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EVALUATION OF THE EFFECTIVENESS OF ECO-FRIENDLY SUBSTANCES AND MICROBIAL ANTAGONISTS AGAINST FUNGAL PATHOGENS OF AGRICULTURAL CROPS AND FOREST PLANTS

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ABSTRACT

Plants are exposed to both biotic and abiotic stresses of various origin and nature. Among biotic stresses, infections by fungal pathogens are particularly relevant while the main abiotic stress factors include pollutants. Of the latter, synthetic chemical pesticides play a remarkable role because of their organic residues as well as inorganic toxic molecules, such as heavy metals.

Species of *Trichoderma* are well known as biocontrol agents of fungal plant pathogens and have also been recognized as a potential source of bioactive metabolites. These metabolites can have antimicrobial properties and be useful in the control of the plant pathogens. Moreover, being produced naturally by a microorganism, they are easily degradable and environmentally friendly, which makes them a valid alternative to synthetic chemical pesticides.

Some *Trichoderma* species have been studied for their ability to grow in the presence of heavy metals and absorb them without any toxic effect.

Two selected strains, *T. atroviride* (TS) and *T. asperellum* (IMI 393899), were used throughout this study with the following objectives: i) to evaluate their antagonistic ability against the pathogen oomycete *Phytophthora nicotianae* in the presence of a heavy metal (cadmium) and thus their ability to protect the plant from both stresses (biotic and abiotic); ii) to investigate their ability to produce antimicrobial substances in liquid culture and test the inhibitory activity of these metabolites against different fungal and oomycete plant pathogens; iii) to determine the active concentration of these metabolites on different plant matrices inoculated with mycotoxigenic fungi.

The antagonistic ability of the two selected strains of *Trichoderma* in the presence of cadmium (Cd) was tested *in vitro* using the dual culture assay and *in vivo* in tomato plants. The *Trichoderma* genes activated by the presence of heavy metals were identified and their expression was quantitatively determined by RT-qPCR.

The bioactivity of culture filtrates from 10- and 30-days-old cultures extracted with ethyl acetate (EtOAc) was evaluated *in vitro* against several pathogenic fungi and oomycetes, to determine

the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC). Culture filtrates were analyzed by GC-MS and HPLC-Q-TOF-MS for the identification of volatile organic compounds (VOCs) and non-volatile organic compounds (nVOCs).

Bioactive metabolites produced *in vitro* by the two selected strains of *Trichoderma* were tested on tomato fruits (inoculated with *Fusarium verticillioides* and *Fusarium graminearum*), corn kernels (inoculated with *Aspergillus flavus*) and wheat kernels (inoculated with *Penicillium verrucosum*). Three concentrations of each metabolite were tested and the minimum dose was MFC. The effects of *in vivo* treatments were evaluated in terms of shelf-life of these three different plant matrices and reduction of mycotoxins produced by these fungi.

In vitro, the inhibitory effect of Cd on *Trichoderma* spp. growth was low. *Trichoderma* was able to inhibit the presence of *Phytophthora* both in presence and absence of Cd. *In vivo*, *Trichoderma* showed no significant plant protection effects against *Phytophthora* in the presence and absence of Cd. The results were confirmed by the analysis of the expression of genes, which were not significantly over-expressed.

Conversely significant results were obtained in the test of active metabolites extracted from culture filtrates of the two selected strains of *Trichoderma*. The extracts from 30-day-old cultures of both strains showed significant cytotoxic effects on the pathogens under study, with minimum fungicidal concentration (MFC) values between 0.19 and 6.25 mg/mL. It was also demonstrated the ability of these metabolites to inhibit the growth of mycotoxigenic pathogens. Overall, these results indicate that these two selected *Trichoderma* strains have antagonistic activity against fungus and oomycete plant pathogens and are a potential natural source of compounds with biological activity.

RIASSUNTO

Le piante sono esposte a stress biotici e abiotici di varia origine e natura. Tra gli stress biotici sono particolarmente rilevanti gli attacchi di agenti patogeni fungini. Tra i fattori abiotici, gli inquinanti sono una importante fonte di stress e tra questi quelli derivanti dall'uso di agrofarmaci chimici di sintesi, che contengono come residui sostanze tossiche organiche e inorganiche, quali ad esempio i metalli pesanti. Questi composti tossici (xenobiotici) possono avere effetti nocivi sull'ambiente con possibili conseguenze per la vita degli esseri viventi, compresi gli esseri umani.

Le specie *Trichoderma* sono note come agenti di biocontrollo di patogeni fungini delle piante, ma di recente sono stati studiati anche come potenziale fonte di metaboliti bioattivi, dotati di proprietà antimicrobiche, che possono essere utili nel controllo di agenti patogeni delle piante. Inoltre, essendo metaboliti prodotti da microrganismi, cioè di origine naturale, sono facilmente degradabili ed ecocompatibili, il che li rende una valida alternativa ai pesticidi chimici di sintesi. Alcune specie di *Trichoderma*, infine, sono state studiate per la loro capacità di crescere in presenza di metalli pesanti e di assorbirli senza subirne gli effetti tossici.

In questa tesi sono stati studiati due ceppi selezionati di *T. atroviride* (TS) e *T. asperellum* (IMI 393899), con i seguenti obiettivi: i) valutarne la proprietà antagonista nei confronti dell'oomicete patogeno *Phytophthora nicotianae* in presenza di un metallo pesante (cadmio) e la capacità di proteggere la pianta da stress biotici e abiotici; ii) accertare la loro capacità di produrre metaboliti con attività antimicrobica in coltura liquida e saggiare l'attività inibitoria di questi metaboliti contro diversi funghi e oomiceti patogeni di piante agrarie e forestali; iii) determinare la dose efficace di questi metaboliti su diverse matrici vegetali inoculate con funghi micotossigeni.

La proprietà antagonista dei due ceppi di *Trichoderma* in presenza di cadmio (Cd) è stata saggiata *in vitro* in coltura duale e *in vivo* su piante di pomodoro. Sono stati individuati i geni

di *Trichoderma* coinvolti nell'interazione con i metalli pesanti e la loro attività è stata determinata quantitativamente mediante RT-qPCR.

È stata determinata la bioattività del filtrato colturale dei due ceppi in colture di 10 e 30 giorni. L'estrazione è stata effettuata con acetato di etile (EtOAc). L'attività è stata valutata *in vitro* nei confronti una serie di funghi e oomiceti patogeni, determinando la minima concentrazione inibitoria (MIC) e la minima concentrazione fungicida (MFC). I filtrati colturali sono stati analizzati con GC-MS e HPLC-Q-TOF-MS per l'identificazione dei composti organici volatili (COV) e non volatili (nVOC).

I metaboliti bioattivi prodotti in coltura liquida dai due ceppi di *Trichoderma* sono stati saggiati su frutti di pomodoro (inoculati con *Fusarium verticillioides* e *Fusarium graminearum*), cariossidi di mais (inoculate con *Aspergillus flavus*) e cariossidi di frumento (inoculate con *Penicillium verrucosum*). Sono state saggiate tre concentrazioni di ciascun estratto: la concentrazione minima utilizzata per ciascun metabolita è stata la MFC. È stato valutato l'effetto del trattamento sulla durata di conservazione delle tre matrici vegetali e l'eventuale riduzione della presenza di micotossine prodotte da questi funghi.

In vitro, l'effetto inibitorio del Cd sulla crescita di *Trichoderma* spp. è stato basso. *Trichoderma* è stato in grado di inibire *Phytophthora* sia in presenza che in assenza di Cd. *In vivo*, *Trichoderma* non ha mostrato effetti rilevanti di protezione delle piante nei confronti di *Phytophthora* sia in presenza che in assenza di Cd. I risultati sono stati confermati dall'analisi dell'espressione genica, che non ha mostrato una over-espressione dei geni presi in esame.

