005; Brian & Hemming, 1945; Vargas et al., 2014; Haraguchi et al., 1996
	Contamination of air	Inhibits production of enzymes, growth and seed germination of plants	Sustainable alternative to chemical pesticides	In fungi, induces redox stress and interferes with protein modification and can inhibit germination of fungal spores and mycelial growth	
			Remediation of heavy metals, toxins and xenobiotics	Low bioaccumulation rate	
			Can improve plant defenses against biotic and abiotic stress	Can inhibit bacterial multiplication	
			High reproductive capacity	Antibiotic activity	
			Ability to survive under very unfavorable conditions		
			Antibiotic activity		
Industry	Exposure and hazard to workers		Biofuels industry, as a producer of enzymes and chemicals		Mukherjee et al., 2013; Schuster and Schmoll, 2010; Konstantinovas et al., 2017
			Food industry, as aroma compounds and food additives		
			Biotechnology industry, as producer of nanoparticles		
			Textile, pulp and paper industry, as cellulase producer		
			T. reesei is a model system of plant cell wall degradation		
Health	*T. longibrachiatum* is considered a human pathogen to immunosuppressed individuals	Can affect cell mechanisms, leading to death of cells	Biodegradation and recycling complex polymers	Effects on different types of tumor cells	Kredics et al., 2014; Schuster and Schmoll, 2010; Mukherjee et al., 2013; Konstantinovas et al., 2017; Grove et al., 2006; Scharf et al., 2016; Dolan et al., 2005; Lewis et al., 2005; Waring & Beaver, 1996; Kwon-Chung & Sugui, 2009;
	Can produce toxins	Immunosuppressive agent	Can produce medicines	Effects against some human pathogens	

T. citrinoviride and *T. harzianum* can cause infection in immunosuppressed individuals	Can reach high concentrations on food and, if ingested by humans or animals, will affect their health	Antibiotic activity
Trichoderma spp. can cause infections in healthy individuals	Toxic for humans, animals and plants Inhibits the growth of cell lines and phagocytosis May play a role in human yeast infections by *Candida albicans*	Can be cyototoxic against human cancer cell lines

2.5.6. Bis-methyl-gliotoxin

Bis-methyl-gliotoxin (bmGT) is considered an inactive metabolite of gliotoxin (Domigo et al., 2012). It occurs when the methylation of the disulfide bridge happens (Sugui et al., 2017). The production of bmGT can decrease the gliotoxin synthesis, decreasing the amount of gliotoxin available (Domingo et al., 2012; Sugui et al., 2017).

This metabolite in opposite to gliotoxin, is nontoxic and not taken up by cells (Domingo et al., 2012). This property emphasize the possibility that bmGT might be a better biomarker of fungal presence (Domingo et al., 2012). In literature, it is also reported that this compound has generated interest as an Invasive Aspergillosis biomarker (Domingo et al., 2012; Vidal-García et al., 2018).

It is important to refer that this compound is only produced after the germination of the conidia, at the time that the fungi form the hyphae (Domingo et al., 2012). The exposure to this compound doesn't happen via airborne conidia, since the hyphae have to grow for the biosynthesis of bmGT (Domingo et al., 2012).

An enzyme, called GtmA (S-adenosylmethionine dependent methyltransferase), is responsible for the bmGT biosynthesis and it has been found in *A. fumigatus*, which is an S-adenosylmethionine (SAM)-dependent methyltransferase (Domingo et al., 2012; Vidal-García et al., 2018).

2.6. Other fungi

2.6.1. Alternaria spp.

Alternaria species are plant pathogens, saprophytes and soil-borne fungi (D'Acunti, 2017; Prelle, Spadaro, Garibaldi & Gullino, 2013). They can be found and develop in decomposing food, usually occurring in fruits and vegetables (D'Acunti, 2017; Prelle et al., 2013). These species are known to be able to grow at low temperatures (Prelle et al., 2013), but the optimal growth temperature is between 18ºC and 32ºC (D'Acunti, 2017; Lee et al., 2015). Other factors affect the growth and the germination rate of *Alternaria* species, including water activity (a_w), strain and their interactions (Lee et al., 2015). The spores of *Alternaria* are small and can spread in the environment easily (D'Acunti, 2017; Lee et al., 2015). The species of this fungus are associated with several damages in field

crops and postharvest decays, such as spoilage of fruits and vegetables during storage, having an important influence in agriculture (Lee et al., 2015; Prelle et al., 2013).

Alternaria can produce many toxic secondary metabolites, such as mycotoxins. The most important *Alternaria* mycotoxins are alternariol (AOH), alternariol methyl ether (AME), altenuene (ALT), altertoxin (ATX), tentoxin (TEN) and tenuazonic acid (TeA) (D'Acunti, 2017; Delgado et al., 1996; Lee et al., 2015; Prelle et al., 2013; Woudenberg, 2015). According to Andersen et al. (2008), ATX was found in more strains of *Alternaria tomatophila*. In a study developed in the Netherlands, TeA was found in high concentrations in one or more food merchandise (Lee et al., 2015). The European Food Safety Authority (EFSA) considers these metabolites as a high concern for public health (Lee et al. 2015; Woudenberg, 2015).