Risultati significativi sono stati ottenuti nel saggio dei metaboliti bioattivi prodotti dai due ceppi di *Trichoderma*. Gli estratti di colture di 30 giorni di entrambi i ceppi hanno mostrato effetti citotossici significativi contro gli agenti patogeni utilizzati nei saggi, con valori minimi di concentrazione fungicida (MFC) tra 0,19 e 6,25 mg/mL. Anche *in vivo* questi metaboliti hanno mostrato un'attività inibitoria significativa sui patogeni micotossigeni.

I risultati complessivamente indicano che questi ceppi di *Trichoderma* hanno un'attività antagonista nei confronti di funghi e oomiceti patogeni delle piante e sono potenziali fonti naturali di composti con attività biologica.

1. GENERAL INTRODUCTION

In recent centuries, the advent of globalization has led to an increase in world population and consequently an increase in the demand for food. In order to meet this demand, the industrial activity and the use of unfair agricultural practices were increased with risks on the environmental and the alteration of the ecosystem.

Environmental pollution is the consequence of the emission into the environment of substances defined as xenobiotic that can alter the normal balance of an organism and/or ecosystem.

Among the main xenobiotic substances that can cause an environmental alteration there are agrochemicals and heavy metals (often contained in the formulations of agrochemicals); these substances can be non-degradable and contaminate water, soil and air but also food, causing risks to health and environment. Moreover, the excessive use of pesticides can affect the normal balance of the rhizosphere, i.e., those microorganisms present in the soil that benefit plants, and lead to resistant pathogenic strains. These problems have led to the search for more environmentally sustainable solutions that can compensate for the environmental damage. A valid alternative is represented by microorganisms, they are able to resist extreme conditions, to proliferate and bringing benefits to plants through the bioremediation of toxic substances and biological control of plant pathogens. In particular, among the beneficial microorganisms are reflected the fungi belonging to the genus *Trichoderma*; many species of *Trichoderma* are known for their properties of antagonism against pathogens and the ability to grow in the presence of toxic substances. This genus is arousing considerable interest in various scientific fields that go beyond the field of agriculture.

1.1. Xenobiotics

A xenobiotic (Greek, xenos “foreign”, bios “life”) is a compound that is foreign to a living organism. Principal xenobiotics include drugs, carcinogens and various compounds that have been introduced into the environment by artificial means (IUPAC, 1997).

Xenobiotics can be inorganic compounds (such as heavy metals) and a large number of organic molecules, which can be of natural, synthetic or semi-synthetic (a compound that partially maintains the original structure) origin. They can influence organisms in many different ways. Among the xenobiotics of natural origin, there are the molecules synthesized by an organism. These molecules are able to modify the metabolic processes of another organism. For example, microorganisms and plants are able to produce secondary metabolites that can interact with other microorganisms as defence mechanisms (El-Moneim and Afify, 2010).

Many of the xenobiotics of natural origin are easily degradable molecules that do not persist in the environment. The principal sources of these substances are the industrial, agricultural and domestic sectors. Many xenobiotic substances can be slowly transformed by living organisms, especially decomposers, until they are completely demolished and are defined biodegradable, while other substances are not biodegradable (or very slowly) (Joutey *et al*., 2013). Most of the pollutants poured into water and soil are able to accumulate in organisms because they are predominantly hydrophobic. Through the trophic chain, they are transferred from one organism to another, increasing their concentration through the process of "biological amplification" (Kay, 1984). Initially, the toxic substance present in soil or water accumulates first in plants, then through nutrition in herbivores and in carnivores.

Inorganic, synthetic and semi-synthetic xenobiotics can be resistant to degradation processes and they can remain in the environment. These substances can be a serious problem for environmental pollution causing dangerous negative effects on living organisms, including humans (Embrandiri *et al*., 2016).

The bioaccumulation is the progressive increase in the amount of a substance in an organism or part of an organism which occurs because the rate of intake exceeds the organism's ability to remove the substance from the body (IUPAC, 1993).

Bioaccumulation can occur through two different mechanisms herewith explained (Alexander, 1999).

- Bioconcentration - direct capture of xenobiotics dissolved in water. It is a phenomenon of physical nature that depends on the lipophilicity of the compound (liposoluble substances are absorbed more easily than water-soluble substances).
- Biomagnification - increasing accumulation of the pollutant through food, so that higher concentrations are reached in organisms at higher levels in the food web.

To produce a toxic manifestation, a chemical agent or its metabolite must be able to interact with specific sites of the organism and be present in an appropriate concentration for a sufficiently long period of time. Therefore, the possible toxic manifestation depends on the chemical-physical properties of the chemical agent, on the exposure and the sensibility of the biological system.

1.1.1. Environmental contamination

Environmental contamination can be defined as the consequence of human activity capable of modifying the properties of conditions or the availability or quality of resources in a given space and time interval (Srivastava, 2012). Contamination becomes pollution when it reaches a level that causes negative effects on organisms, populations, ecosystems. The processes of environmental pollution can be of natural or artificial origin: the first are associated with phenomena of decay of materials, natural combustion and eruptive processes, while the second are closely related to industrialization. As the years passed, such problems have grown with the increase of the population, the industrial growth and the remarkable differentiation of the productive processes (Khan and Ghouri, 2011).

All countries in the world have had to acknowledge the existence of problems such as air, water and soil pollution, acid rain, endangered species, climate change, hazardous waste, deforestation. Despite this, production activities continue to release substances of various kinds into the environment with consequent risks of contamination of air, soil, subsoil, surface water and groundwater.

In recent years, the problem of safeguarding the environment related to that of economic development is one of the most relevant issues that the industrialized world has to face. It touches the individual conscience of each one, it is very much felt by public opinion in all countries and increasingly affects the policies of governments and international organizations (Jordan and Lenschow, 2010).

In fact, anthropogenic activities through industrial processes (chemical and petrochemical), mining, urban and agricultural activities have determined a consistent increase in the concentration of heavy metals and other inorganic and organic pollutants into water and soil. This situation has resulted in progressive damage to natural resources, the degradation of the territory and a danger to public health.

The greatest effects on human health are related to direct contact with contaminated and particularly frequented areas. The intake of contaminated water, the inhalation of vaporized compounds and the entry of toxic substances into the food chain have toxicological importance. Among the main causes of environmental pollution, there are incorrect agricultural practices, due to the excessive use of agrochemicals, such as pesticides and fertilizers (Merrington *et al.*, 2002). These compounds are hardly biodegradable and remain in the soil, through the leaching of rainfall they reach the subsoil, the aquifers and flow into water bodies (rivers, lakes and oceans). The use of contaminated water (recycling of water from rivers, lakes and aquifers) to irrigate crops can bring the pollutant back into the soil and crops. Moreover, the contamination of water bodies can alter the present aquatic ecosystem.

1.2. Agrochemicals

The primary environmental contaminants produced by agriculture are agrochemicals, especially pesticides. These are deliberately introduced into the environment by farmers to protect crops and improve yields.

Agrochemicals are the various chemical products used in agriculture production to improve productivity and control pests and diseases. In most cases, the term agrochemical refers to the wide range of chemical pesticides and the term may also include synthetic fertilizers, hormones and other growth chemicals (Mandal *et al*., 2020).

Pesticides are chemically different substances. About 100 different fungicides are now in use, along with about 90 herbicides, 70 insecticides and other pesticides (Ministero della salute, 2020). However, each specific pesticide chemical (also known as the active substance) can be marketed in a variety of formulations, which contain additional substances that act to increase the effectiveness of the actual pesticide. These so-called "inert" ingredients in the formulation may include solvents, detergents, emulsifiers and chemicals that allow the active ingredient to better adhere to the foliage. In total, there are more than 5000 different pesticide formulations.

Pesticides can also be classified according to their chemical structure. Inorganic pesticides, for example, are simple compounds of toxic elements such as arsenic, copper, lead and mercury.

Inorganic pesticides were previously used widely, mainly as fungicides. However, their use has been limited due to their high degree of environmental contamination. Organic pesticides (containing carbon) are the most widely used. They can be natural (produced by plants or microorganisms) or they can be semi-synthetic (using chemical compounds isolated from natural sources as starting materials to produce other new compounds with distinct chemical characteristics) or synthetic.

Most organic pesticides, however, have been synthesized by chemists. Synthetic organic pesticides include well-known groups such as chlorinated hydrocarbons (including the insecticide DDT, and the herbicides 2,4-D and 2,4,5-T), organophosphates (such as parathion and malathion), carbamates (for example, carbaryl and carbofuran), and triazine herbicides (such as atrazine and simazine).

1.2.1. Excessive use of agrochemicals

Over the last 100 years, with the exponential increase in population, traditional agriculture has been replaced by intensive agriculture that aims to maximize the productive capacity of the land to meet the growing demand for food.

In modern intensive agriculture (or intensive farming) the greatest exploitation is given by the use of technological innovations, chemical fertilizers and pesticides, as well as machinery suitable to make the working processes faster.

Moreover, intensive farming is based on the cultivation of monocultures, which consists of using large areas of land to cultivate a single plant species, in an intensive and standardized manner, in order to maximize yields and obtain maximum profit. Often this standardization is accentuated by the use of a few varieties, very productive but very demanding, and the massive use of synthetic fertilizers and pesticides. However, these intensive agricultural practices have significantly negative impacts on the environment and the ecosystem (Zhang *et al*., 2018).

1.2.2. Health and environmental risks

In the field, pesticides can be applied directly into plants, to the soil surface and to the soil below the surface. Pesticides can be lost through degradation, evaporation and other dissipation processes. They can reach both soil and surface water through several routes, including surface runoff, erosion, leaching, spray drift, improper container disposal and accidental spills. The physical properties of a given pesticide regulate the extent to which it is absorbed into the soil and prevent its displacement. The application of soluble pesticides to the soil causes the highest rate of loss in terms of surface runoff.

The penetration of pesticides into surface waters at significant levels can lead to the destruction of ecosystems of both flora and fauna.

Some agrochemical products can bioaccumulate in edible organisms and make them unsafe for human consumption. In addition, contamination of drinking water is becoming a major

problem, especially at sites without treatment, where without a significant degradation process they can continuously permeate the water. The World Health Organisation (WHO) reports that 500,000 people are poisoned by pesticides every year, of which at least 5,000 die. The rate of biocide poisoning is 13 times higher in developing countries (Weir and Shapiro, 1981).

Pesticides can represent a serious danger to all forms of life. They have a high affinity for lipids in biological tissues. Even when present only in traces, bioaccumulation processes can lead to high and even lethal concentrations in the tissues of higher predators, including humans (Hindin, 1970).

Some studies show that foods often contain different types of residues. Residues from pesticide application may persist in tissue or on the surface of crops when they reach the market. Some substances interact with each other in a synergistic way and their combined effect is greater than the sum of the individual components (Reffstrup *et al*. 2010).

A concern is the repetitive intake of pesticides through food, especially lyophilic pesticides (which bind to fats) and that over time accumulate in the body giving significant toxic effects, such as neurodegenerative diseases, tumours, fetal malformations, autoimmune and metabolic diseases.

1.2.3. Effects on non-target microorganisms

Many pesticides are capable of targeting a wide range of non-target microorganisms (Zaller and Brühl, 2019).

The value of pesticides lies in their ability to kill harmful or unwanted organisms, but they are rarely selective. Most act by interfering with fundamental biochemical and physiological processes that are common to a wide range of microorganisms, not just parasites.

In particular, fungicides and bactericides can interfere with the microflora population, eradicating beneficial fungi and bacteria present in the soil, which contribute to the natural defence of plants and the bioavailability of microelements. These microorganisms are also

responsible for the processes of removal of pollutants present in the soil, including pesticides, through biotransformation into non-polluting substances (Chaudhry *et al*., 2005).

1.2.4. Resistance to agrochemicals

The excessive use of pesticides has led to the onset of resistance, i.e. the resistance of parasites to pesticides developed to control them. This is exacerbated by the non-selectivity of pesticides that compromises the microflora or more seriously affects the presence of beneficial bacteria and/or fungi able to naturally control the population of pathogens.

The excessive and repeated use of the same active substance exerts a favourable selective pressure on resistant individuals, giving them an advantage over sensitive ones.

In particular, if active substances with the same specific mechanism of action are used, it facilitates the establishment of these phenomena that occur with the total or partial lack of activity of the applied formulation.

For example, fungi react in many ways to the presence of the fungicide in the environment, so as not to succumb to the toxic action of the chemical molecule they develop metabolic processes. In general, there are phytopathogenic fungi that can biochemically alter the receptor of the active site in such a way that it is no longer compatible with the fungicidal molecule, others that are able to develop an alternative metabolic pathway in order to avoid the action site; in other cases they are able to metabolically detoxify the active substance, or manage to exclude it from the cell by reducing the permeability of the membrane or increasing the rate of expulsion from the cell itself.

1.3. Heavy metals

Metals are solid chemical elements, with the exception of mercury, at room temperature, with an atomic number higher than 20, mainly are the transition metals reported in the periodic table.

In general, heavy metals are defined as elements that have the following characteristics (Lapedes, 1974):

- density higher than 5 g/cm³
- behave like cations
- low solubility of their hydrates
- ability to form complexes
- high affinity for sulfides
- change their oxidation state according to pH

Some metals, defined as essential, are part of the biological cycles of living beings.

Are defined as "essential" metals those essential for the physiological functions of the organism, for example, iron (present in the EME) is part of the constitution of haemoglobin, it is a protein involved in redox reactions essential for breathing. However, the iron taken through diet or supplements must not exceed certain concentrations otherwise it too is toxic to the body.

In fact, all metals are considered toxic if the increase in dosage taken can create damage to health; the same metal can be essential at low doses and be toxic at higher doses.

However, there are heavy metals that are not among those indispensable for organisms, these include mercury, cadmium, lead and chromium, and they are also the major polluters.

Metals are toxic to the body due to their ability to accumulate in the organs and to interact with sulphur groups (-SH) of enzymes, whose metal-protein binding inhibits catalytic activity, affecting normal metabolic processes (Matts *et al*., 1991).

The toxicity can be acute or chronic depending on the period of exposure to the metal; in chronic, it can have symptomatology with organ failure, brain deficit, the onset of psychic diseases, cardiovascular disorders, etc., in addition to a higher incidence of cancer and in pregnant women can lead to fetal malformations or abortion (Honda *et al*., 2006).

1.3.1. Heavy metal pollution

Heavy metals have a tendency to accumulate in the soil and to re-enter the food chain of living beings, giving toxic effects even at small concentrations.

Heavy metal contamination can be of natural or anthropogenic origin. The main natural source is the rocky substrate. Among the sources of anthropic origin, the most relevant are caused by civil and industrial activities which also include agricultural practices.

Heavy metals are normally present on the earth crust in traces with very low concentrations ≤ 0.1%, in the order of part per million (ppm) and have natural origin from the disintegration of the rocky substrate and the environmental conditions in which the pedogenesis took place.

About 80% of crustal rocks are formed by a series of low-density minerals, mostly silicates and aluminosilicates; within the crystalline lattice of these primary minerals can be inserted, for isomorphic replacements and vicariance, some trace elements including heavy metals.