Alternaria species are known as human allergens, usually resulting in hypersensitivity pneumonitis, asthma, allergic sinusitis and rhinitis (Lee et al., 2015; Woudenberg, 2015) and their toxins have also been suggested as mutagenic and carcinogenic (Lee et al., 2015). It can also cause mycosis, specially affecting immune-compromised individuals (Woudenberg, 2015). Despite the lack of regulation, the hazard related to these fungi should be considered and, according to Lee et al. (2015), efforts have been made to understand the risks of these species to public health and the environment. The contamination of various groups of food (vegetables, fruits and plants) and the omnipresence of *Alternaria* species in the environment are other factors for the major concern about *Alternaria* species and their toxins.

The genus *Alternaria* is divided into 24 sections (Lee et al., 2015; Woudenberg, 2015), being the largest section the *Alternaria* section *Porri*, where is included *Alternaria tomatophila* (*A. tomatophila).* This can cause diseases in diverse types of plants, but is mostly known to cause early blight of tomato (Woudenberg, 2015). Other *Alternaria* species are associated with other plant diseases, such as *A. solani*, which causes potato early blight, *A. arborescens*, which causes tomato stem canker and *A. dauci*, which causes carrot leaf blight (Woudenberg, 2015). This genus of fungus is related to great losses on the production, because of the post-harvest diseases caused by its different species (D'Acunti, 2017; Prelle et al., 2013; Woudenberg, 2015).

2.6.2. Rhizoctonia solani

R. solani is a ubiquitous fungal species and it has been identified as a plant pathogenic fungus, attacking many horticultural plants (Atanasova et al., 2013; D'Acunti, 2017). Therefore, this fungus species has a severe influence in the damages made on a wide range of crops all over the world (Atansova et al., 2013).

R. solani pathogenic process involves the attack of the plant seeds, stem and roots, but it also includes phases as the secretion of invasive enzymes, the production of mycelium and spores in the plant surface and the necrosis of the host plant (D'Acunti, 2017).

It is also known as a model prey fungus for the *Trichoderma*-based products, being used in many studies of a formulation of new *Trichoderma*-based biocontrol products (Atanasova et al., 2013).

3. Materials and Methods

3.1. Dual Cultures

Considering the principal results obtained by D'Acunti (2017), in which only *T. virens* produced gliotoxin when in interaction with plant-pathogen fungi *Rhizoctonia solani* (RS) and *Alternaria tomatophila* (AT), in this study the effect of the presence of those plant pathogens, in the growth of *T. virens* (strain GV 29-8) and the effect of *T. virens* on these plant-pathogens were performed. All the fungi used in this assay belong to the Fungal Collection f the Agrifood Laboratory of the Università degli Studi di Bréscia (USB). All the experiments were developed in Agriffod Laboratory of USB. The treatments performed in this assay and the respective conditions are presented in Table 4.

Table 4 - Treatments and conditions of the Dual Cultures Assay.

Treatment Code	Identification of species	Treatments – mixture of strains	Media (solid)	Number of Replicates
1	*T. virens* + *T. virens*	GV 29-8 + GV 29-8	Potato Dextrose Agar (PDA) (Sigma-Aldrich)	5 replicates
2	*T. virens* + *R. solani*	GV 29-8 + RS		5 replicates
3	*T. virens* + *A. tomatophila*	GV 29-8 + AT		5 replicates
4	*R. solani* + *R. solani*	RS + RS		3 replicates
5	*A. tomatophila* + *A. tomatophila*	AT + AT		3 replicates

In this assay, the plugs of fungi were not in direct contact with the medium, since it was used cellophane paper to separate the plugs from the medium. A fungi plug of 6 mm was added to the edge of each plate, as it is shown in the Figure 3. The plugs used were withdrawn from a week old plates, where it was obvious the growth of conidia. The plates were sealed with parafilm to secure the hydration of the media.

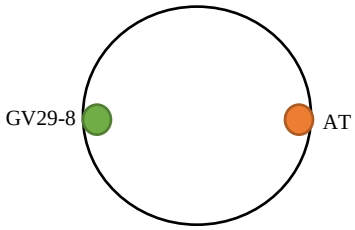

Figure 3 - Example of the layout of the 9cm petri dishes for the Dual Cultures assay.

In this experiment, the plates were incubated at 26 ºC, for one week. At the end of the week, pictures of all the plates were made and it was verified whether or not the fungi colonies came into contact with each other.

3.2. Antagonism Experiment with *Alternaria tomatophila*

In this assay, it was evaluated the ability of three *Trichoderma* strains (GV29-8, T6776 and RUTC30) to reduce the growth of *A. tomatophila* (AT), in a dual culture assay on PDA. The assay was made in agreement with results obtained in the "Dual Cultures" assay (section 3.1.). The colonies were added to the PDA plates in the form of a 6 mm plug. All the fungi used in this experiment belong to the Fungal Collection of the AgriFood Laboratory of the USB. The treatments performed in this assay and the respective conditions are presented in Table 5.