In order for the substitution to happen, the vicarianz elements must have similar ionic radius, charge and electronegativity. The isomorphic substitution occurs when the difference between the radius of the greater element and that of the heavy metal does not exceed 15% and the difference in charge is less than the unit.

The sedimentary rocks, which alone make up 75% of the outcropping rocks, make up the main parental substrate for pedogenesis.

They are formed by the lithification of sediments that may consist of fragments of rocks igneous, metamorphic or sedimentary, from minerals resistant to alteration, from secondary minerals (e.g. clays) or by chemical precipitation (chalks, carbonates).

The concentration of heavy metals in sedimentary rocks, in addition to the mineralogical composition of terrigenous constituents, also depends on the adsorbing capacity of the sediment, as well as the concentration of the trace elements present in the water in which the sediments deposited or subsequently came into contact (Sheppard *et al.*, 1992). Usually, silts and clays tend to adsorb high amounts of metal ions and metalloids. On the contrary,

sedimentary rocks such as sandstones, being mainly composed of quartz granules have a lower probability of containing trace elements.

However, contamination by heavy metals rarely occurs from natural sources.

The emergence of environmental pollution from heavy metals has increased due to their greater use in industrial processing. Many studies indicate that the heavy metals that have undergone a greater increase due to anthropisation are Cd, Pb and Hg. The average cadmium concentration in soil is six times higher than the average crustal concentration; lead and mercury are about twice the average crustal value (Mirsal *et al*., 2008).

The causes leading to such an increase are mainly due to the fallout of airborne pollutants emitted from various anthropogenic sources, such as chemical and petrochemical industries, waste incineration, vehicle traffic, etc. Moreover, some agricultural practices introduce significant amounts of heavy metals through the use of chemical fertilizers, sewage sludge, other organic soil improvers, pesticides (Battaglia *et al*., 2007) and the use of water with low-quality requirements.

Phosphate fertilizers are often obtained by processing phosphate rocks that contain varying amounts of heavy metals. This is believed to be the main anthropogenic source of Cd found in European soils (Morselli, 2003). Many are the impurities contained in these chemical compounds and more frequently we find elements such as Cu, Hg, Mn, Pb, As, Sn, Co and Zn. Co, Cu and Zn are contained, even in considerable quantities, in animal manure. Although they are assimilated in very low percentages, they are particularly concentrated in faeces and urine (Mirsal, 2008). Very important is the reuse in agriculture of sludge rich in organic and mineral substances, produced by the purification of urban wastewater (wastewater), whose main limitation of use depends on their content in heavy metals. The same limitation is adopted for compost, which is the end result of an organic solid urban waste treatment process. These can be particularly rich in Cr, Cu, Cd and Zn.

Among the sources of environmental pollution should be considered the possible releases of toxic substances by old landfill sites without any consideration of environmental pollution. The landfill of waste is a source of soil pollution often associated with the release of heavy metals such as Zn, Cd, Ni and Pb (exhausted batteries). The incineration of waste, being a process involving combustion at high temperatures, contributes to the emission of dust and metals among which the most frequently found are Zn, Pb, Cd and Hg.

1.3.2. Cadmium

Cadmium is a silvery-white metallic element, ductile and malleable, with the symbol Cd and atomic number 48; it is one of the transition elements and belongs to the IIB group of the periodic table. This metal was discovered in 1817 by the German chemist Friedrich Stromeyer who identified it in the incrustations present inside a zinc furnace. it melts at 321 °C, bubbles at 765 °C, has a relative density of 8.64 g/cm3 and atomic weight 112.40. At room temperature, it keeps unchanged for a long time, but if heated it ignites easily in the presence of air, generating a bright flame, and quickly turns into oxide, CdO. In nature, Cd is never found in its native state and is generally not abundant in the Earth's crust, where it is associated with an excess of Zn in rock formations. It is mainly obtained as a by-product of Zn ore refining, from which it is separated by fractional distillation or electrolysis. Cd is released into the environment by refineries, heating systems, metallurgical industries, waste incinerators, urban traffic, cement industries and the use of phosphate fertilizers.

Solutions of an unpolluted soil have a Cd concentration between 0.04 and 0.32 μM, moderate concentrations are between 0.32 and 1 μM, with higher concentrations we speak of soils polluted by cadmium (Wagner, 1993).

1.3.2.1. Phytotoxicity of cadmium

Duxbury (1985) classifies the Cd as an intermediate element of toxicity to soil organisms and microbial processes. High concentrations in the soil can be mutagenic and teratogenic for many animal species (Degraeve, 1981).

Only a fraction of the total Cd present in the soil is available for absorption by the plants, and its magnitude is largely influenced by the pH of the soil and its content in the organic substance. Cd is a metal that is easily absorbed by the roots of the plant, and then in minimum part be moved, through the xylem, to the leaves and fruits (Wagner, 1993).

In plants, exposure to high concentrations of Cd leads to the inhibition of the growth of both the roots and the aerial part, the appearance of foliar chlorosis, the alteration of the water balance (Dixit *et al*., 2001; Pietrini *et al*., 2010; Gaudet *et al*., 2011) and inhibition of the stomatic opening. The reduction in growth is linked to the decrease in the rate of elongation of plant cells, especially those of the stem, caused by inhibition irreversible exerted by the metal on the proton pump responsible for this process (Aidid e Okamoto, 1992). Numerous studies have, in fact, shown that the Cd reduces photosynthesis as it determines:

- direct effects on electronic transport in Photosystem II (PSII) (Clijsters and Van Assche, 1985; Baszynski, 1986; Pietrini *et al*., 2010b);
- reduction in plastoquinone content in the chloroplast (Krupa *et al*., 1992);
- inhibition of the Calvin cycle (Weigel, 1985);
- decrease in chlorophyll biosynthesis (Padmaja *et al*., 1990).

Many studies have demonstrated the negative interference exerted by the Cd against the content and activity of many metabolic enzymes (Van Assche and Clijsters, 1990; Mattioni *et al*., 1997; Siedlecka *et al*., 1997; Iori *et al*., 2012).

Plants respond to the presence of an excessive concentration of Cd in the soil with a series of mechanisms to counteract the absorption of the metal and/or defend against its toxic effects (Sanità di Toppi *et al*., 1999).

The main defence mechanisms that the plant puts in place when subjected to stress from Cd involve processes for the complexation of the Cd in the cytosol through glutathione and in the vacuole through organic acids when the plant is subjected to low levels of metal. While the presence of higher concentrations also requires other mechanisms, such as synthesis and complexation through phytochelatins and sulfide (Vogeli-Lange and Wagner, 1996). Stress proteins seem to only be induced by high or very high Cd concentrations and can act to limit and repair the damage to cellular proteins and perhaps also exert a protective action of the membranes (Panaretou and Piper, 1992).

1.3.2.2. Health risk

The risk of cadmium poisoning in humans is due to ingestion of contaminated food or inhalation of cadmium particles. The latter case occurs in particular during professional exposure.

The main sources of cadmium from food are vegetables due to their ability to accumulate it. In fact, a significant linear correlation between cadmium content in plants and cadmium content in soil has been reported (Alloway, 1997). The concentration of anthropic cadmium in plants would be 10-50% higher on average, indicating a tendency to a strong accumulation of the metal in plant biomass. Cadmium is an element that accumulates in the body that increases with age. About 80% of cadmium is retained in the liver and kidneys. The half-life, i.e. the time it takes for a substance to halve its concentration in the blood or in an organ of the body, of cadmium in the body is 10-30 years, so the concentration of the metal in tissues increases throughout life. The effects of cadmium on terrestrial and aquatic animals include acute and chronic toxicity. The main signs of cadmium poisoning in mammals are anaemia, reduced productivity, reduced growth and liver and kidney damage. Toxic effects on soil microorganisms such as growth slowing and inhibition are also known, even at relatively low concentrations.