Table 5 - Treatments and conditions of the Antagonism Experiment with *Alternaria tomatophila*.

Treatment Code	Identification of species	Treatments – mixture of strains	Number of Replicates
1	*T. virens + A. tomatophila*	GV 29-8 + AT	3 replicates
2	*T. afroharzianum + A. Tomatophila*	T6776 + AT	3 replicates
3	*T. reesei + A. Tomatophila*	RUT C30 + AT	3 replicates
4	*A.tomatophila + A. tomatophila*	AT + AT	3 replicates

The plugs were placed as shown in Figure 4. The duration of the assay was one week and the growth rate was determined by measuring the radius of the colony every 12 hours (1st day, 2nd day, 3rd day, 4th day, 5th day and 7th day). The cultures were incubated during all experiment at temperature was 26ºC, always in dark.

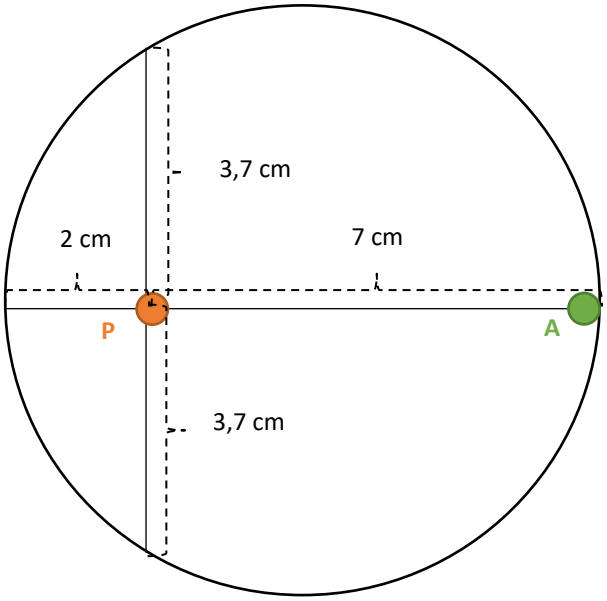

Figure 4 - Example of the layout of the 9cm petri dishes for the Antagonism Experiment with Alternaria tomatophila, where P is the pathogen (*A. tomatophila*) and A is the Trichoderma species (*T. virens, T. reesei or T. afroharzianum*), depending on the treatment.

The values of growth were transformed to regression lines and the slopes obtained were compared to determine the effect of *Trichoderma spp*. on the growth of AT.

3.3. Production of Gliotoxin and Bis-methyl-gliotoxin in the interaction of *Trichoderma spp.* with *Alternaria tomatophila*

This assay aimed the quantification of the production of Gliotoxin and bmGT, in the interaction of T*richoderma spp.* with *A. tomatophila*. The treatments performed in this assay and the respective conditions are presented in Table 6.

Table 6 - Treatments and conditions of the experiment to quantify the production of gliotoxin and bis-methyl-gliotoxin in the interaction of *Trichoderma spp*. with *Alternaria tomatophila*.

Treatment Code	Identification of species	Treatments – mixture of strains	Number of Replicates
A	*T. virens*	GV 29-8	6 replicates
B	*T. virens* + *A. tomatophila*	GV 29-8 + AT	6 replicates
C	*A.tomatophila* + *A. tomatophila*	AT + AT	3 replicates
D	*Weindling medium*	WDL	3 replicates
E	*T. virens* + *T. afroharzianum*	GV 29-8 + T6776	6 replicates
F	*T. virens* + *T. reesei*	GV 29-8 + RUT C30	6 replicates

The medium used in this assay was Weidling medium (WDL), which according to Weidling (1934), is the best substrate for gliotoxin production by *Trichoderma*. Only the strain GV 29-8 was put as a liquid suspension, the other fungi (AT, RUT C30 and T6776) were added in the form of a 4 mm plug. The suspension was prepared with sterile deionized water, as described in the supplemental material 7.1.. Both the plug and the suspension of mycelia were added at the same time. The conditions of the experiment were: final volume of 10 mL; incubation temperature 25 ºC, shaking of 200 rpm; duration of the experiment was 48 hours; and 16/8 hours of light/dark.

After the experiment, the replicates were filtrated (normal filter paper) and sampled. For the treatments A, B, E and F, the replicates were joined to make one sample. In this way, for each treatment, only 3 samples were made. After that, the samples were frozen at -20 ºC, until the further extraction.

3.4. Extraction and Quantification Procedure

The extraction was performed with ethylacetate: diethylether (50:50). The volume used of the mixture of solvents for each extraction was 5 mL. To each sample, was added miliq water, in the ratio of 1:1. The extraction protocol (Supplemental Material 7.2.) was performed. After it was added 3g of MgSO4, in order to remove the water of the sample. The samples were filtrated with filter paper and then dried in a HB10 rotavapor (IKA Works Inc., NC, USA), with bath temperature of 40ºC and 80 rpm.

After the extraction, the samples were resuspended in one mL of methanol and then centrifuged in a speed-vacuum centrifuge (Savant SpeedVac 100, ThermoFisher Scientific, MA, USA) for one hour, until the samples were completely dried. The samples were kept at -20ºC until the HPLC analysis, which was made by the Proteomica Laboratory of the Università degli Studi di Bréscia.

For HPLC analysis, each samplewas resuspended in 100 µL of methanol. Five µL of each biological sample or standard were analyzed three times by RP-HPLC-UV, using a DionexTM UltiMateTM 3000 Thermo Fisher Scientific S.p.A (Milan, Italy), equipped with a LPG-3400SD quaternary analytical pump, a WPS-3000SL analytical autosampler, a VWD-3100 UV–Vis detector, a TCC-3000SD thermostatted column compartment and an AFC-3000 automatic fraction collector. Chromatographic separation was performed using a Waters XSelect CSH C18 column (150mm×2.1mm ID, particle size 3.5 µm). The specific liquid chromatographic (LC) parameters were: mobile phase (A) water: TFA (99.95:0.05 v/v); (B) acetonitrile; the mobile phase flow rate was 0.3 ml/min; gradient program: 10% B for 2 min, from 10% to 90% B in 38 min, 90% B for 1 min and then from 90% to 10% B in 1 min and re-equilibration to 10% B for 3 min. All analyses were performed at 30 °C. The detection wavelength (ƛ) was set at 254 nm.

For the quantification, a solution of luteolin (25 µg/ml) was used as internal standard. Calibration curve of luteolin was done using six different dilutions of the luteolin reference standard (100.00 µg/ml; 50.00 µg/ml; 25.00 µg/ml; 10.00 µg/ml; 5.00 µg/ml; 1.00 µg/ml). Calibration curve of gliotoxin was done using five different dilutions of the gliotoxin reference standard (550.00 µg/ml; 275.00 µg/ml; 55.00 µg/ml; 27.5.00 µg/ml; 5.50 µg/ml). Calibration curve of bmGT was done using five different dilutions of the bmGT reference standard (250.00 µg/ml; 50.00 µg/ml; 25.00 µg/ml; 12.50 µg/ml; 2.50 µg/ml).

The calibration curves were plotted using the area under peak *versus* standard reference concentrations (µg/ml). Biological sample concentrations were calculated by determining the ratio between the gliotoxin or bmGT area under the peak and area under peak of internal standard and comparing them to the area with the correlated calibration curves.

3.5. Statistic Analysis

The statistic analyses in this thesis were made by the software Excel. The program GraphPad Prism 5 was used to make the regression analysis and the analysis of covariance of the assay “Antagonism Experiment with *A. tomatophila*”.

4. Results and Discussion

The production of gliotoxin by *Trichoderma spp.* is a known mechanism of biological control of pathogens, such as *Alternaria spp*. and *Rhizoctonia spp.* (Elvidge and Spring, 1949; Scharf et al., 2016). The aim of this study was to perform the interaction between some *Trichoderma spp.* and to evaluate the potential of production of gliotoxin and its methylated form, bmGT, in the interaction between *Trichoderma spp.* and plant-pathogens, such as *A. tomatophila* and *R. solani*.

4.1. Dual Cultures

In this Dual Cultures assay, it was verified that *T. virens* seems to affect negatively the growth of the plant-pathogen, *A. tomatophila*. As is shown in Figure 5, it was created an inhibition zone between the two fungi (row 3). It is believed that in this zone, there is the major quantity of secondary metabolites of both fungi. Also, this zone is where the most important reactions occur, so that the inhibition of *A. tomatophila* by *T. virens* can succeed. Our results are corroborated with the data reported by D'Acunti (2017), which show that the production of gliotoxin by *T. virens* is superior when the fungus is in interaction with the plant-pathogen, *A. alternata* (D'Acunti, 2017).

However, the Dual Cultures assay had some kind of contamination, as it is shown in Figure 5. It was possible to verify that not all the colonies of *Rhizoctonia solani* have the same color (row 2 and row 4), indicating that contamination occurred. According to the fungi collection used, colonies of *Rhizoctonia solani* are supposed to be white (as it is possible to see in picture 4B of Figure 5). For this reason, in this study it was not in considered the replicates that envolved the plant-pathogen *Rhizoctonia solani*.