1.4. Remediation techniques

The need to rehabilitate polluted areas is a very strong issue of the modern era. With the help of the awareness of the population and the state, several methods (management of waste separation and recycling), laws (Legislative Decree 152/2006) and standards of social behaviours, which are contributing to the environmental balance, have been introduced. However, there is still the need to purify polluted areas or areas subject to pollution.

The remediation of a contaminated site is divided into two categories, *ex situ* treatment where the contaminated material is removed and moved to installation, and *in situ* treatment where the contaminated material is treated in the contaminated site.

Ex situ treatments can be divided into on-site and off-site; in the first case, the material is treated close to the site of removal. In the second case, it is transported to specialized installations far from the site of removal.

Although in the past *ex situ* treatment has been preferred because it was less time-consuming, today *in site* treatment is preferred for several aspects such as cost reduction, the possibility to clean up deeper areas, which often cannot be reached by excavation, release from infrastructure and are also accepted by the public because there is less disfigurement of the landscape.

The remediation technologies use processes on the contaminant based on three types:

- transformation: it consists of the attack of organic compounds that pass from substances with a complex structure to simpler intermediates until eventually the complete mineralization;
- removal: this is the typical case of heavy metals (in the elementary form) that cannot be further degraded but only separated and removed from the contaminated matrix;
- immobilization: techniques based on this principle apply when any other alternative can be used and it is necessary to immobilize the pollutant in the contaminated site to avoid the extension of pollution.

The remediation of polluted sites can be realized by mechanical, chemical-physical and biological techniques.

The mechanical techniques consist in using instrumentation (e.g. grating in a wastewater treatment installation) that allows the separation of large particles from the soil or contaminated water; these are followed by chemical-physical or biological techniques that allow removing small particles and substances present in the system to be purified.

The chemical-physical treatments consist of the use of chemical additives that allow the removal of pollutants, generally poorly biodegradable. These treatments can be extractive, detoxifying, immobilization; they are herewith explained.

- Extractive treatments consist in the separation and removal of the contaminant from the matrix, through the transformation into more easily removable compounds or through a phase transfer of the pollutant.

- Detoxification treatments allow the transformation into less toxic substances, altering their chemical structure.

- Immobilization treatments, including stabilization and confinement of the contaminant in the matrix, is used when it is not possible to apply the first two to avoid the extension of the pollutant. In heavy metal pollution, the techniques used are aimed at reducing the availability of the metal:

- through the use of reagents that operate oxidation, reduction or neutralization reactions, allowing to transform the metal in its less toxic or less soluble forms;

- by precipitation as hydroxides, sulphides or carbonates and then separated by sedimentation;

- by complexation by chelating agents, such as EDTA, allowing the solubilization of metals in the matrix.

The chemical-physical treatments are those considered more effective in terms of cost and time than the biological ones, however, the use of chemical reagents can lead to the formation of secondary substances as much or more toxic than the starting compound, or the same reagents can be polluting.

The biological treatments (or bioremediation) are a series of purification technologies that exploit the metabolic capacity of some microorganisms and plants to biodegrade or detoxify pollutants.

They are among the main environmental remediation techniques that can be used *in situ*, at low cost, with less handling and risk of secondary pollution, however, they require long time and not all pollutants can be removed with this methodology (Bonomo and Sezenna, 2005).

The bioremediation techniques that are used in the remediation of polluted sites are multiple and depend on the type of pollutants.

1.4.1. Bioremediation of heavy metals

The removal of heavy metals can be done by different bioremediation techniques, such as bioleaching, biosorption, phytoremediation and mycoremediation.

Bioleaching is a technique that consists of the use of microorganisms that, through a process of biological leaching of contaminated soil, make it possible to move into solution the metal species, which will be subsequently extracted from the leachate by chemical-physical or other biological methods. Some bacteria are able to obtain energy through the oxidation of metal sulphides, contributing to the solubilization of metals from contaminated substrates. Examples of these bacteria are *Acidithiobacillus ferrooxidans* and *A. thiooxidans* (Wang *et al*., 2009).

In general, Fe^{3+} ions are used to oxidize the ore independently from the bacterium, in fact, the role of bacteria is the further oxidation of the ore and the regeneration of Fe^{3+} through the oxidation of Fe^{2+}.

An example of this process is the breakdown of pyrite ore (FeS_2) through the oxidation of sulphur and metal, making them soluble.

In the first phase, the disulphide is spontaneously oxidized to thiosulfate by the ferric ion (Fe^{3+}), which in turn is reduced to ferrous ions (Fe^{2+}) (Brandl, 2001):

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow 7Fe^{2+} + S_2O_3^{2-} + 6H^+$$

The ferrous ion is oxidized by the bacterium back into the ferric ion (Fowler *et al*., 1999; Brandl, 2001):

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$$

Thiosulphate is also oxidized to give sulphate (Fowler *et al*., 1999; Brandl, 2001):

$$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2Fe^{2+} + 4SO_4^{2-} + 4H^+$$

The microbial oxidation process takes place on the cell membrane of bacteria, where electrons pass inside the cell and are used in biochemical processes to produce energy for the bacteria, reducing oxygen in the water.

Several species of fungi can be used for bioleaching, different studies have shown that two fungal strains *Aspergillus niger* (Mulligan, Kamali and Gibbs, 2004) and *Penicillium simplicissimum* (Amiri, Mousavi and Yaghmaei, 2011) were able to mobilize Cu and Sn by 65%, and Al, Ni, Pb and Zn by 95%. This form of bioleaching is not based on metal oxidation, but exploits the fungal metabolism capable of producing organic acids (such as citric acid) that dissolve metals (Franz *et al*., 1991).

Biosorption consists in the use of living or dead biomasses or their derivatives able to adsorb or complex metals on their surface, through interaction with various ligands or functional groups present on cell membranes, transforming soluble metals into insoluble metals (precipitate) (Volesky and Holan, 1995).

Several commercial products have been developed based on the characterization of the properties of various species of yeasts, algae, fungi and bacteria.

Biomasses are immobilized on different types of matrix, the most common ones are alginate, polyacrylamine, polysulphone, silica gel and glutaraldehyde.

Phytoremediation is a technique that consists in the use of plants able to purify soil, air and water from toxic substances, exploiting the ability to concentrate and metabolize various molecules in their tissues (Reichenauer and Germida, 2008).

This innate ability of some plants, called hyperaccumulators, makes them possible to bioaccumulate, degrade or make harmless contaminants in soil, water and air, thus the possibility to use plants in strategies against environmental pollution (Salt *et al*., 1998).

Many plants have the ability to absorb, from soil and water, heavy metals essential to their development, such as Fe, Mn, Zn, Cu, Mg, Mo and Ni, and to use them for their growth, but also to incorporate metals considered non-essential such as Cd, Hg, Pb, As, Ag and Cr.

The plant that removes the contaminant from the environment can use a number of processes (K. Oh *et al*., 2013; Cunningham *et al*., 1995; Lofty and Mostafa, 2014):

- Phytosequestration - consists of phytochemical complexation at the root level, reducing the bioavailable fraction of the contaminant, through sequestration at the cell membrane level and subsequent storage in the vacuoles present in the root cells.

- Phytoextraction - the absorption and concentration of the substances in the plant biomass.

- Phytostabilization - the contaminant is rendered harmless by immobilization in plant organs.

- Phytotransformation - chemical modification of substances through plant metabolism that can occur by inactivation, degradation (phytodegradation), or immobilization (phytostabilization).

- Phytostimulation - the strengthening of the microbial activity present in the soil, for the degradation of the contaminant, at the root level; this process is also known as rhizosphere degradation.

- Phytovolatilization - removal of substances in soil or water through a process of transformation into more volatile substances.