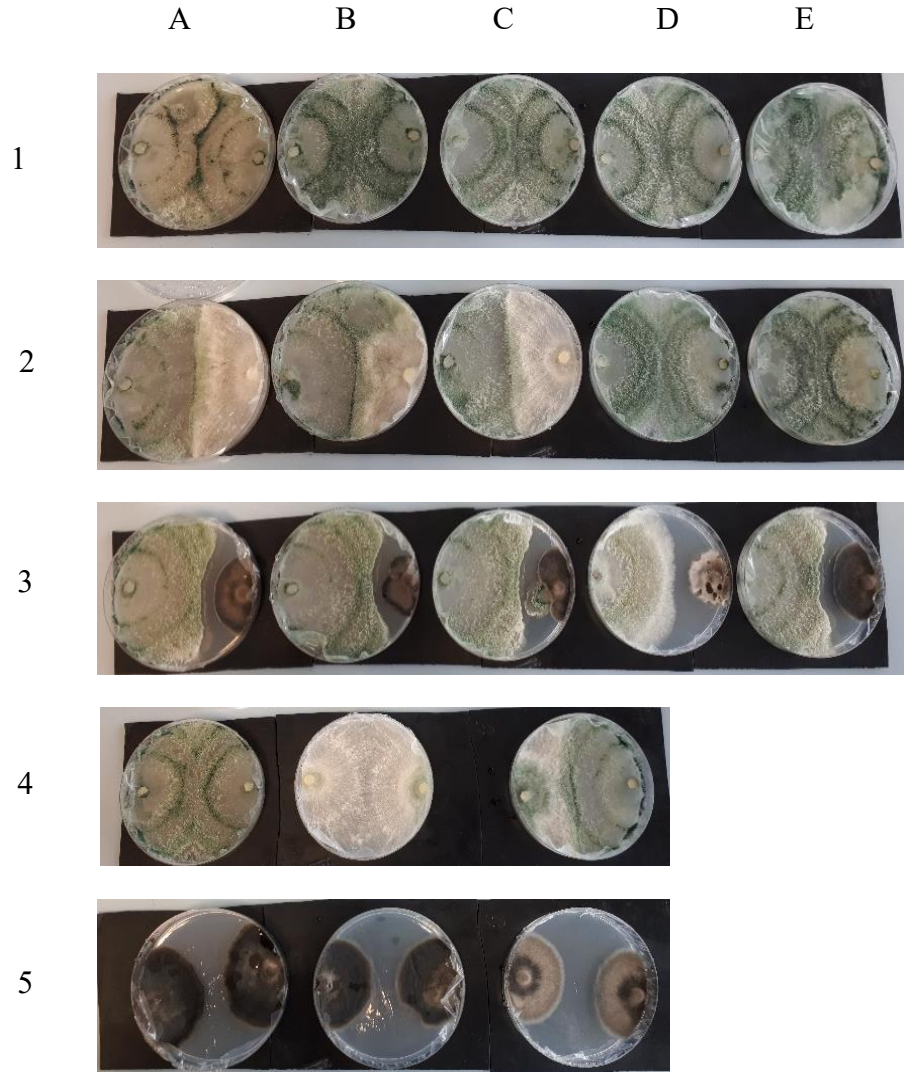

Figure 5 - Results of the Dual Cultures assay, where it was studied the effect of *Trichoderma virens* (GV29-8) in the growth of plant-pathogens, *Rhizoctonia solani* (RS) and *Alternaria tomatophila* (AT). The effect of the mentioned plant-pathogens on *T. virens* was also studied. The numbers correspond to the treatments (1: GV 29-8 + GV 29-8; 2: GV 29-8 + RS; 3: GV 29-8 + AT; 4: RS + RS; 5: AT + AT) and the letters indicate the replicates made (A to E).

4.2. Antagonism Experiment with *Alternaria tomatophila*

In order to analyse the effectiveness of *Trichoderma* species (*T. virens, T.reesei* and *T. afroharzianum*) in the reduction of the growth of *A. tomatophila*, an antagonism

experiment was performed and the results are presented in Figure 6. The results show that the growth of *A. tomatophila* was significantly reduced, when in dual culture with *T. virens* and *T. afroharzianum*. The growth of *A. tomatophila* was not affected by *T. reesei*.

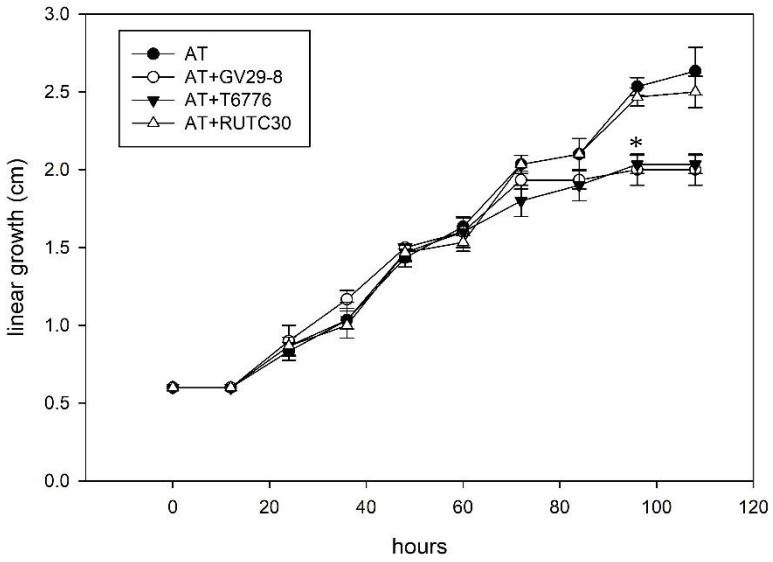

Figure 6 - Linear growth of *A. tomatophila* (AT), during the time in dual culture in interaction with itself (AT); in interaction with *T. virens* (GV 29-8 + AT); in interaction with *T. afroharzianum* (T6776 + AT); and in interaction with *T. reesei* (RUT C30 + AT). Values are expressed as the mean ± SD (n=3).

*Indicate significant differences ($p_{value}<0.05$)

These differences are confirmed by the significance level of the three comparisons, resulting from the linear regression of the growth of *A. tomatophila* (Table 7).

Table 7 – Values of the slope (mean ± SD), r^2 and p_{value}, resulting from linear regression of the growth of *Alternaria tomatophila* (AT), in the dual culture, in interaction with itself (AT + AT); in interaction with *Trichoderma virens* (GV 29-8 + AT); in interaction with *Trichoderma afroharzianum* (T6776 + AT); and in interaction with *Trichoderma reesei* (RUT C30 + AT).

Treatments	Slope (mean±SD)	R^2	p_{value}
GV 29-8 + AT	0.01513±0.001439	0.9326	< 0.0001
T6776 + AT	0.0154±0.001170	0.9560	< 0.0001
RUT C30 + AT	0.01995±0.001130	0.9750	0.3013
AT +AT	0.02089±0.001010	0.9817	

The inhibition of *A. tomatophila* was, on average, 0.5 cm in the cases of the interaction with *T. virens* and with *T. afroharzianum*. As it is possible to verify by the values obtained in the slope of the interaction of *T. reesei* with *A. tomatophila*, there was no inhibition of the plant-pathogen.

In this experiment it was also possible to verify the appearence of the inhibition zone, like in the “Dual Cultures” experiment. However, in this zone, the hyphae of both fungi eventually ceased. In the replicates of *T. virens* with *A. tomatophila* and *T. reesei* with *A. tomatophila*, the filaments appeared on the 7th day of the experiment. On the other hand, in the replicates of *T. afroharzianum* with *A. tomatophila* the filaments grew in the inhibition zone at the 4th day of the experiment, reaching a total overgrow at the 7th day. This may indicate that strain *T. afroharzianum* has a major inhibition effect over *A. tomatophila* than *T. virens* or *T. reesei*.

Hereupon, it can be afirmed that the inhibition of *A. tomatophila* by *Trichoderma* species occurs, but it is not effective long-term, since both fungi started to grow on the inhibition zone in all of the treatments performed.

4.3. Production of Gliotoxin and Bis-methyl-gliotoxin in the interaction of *Trichoderma spp.* with *Alternaria tomatophila*

The results of the experiment regarding the production of Gliotoxin and bmGT in the interaction of *Trichoderma spp.* with *A. tomatophila* are presented in Figure 7. It is possible to afirm that strain GV 29-8, when in interaction with *A. tomatophila*, produces less gliotoxin than when by itself, probably due to the fact that *A. tomatophila* "overcomed" the inhibition phase. D’Acunti (2017), obtained the opposite results, in which strain GV 29-8 by itself produced less gliotoxin than when in interaction with *A. tomatophila*.

It was reported in some studies that *Trichoderma spp.* was able to overgrow plant-pathogens, such as *A. porri* and *A. alternata* (Abo-Elyousr, Abdel-Hafez and Abdel-Rahim, 2014; Chethana, Ganeshan, Rao and Bellishree, 2013; Gveroska and Ziberoski, 2012; Imtiaj and Lee, 2008; Prakasam and Sharma, 2012). This ability of *Trichoderma spp.* may be related to the competitive capability and the mycoparasitic activity of these fungi. It is known that the main mechanisms of biological control of *Trichodeerma spp.* are associated to mycoparasitism, fungistasis, antibiosis and nutrient competition, as reported before (Bezuidenhout, 2012; Gajera et al., 2013; Mukherjee et al., 2012). With is properties, *Trichoderma spp.* is able to grow faster than other fungi, such as *Alternaria spp.* and overgrow the pathogens.