- Rhizofiltration - is the capture by plants of pollutants present in dissolved form in groundwater; it occurs in the root zone, through processes of adsorption, concentration or precipitation of contaminants.

Heavy metal phytoremediation uses the extraction and accumulation capacity in plant tissues or their immobilization in the rhizosphere.

Plants that accumulate metals are capable of accumulating quantities of a certain metal more than the average. This type of plant usually grows on metalliferous soils and is able to complete its life cycle without showing any symptoms of phytotoxicity to metals (Baker *et al*., 2000).

The concept of hyperaccumulation has been introduced for plants that can absorb these elements in high concentrations, more than 0.1% of dry weight (1000 mg/Kg), depending on the specific element. (Tsao, 2003). Among the hyperaccumulator plants, many belong to the family of *Brassicaceae Euphorbiaceae*, *Asteraceae*, *Lamiaceae* and *Scrophulariaceae* (Kumar *et al*., 1995; Salt *et al*., 1995).

The phytopurification capacity of a certain metal can vary from species to species.

Mycoremediation is a bioremediation technique that uses fungi to degrade or sequester contaminants in soil and water.

The removal of metals by fungi can be done with three different mechanisms (Singh and Harbhajan, 2006):

- bioabsorption of metals on the cell wall
- intracellular capture
- chemical transformation.

An important role in the detoxification of heavy metals is played by a category of enzymes called metallothioneins.

Metallothioneins are low molecular weight proteins (500 to 14,000 Da) with a high cysteine content, responsible for resistance to heavy metals (Park *et al*., 2001).

They were discovered in 1957 by a research group interested in the purification of a cadmium-binding protein extracted from the renal cortex of equine origin (Margoshes and Vallee, 1957). From this discovery other proteins, with characteristics similar to equine metallothionein, have been identified in several living organisms such as protozoa, prokaryotes, plants, fungi and in several phylum belonging to the animal kingdom, including man.

Metallothioneins have been conventionally divided into three categories (Fowler *et al*., 1987):

- Class I: metallothioneins with primary structure correlated to those of mammals.

- Class II: metallothioneins whose sequence does not have homologies with those of mammals.

- Class III: defined phytochelatins, they are polypeptides analogous to metallothioneins, constituted by γ-glutamyl-cysteinyl units, present in plants and fungi.

This type of classification dating back to 1987 has been replaced by a new classification based both on structural analogies of cysteine residues and on phylogenetic relations, dividing the superfamilies of metallothioneins into families and subfamilies (Binz and Kägi, 1999).

Mainly metallothioneins are Cys-Cys, Cys-X-Cys and Cys-X-Y-Cys in which X and Y are amino acid residues other than cysteine. A further classification by Cobbett and Goldsbrough (2002) into 4 types (1,2, 3 and 4) is based on the distribution of their Cys residues and a Cys-devoid region (called spacers), which is characteristic of plant metallothioneins.

The phytochelatins of plants and fungi, whose general structure is (γ-Glu-Cys)n-Gly n=2-11, are induced in the presence of the metal and operate the chelation of this and the subsequent storage inside the vacuoles.

The thiol or sulfhydryl groups (-SH) of cysteine allow the chelation of heavy metals preventing their interaction with cellular components and therefore their toxic action.

1.5. Plant pathogens

Plants are constantly exposed to the action of multiple adversities that can negatively affect their development, growth and reproduction, and in extreme cases the death of the plant. These problems can lead to significant drops of productivity and/or product quality. Diseases affecting plants can be divided into biotic plant diseases and abiotic plant diseases (Gull, Lone and Wani, 2019). Biotic plant diseases are determined by the competitive action of other living organisms or living entities, such as nematodes, fungi, oomycetes, bacteria, viruses, etc., which fall into the same biotic category. Abiotic plant diseases are caused by the adversity of physical-chemical environmental factors, such as reduced water availability, nutrient deficiency, excess

salts, extreme temperatures, pollutants, etc., which fall into the category of abiotic stress. Pathogens act through mechanisms that promote their proliferation and induce changes in the plant that affect physiological activities and basic metabolic processes. Pathogens are classified on the basis of their particular mode of action against the host in necrotrophs, which kill cells by means of toxic molecules and decompose plant tissues through lytic enzymes before feeding on them for their growth, and in biotrophs which, on the contrary, draw nourishment from cells that are still viable (Glazebrook, 2005). Hemibiotrophs show both previous behaviours according to the particular stage of the biological cycle.

The pathogenesis stage occurs when the pathogen penetrates the plant directly (tissue penetration) or indirectly through wounds or natural openings, such as stomata. Fungi can have both means of penetration listed above. They can penetrate the tissues of the plant by means of specialized structures (penetration hyphae) and the release of lytic enzymes (Kolattukudy, 1985). Bacteria and viruses, which do not have these specialized structures, exploit natural openings, pre-existing wounds or are inoculated by vectors (such as insects).

1.5.1. Fungi

The fungi kingdom consists of nine phylum-level clades: Opisthosporidia, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Zoopagomycota, Mucoromycota, Glomeromycota, Basidiomycota and Ascomycota (Naranjo-Ortiz *et al.*, 2019).

Fungi are eukaryotes, unicellular or multicellular organisms; they can reproduce by asexual and sexual mechanisms. Fungi are heterotrophic, i.e. they feed on organic matter produced by other organisms. In fact, they do not have differentiated tissues and a lymphatic system as in plants. All are achlorophyllous, i.e. they do not have chlorophyll pigments and are incapable of photosynthesis.

Most fungi are filamentous, consisting of hyphae that show an apical and branched growth whose structure is called mycelium (Papagianni, 2004). Some are unicellular, such as yeasts.

The single cell or hyphae are surrounded by a cell wall consisting of chitin (a polymer of the amino sugar N-acetylglucosamine) and glucans (Free, 2013). The nuclei are typically haploid. The cells that make up the hyphae can be mono or polynucleated and can be divided by septa. Septa have pores that allow the passage of protoplasm and cellular organelles.

The fungi can reproduce asexually or sexually (Taylor, Jacobson and Fisher, 1999; Hetman, Sun and James, 2013).

The asexual reproduction does not involve the union of sexual organs (gametangia) or sexual cells (gametes) or nuclei. It can take place through different mechanisms, herewith shortly described:

- mycelial fragments, if the conditions are favourable, a new individual mycelium can be formed from a fragment of hypha;
- fission, it is the cell division of the mother cell into two individual daughter cells, which carry the same genetic heritage as the parental cell;
- budding, it is the production of an outgrowth or bud from a parent cell, this mechanism is common in yeasts; the nucleus of the parent cell splits into the two daughter cells and migrates into the bud, which increases in volume until it explodes and gives birth to a new individual;
- production of asexual spores, this reproductive mechanism is really common in many fungi.

Asexual spores are commonly called conidiospores or conidia. They are asexual reproductive structures that form in particular structures, called conidiophores. The fungi that present only this mode of asexual reproduction are called mitosporic fungi (Kiffer, 2011). Conidia can be single or in chains or clusters. They can be unicellular (example in *Colletotrichum* spp.), bicellular, microconidia (in *Fusarium* spp.) and multicellular (*Pestalotiopsis* spp., *Cercospora* spp.). Single-cell spores are called amerospores, two-cell spores are didymospores and multi-cellular spores are called phragmospores. The multi-cellular conidia can be divided by septa into one or three planes. In *Alternaria* spp., conidia are with both transverse and longitudinal septa and are called dictyospores. The conidia can have different shapes and colours; they can

be globose, elliptical, ovoid, cylindrical, branched, spiral or star-shaped; they can have a hyaline, pink, green or dark colour.

The colouration of conidia and conidiophores are important characteristics in morphological identification.

The conidia can be free, simple or branched, distinct from each other or can be aggregated to form compound sporophores or fruiting bodies such as synnemata, sporodochia, acervuli and pycnidia.

The fungal colonization occurs by landing the spores on the substrate. The spores normally appear as a dehydrated structure, this is a normal characteristic of the cell that must colonize the surfaces of plants (often they have a hydrophobic nature). Some spores have a drop of adhesive material on the conidia that attaches the spore to the surface of the leaf when transported to it, for example *Magnaporthe grisea* (Hamer *et al*., 1988). The subsequent hydration of the spore makes the adhesion to the leaf surface even more stable. Adhesion is a two-step process with passive adhesion mediated by hydrophobic and electrostatic interactions, followed by subsequent adhesion during swelling and germ cell formation.

The sexual reproduction mechanism is important for genetic variability and makes it possible the fungus to adapt to new environments.

Sexual reproduction by meiosis exists in several phyla of fungi. There are many differences in the morphology of sexual structures and spores within fungal groups. These differences are used for the classification of fungi.

Fungal sexual reproduction differs in many ways from sexual reproduction in animals or plants. While the nuclear division in other eukaryotes, such as animals, plants and protists, involves the dissolution and reformation of the nuclear membrane, in fungi the nuclear membrane remains intact throughout the process. The nucleus of the fungus becomes pinched at its central point, and the diploid chromosomes are separated from the spindle fibres that form within the intact nucleus.

Sexual reproduction in fungi occurs in three sequential stages: plasmogamy, karyogamy and meiosis. Diploid chromosomes are separated into two daughter cells, each containing a single set of chromosomes (a haploid state).

In plasmogamy, the anastomosis of two cells or gametes and the fusion of their protoplasts occur. In the process, the two haploid nuclei (compatible nuclei) of opposite sex are reunited, but the nuclei do not fuse.

Karyogamy leads to the fusion of these haploid nuclei and the formation of a diploid nucleus (i.e. a nucleus containing two sets of chromosomes, one for each parent). The cell formed by karyogamy is called zygote. In most fungi, the zygote is the only diploid cell of the entire life cycle.

In lower fungi, karyogamy usually follows almost immediately the plasmogamy, while it can be delayed in higher fungi. In the higher fungi, plasmogamy results in a binucleated cell containing one nucleus from each cell. This pair of nuclei is called dikaryon. The dikaryotic state resulting from plasmogamy is often a prominent condition in fungi and can be prolonged for several generations.

Nuclear fusion is the last stage of sexual reproduction that occurs in all fungal sexual reproductions, it is called meiosis. During meiosis, cell division occurs that reduces the number of chromosomes to one set per cell, in this way the haploid phase is restored. The haploid nuclei will be incorporated into spores called meiosis.

Fungi in which a single individual carries morphologically distinguishable male and female sex organs are called hermaphrodites.

When the female and male organs are produced on two different individuals, they are called dioecious or bisexual. Dioecious species usually produce sexual organs only in the presence of an individual of the opposite sex.

Fungi use a variety of methods to bring together two compatible haploid nuclei (plasmogamy). Some produce specialized sex cells (gametes) that are released from differentiated sex organs

called gametangia. If the gametes and gametangia produced are morphologically identical or similar they are called isogametes and isogametangia respectively. When the gametes and gametangia produced differ in size and structure (morphologically different) they are called heterogametes and heterogametangia, respectively. In the latter case, the male gametangio is called antheridium and the female gametangium is called oogonium. The male gamete is known as antherozoid or sperm and the female as egg or oosphere.

In some fungi, two gametangia come into contact, and the nuclei pass from the male to the female gametangium, thus assuming the function of gametes. In some fungi, the gametangia themselves can merge to bring their nuclei together. Finally, some of the more advanced fungi do not produce any gametangia; somatic (vegetative) hyphae take over the sexual function, come into contact, fuse and exchange nuclei.

1.5.1.1. Mycotoxins

Fungi are omnipresent in nature and have evolved over time to colonize a wide range of ecosystems. Part of this evolutionary process has been the development of the ability to produce secondary metabolites. Secondary metabolites can be defined as molecules produced by microorganisms, and other organisms such as plants, which are not directly essential for growth (Magan and Aldred, 2007a).

They are, therefore, metabolic products that differ from primary metabolites that are essential for growth by providing energy, synthetic intermediates and key macromolecules (Betina, 1989).

Secondary metabolites are produced by the main groups of microorganisms, bacteria (such as actinomycetes) and fungi (such as ascomycetes). Many hundreds of secondary metabolites are known but many are still unknown. In fact, the individual microbe may be able to produce a large number of metabolites, and the production profile may change depending on growth conditions, such as nutrient and water availability.

Some secondary metabolites produced by fungi have been used in pharmaceuticals, such as penicillin. However, there are secondary metabolites defined as toxic, this group is collectively known as mycotoxins.

Mycotoxins are produced during the growth process of the plant, by endophytic fungi, while during food storage they are produced by saprophytic fungi (Hussein and Brasel, 2001).

The principal factors involved in mycotoxin production may be extrinsic and intrinsic. Extrinsic factors are the set of conditions that can promote fungal development and consequently the production of their metabolites. Among these, we can classify physical, biological and chemical factors. The physical factors that can influence the synthesis are temperature, humidity, pH, oxygen, the nature of the substrate. Biological factors may be due to competition with other microorganisms and host resistance. Chemical factors can also influence the synthesis of mycotoxins, in fact, the use of fungicides can increase the production of mycotoxins, supporting the thesis that mycotoxins are produced in response to a stress stimulus.

Intrinsic factors are related to the ability of the fungal strain to produce mycotoxins (Huwig *et al*., 2001), i.e. the toxigenic factor. The presence of mycotoxigenic fungi in a matrix is not an absolute index of the presence of mycotoxins.

Each fungal species is linked to well-defined environmental conditions and particular nutritional needs. The conditions that make it possible the development of mycelium often differ from those that lead to the production of mycotoxins.

The fungi that produce mycotoxins are commonly called mycotoxin fungi.

The main filamentous fungi producing mycotoxins are *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp. (Greeff-Laubscher *et al*., 2020). It should be noted that one mycotoxin can be produced by different fungal species or one fungal species can produce different mycotoxins. The most important mycotoxins produced by *Aspergillus* spp. or *Penicillium* spp. are ochratoxin A (OTA) (Varga et al., 1996; El Khoury and Atoui, 2010) and aflatoxins (AFs), of which aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2

(AFG2) are the most common (Richard, 2008). The most common mycotoxins of *Fusarium* spp. are fumonisins (FBs) of which fumonisin B1 (FB1) and fumonisin B2 (FB2) are predominant, zearalenone (ZEA) and trichothecenes (TCT), of which the most known are deoxynivalenol (DON) and the toxins HT-2 and T-2 (Munkvold, 2017). These types of fungi are generally associated with the climatic and cultural phases of different geographical regions. The genus *Fusarium* is common in field contamination, and *Penicillium* and *Aspergillus* are common for crop conservation. Consequently, mycotoxins such as FBs and DON are mainly produced before harvest, while AFs and OTA are mainly produced during post-harvest phases. Depending on when they are produced, their amount can be reduced in various ways. Mycotoxins before harvesting can be reduced by applying Good Agricultural Practices (GAP), using control methods, developing resistant crop varieties, using crop protection chemicals, etc. Post-harvest mycotoxins can be reduced by strategies, such as proper drying, handling, packaging, storage and transport conditions, application of detection and detoxification methods and removal of damaged grain (Magan and Aldred, 2007b).