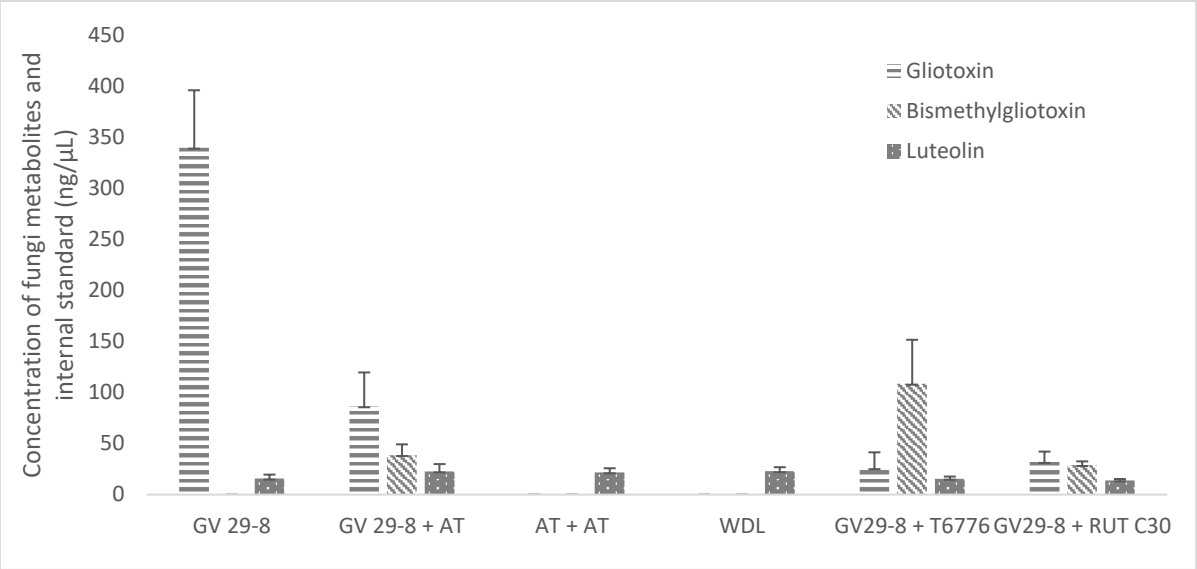

Figure 7- Production of gliotoxin and bis-methyl-gliotoxin by *Trichoderma virens* (GV 29-8), when in interaction with *Alternaria tomatophila* (AT), *Trichoderma reesei* (RUT C30) and *Trichoderma afroharzianum* (T6776). The control used was the internal standard, Luteolin (LUT).

It is important to refer that bmGT is produced in the interaction between *T. virens* and *A. tomatophila*, but in the treatment where *T. virens* is by itself this compound was not produced. This could mean that the interaction of *T. virens* with *A. tomatophila* enhance the methylation of the disulfide bridge and, according to Domingo et al. (2012), the production of bmGT decreases the gliotoxin synthesis, being this fact the potential cause for the decreasing of gliotoxin amount in this interaction.

In the treatments where it was studied the interaction between two *Trichoderma* species (treatments E: GV 29-8 + T6776; and F: GV 29-8 + RUT C30), the production of gliotoxin was lower and the production of bmGT was increased. A possible explanation for this, as reported before, could be the fact that the production of bmGT can decrease the gliotoxin synthesis, decreasing the amount of gliotoxin available, since there is no bioaccumulation of gliotoxin in the environment (Domingo et al., 2012; Sugui et al., 2017; Santa Cruz Biotechnology, Inc., 2016).

It should be distinguished the two treatments analysed above, since one of them presented more intense results than the other one. In the treatment of the interaction of *T. virens* with *T. afroharzianum*, the amount of bmGT is more pronounced than in the interaction with *T. reesei*. These results corroborate that *T. reesei* (strain RUT C30) is not considered a biocontrol agent (Contreras-Cornejo et al., 2016; Corsi, 2016; D'Acunti, 2017). On the

other hand, *T. afroharzianum* is considered a fungicide and a biocontrol agent (Atanasova et al., 2013; Mukherjee et al., 2012; Phupiewkham et al., 2015). Despite of the fact that *T. afroharzianum* was not able to produce a high amount of gliotoxin in the conditions of this study, there is proof of the antibiotic and fungistatic properties of this fungus (Fiorini, 2016). Therefore, the research for secondary metabolites with antibiotic properties against pathogens should continue for this species.

As it was expected, in the treatments of control (AT + AT and WDL), there was no production of compounds, gliotoxin and bmGT.

The results show that the production of secondary metabolites by *T. virens* and by *T. afroharzianum* may affect the gorwth of plant-pathogen *A. tomatophila*. Furthermore, it was also confirmed that *T. reesei* probably is not able to inhibit the growth of *A. tomatophila*. However, it is not possible to assert that the gliotoxin is responsible for that inhibition, at least in the interaction of the pathogen with *T. afroharzianum*. Another metabolite can be the cause of the fungistasis, such as bmGT. Therefore, it just can be affirmed that the presence of the plant-pathogen *A. tomatophila* has an effect on the production of gliotoxin and bmGT, by *Trichoderma spp.*.

The knowledge about the effects of secondary metabolites is of major importance, in order to optimize its presence and use in the environment. The negative effects associated with mycotoxins are, usually, numerous, but there are some properties of these compounds that make their use in different environments appealing. It should be considered the risks and the benefits of the presence of gliotoxin and bmGT in the environment, as well as in occupational environments. A study of dose-response of these metabolites should be performed to enable the use of these compounds for its therapeutic potential. The exposure of workers to mycotoxins can have nefarious health consequences, but the conditions of the exposure, such the dose, are crucial to the type of the effects that arise, positive or negative. The presence of gliotoxin or bmGT could be used as bioindicators of air quality, indicating the presence of one of their producers when detected in an environment. This could facilitate the detection of fungi responsible for occupational diseases related to these metabolites, such as *A. fumigatus*, and possibly prevent the appearence of some of these diseases. Despite the negative consequences of the use of biocontrol agents, this practice is more suitable than the use of synthetic pesticides, not only for the environment, but also for human health.

5. Conclusions

The production of gliotoxin and bmGT by *Trichoderma spp.* is considered to be a mechanism of biological control. By the production of secondary metabolites, *Trichoderma spp.* are able to inhibit the growth of other fungi or even kill them. The ability of mycoparasitism is one of the properties that increase the worldwide use of *Trichoderma* species as microbial biological control agents against bacteria, viruses and fungi.

The Integrated Pest Management, a sustainable and effective approach of management for crop pests and natural enemies, was implemented, in order to increase the use of biocontrol agents as an alternative to chemicals, increasing the security for consumers and reducing the risk for public health. However and considering that some of the organisms used in this practice produce secondary metabolites, there should have regard to the effects of these secondary metabolites and how can they affect humans. Therefore, the regulation of them, such as gliotoxin and bmGT, should exist, considering the effects of these compounds on human health.

In this thesis, it was evaluated the production of gliotoxin and bmGT by *Trichoderma spp.* in the presence of *A. tomatophila*. *A. tomatophila* is a known plant-pathogen, usually related to plant diseases in tomato and potato. The growth of this fungus is affected by the presence of *Trichoderma spp*. (specifically *T. virens* and *T. afroharzianum*), meaning that *Trichoderma spp.* is effective as a biocontrol agent regarding *A. tomatophila*. Despite of the positive effects of *Trichoderma spp*., as the biological control properties against pathogenic fungi, there are a species, such as *T. longibrachiatum*, that are known to be a human pathogen to immunosuppressed individuals.

The production of secondary metabolites by *Trichoderma spp.* can be affected by the presence of *A. tomatophila*. The results of this work showed that, depending on the species of *Trichoderma*, the production of secondary metabolites can increase, as a mechanism of defense, which can result in the biological control of plant-pathogens. It was also possible to attest that the metabolite produced in great concentration is species dependent.

Further studies may focus on the production of bmGT and in its production only when the pathogen is present. It should also be studied deeply the interaction of *T. afroharzianum* with *A. tomatophila*, considering that in dual culture, this species of

Trichoderma was able to inhibit the growth of the pathogen quicker than *T. virens*. Studies should be performed in order to identify and quantify the presence of secondary metabolites in the inhibition zone created in the interaction of *Trichoderma spp.* with pathogenic fungi, in order to clarify the mechanism of action of *Trichoderma* secondary metabolites.

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7. Supplemental Material

7.1. Fungal suspension protocol

PREPARATION OF FUNGAL SUSPENSION

1. Add 15 mL of Weindling medium to a 10 days old petri dish.
2. With a spatula, release the spores from the solid medium.
3. Move the liquid medium to a previously labled 15 mL falcon.
4. Make a suspension of double deionized water (ddH_2O): fungal suspension (10:1), meaning that it should be added 900 µL of ddH_2O and 100 µL of fungal suspension to a previously labled eppendorf.
5. Vortex the Eppendorf.
6. Wash the Bucker chamber and the coverslip with alcohol.
7. Put a drop of the suspension (10:1) in the square of the chamber and put the coverslip.
8. Count the conidia at the microscope.
9. Make the accounts with the following equation:

$$Concentration\ of\ the\ eppendorf\ \left(\frac{spores}{mL}\right) = \frac{n^{o}\ spores\ counted}{n^{o}\ squares\ counted} \times 4000 \times 1000$$

10. Since it was med a dilution of 10:1, the original concentration of the suspension (15 mL falcon) will be:

$$Concentration\ of\ falcon = Concentration\ of\ eppendorf\ \times 10$$

11. Considering the following equation, calculate the volume of the initial suspension (V_i), with the initial concentration (C_i) and of Weindling medium that it has to be added to reach the final concentration (C_f) and the final volume (V_f).

$$Ci \times Vi = Cf \times Vf$$

7.2. Gliotoxin extraction procedure

GLIOTOXIN PROTOCOL EXTRACTION

1. Defrost the samples, leaving them in fridge over night;

2. Take the samples from the fridge ans vortex them, paying attention to dissolve any deposite in the bottom of the falcon;

3. Transfer 5 mL of sample in a previously labeled glass becker;

4. Dilute the sample with miliQ water in ratio 1:1 (5 mL of sample and 5 mL o miliQ water);

5. Put a magnetic anchor in the becker with the sample;

6. Go under chemical cabinet and add half of the total volume of the solvent mixture (Ethylacetate: Diethylether) - add 5 mL of Ethylacetate: Diethylether;

7. Cover the becker with alluminium foil and put the sample in rotary shaker and keep it in agitation for 10 minutes;

8. Put a labeled separating funnel on the support under the chemical cabinet and put a clean labeled glass becker under it;

9. After the ten minutes, switch off the rotation and transfer the mixed sample in the separating funnel;

10. In the separating funnel, there will create two layers, the unpolar one (bottom side of the funnel) and the polar one (upper side of the funnel), in which is the gliotoxin. Transfer the upper phasein the clean becker under the funnel;

11. Transfer the unpolar phase (bottom phase) in the first used becker;

12. Repeat from 6 to 11 for 2 times (total of 3 times);

13. Trash way the unpolar phase (biological liquid waste);

14. Add 3g of $MgSO_4$ (Magnesium sulfate) and manually vortex it;

15. Filtrate, under chemical cabinet, the samples to a previously labeled rotavapor balloon, using a filter paper;

16. After the filtration, the sample is ready to be dried by rotavapor.