Although mycotoxins are more commonly found in cereals and cereal products, they can also be found in dairy products, spices, dried fruit, nuts, coffee, vegetable oils, wine and fruit juices. Any processed product manufactured from contaminated raw materials may contain mycotoxins. In addition, mycotoxins commonly associated with cereals, such as AFs, OTA, DON and ZEA, are moderately stable in most food processing systems, such as milling, baking, frying, roasting and boiling, where temperatures are up to 120 °C. They are not eliminated from food processing, although in some cases their concentration is significantly reduced (Nagl *et al*., 2015). This leads to their persistence in the food chain.

Intoxications from mycotoxins are called mycotoxicosis and range from nausea and vomiting (in acute) to carcinogenic and teratogenic consequences (in chronic) (Zain, 2011).

1.5.2. Oomycota

Oomycota or oomycetes form a distinct phylogenetic lineage of eukaryotic fungus-like microorganisms. Oomycota were originally grouped in the fungi kingdom, however, molecular and phylogenetic studies have revealed significant differences between fungi and oomycetes, which means that the latter are now grouped with Stramenopiles (which include some types of algae) in the Chromista kingdom (Van de Peer and Wachter, 1997; Rossman and Palm, 2006).

The cell wall of oomycetes is composed of cellulose and glucans, and not chitin as in fungi. In addition, the nuclei within the hyphae are diploid and not haploid as in fungi (Rossman and Palm, 2006).

Oomycetes are also often referred to as water moulds, this name refers to their previous classification as fungi and their preference for high humidity conditions and running surface water. Although the nature that led to this name is not true for most species, which are terrestrial pathogens.

Oomycetes are filamentous, rarely have septate hyphae. Some are unicellular, while others are filamentous and branched. They are heterotrophic and can reproduce both sexually and asexually. The sexual reproduction of an oospore is the result of the contact between the hyphae of the male antheridium and the female oogonium (Judelson, 2009); the fertilized eggs are converted into non-motile spores, or oospores, which then germinate even in mature individuals, these spores can hibernate for this reason they are also known as spores at rest. Asexual reproduction involves the formation of chlamydospores and sporangia, producing mobile zoospores (Walker and Van West, 2007). The production of zoospores is a characteristic of oomycetes. The zoospores are mobile spores that due to the presence of whip swimming structures known as scourges are ungrateful to move in the water, normally they are biflagellate. Individuals can sprout from these spores.

Oomycetes can be both saprophytic and pathogenic.

1.5.2.1. Phytophthora

The genus *Phytophthora* from the greek phyton (plant) and phtheiro (destructor) includes some of the most destructive pathogens, capable of causing serious epidemics and extensive damage on different species of plants in both agricultural and forest environments. Precisely for this reason, these pathogenic species have caused heavy losses, not only in the forest landscape, but also economically in the case of horticultural plants; for example, *Phytophthora infestans*, the causative agent of potato downy mildew, caused between 1845 and 1849 the great Irish famine. *Phytophthora* species have a branched, hyaline mycelium, coenocytic, set only in old crops. The diameter of hyphae depends on the physical and chemical nature of the culture medium and varies depending on whether the mycelium is superficial, airborne, submerged or in host tissues; it is typically 5-8 μm. Hyphae sometimes appear dilated, knotty with perpendicular branches often choked at the base. Particular hyphal swellings are characteristic of some species, such as *P. cryptogea* (Ribeiro, 1978), which differ from other reproductive structures of the fungus, such as chlamydospores, for the absence of a septum that divides them from the hypha.

Asexual reproduction occurs through sporangia and chlamydospores, depending on the species. Sporangia are carried by sporangiophores branches that are not substantially different from vegetative hyphae. In some species, the sporangiophore gives rise to a new sporangium inside an empty sporangium (internal proliferation), or it continues to grow and comes out through the exit hole and forms a second sporangium at a certain distance from the first (external proliferation). The protrusions have a high variability of shape and size and they can be subspherical, ovoid, limoniform, pyriform, hyaline or straw yellow. They germinate directly with one or more germinating tubules, or indirectly through zoospores. Some species have a papillae, a mucilage area inside the wall of the sporangium that soaks with water and dissolving allows the zoospores to escape.

The zoospores emerge with amoeboid movements through the exit pore, they are kidney-shaped, with two scourges inserted on the concave side. When they find a suitable substrate they encyst, losing the scourges and germinate through the formation of a germinating tubule.

Particular conservation organs of the genus *Phytophthora* are the chlamydospores; they are spherical or ovoid, not papillated, hyaline or slightly brown, with thin or thick walls, terminal or intercalary. They are distinguished from hyphal swellings by the presence of a septum that separates them from the rest of the hypha.

As already metioned, sexual reproduction produces oospores that represent structures of survival. They originate from the fusion of antheridium and oogonium. The oogonium has a spherical and pyriform shape, with a smooth wall and hyaline or straw yellow coulor. It is separated from the hypha by a septum and encloses an oosphere. The oosphere, after fertilization, turns into a smooth and spherical oospore that can partially (aplerotic) or completely (plerotic) fill the oogonium. Antheridium generally originates from an apical swelling of the hypha and it is separated by a septum. Two types are known, the amphigynous/paragynous that closely surround the stem of the oogonium, and those that adhere laterally to the oogonium near the stalk.

Phytophthora species are mainly found in excessively compact soils with low ventilation and a high degree of humidity due to stagnation. The characteristic symptoms caused by *Phytophthora* species mainly concern the roots and the collar, with the appearance of darkening, followed by real rotting processes that can also cause the death of the plant or, in less serious cases, the emission of new adventitious roots.

On the stem, infections occur in the form of brownish lesions, which tend to deepen until they reach the vascular tissues, causing the collapse of the parts above and leading the plant to die. In the case of tree plants, infections are latent and the course of the disease is chronic.

Phytophthora spp. are also responsible for downy mildew in tomato and potato and brown rot of the fruits (citrus fruits), therefore they are cause of great economic damage.

1.5.3. Other biotic stresses

Viruses do not have a cellular structure and organization. The viruses that infect plants are called phytoviruses, they are ungrateful to settle and multiply within the host plant.

They consist of a nucleic acid, DNA or RNA, which in the case of DNA can be single or double-stranded. The nucleic acid is protected within the capsid, which is a protein compartment.

Viruses can spread through the phloem or xylem. Transmission can occur by direct contact of the healthy plant with the diseased plant (e.g. grafting), by transport through the air (wind) or by vectors (such as insects).

Nematodes are obligate biotrophs. They are equipped with a stiletto capable of crossing the cell wall, they can attack the roots of plants using the cells of this tissue to feed, removing nutrients from the plant and bringing structural changes to the root system.

Bacteria are unicellular prokaryotes that can form colonies (mucilaginous masses). They are thermophilic organisms, preferring optimal temperatures between 25-35 °C, have higher humidity and water requirements than fungi, and are not capable of direct penetration into host tissue. Normally they are present in the rhizosphere and can contribute to plant growth. However, if conditions are favourable, they can become pathogenic and due to their rapid rate of reproduction can be extremely infectious.

1.6. Biological control

The term biological control or biocontrol is used in different fields of biology. In plant pathology, the term applies to the use of microbial antagonists to suppress diseases as well as the use of specific host pathogens to control weed populations. In both fields, the organism that suppresses the parasite or pathogen is referred to as the biological control agent (BCA). More generally, the term biological control has also been applied to the use of natural products extracted or fermented from various sources. These formulations can be very simple mixtures

of natural ingredients with specific activities or complex mixtures with multiple effects on the host and the target parasite or pathogen.

In modern research, the term biocontrol is also associated with biotechnological techniques that use natural or modified organisms, genes or genes produced to reduce the effects of unwanted organisms and to promote beneficial organisms for plants (Kevan and Shipp, 2017).

The main objective of biological control is to reduce the inoculum density or pathogenic capacity of the parasite by one or more organisms.

1.6.1. Microbial biological control agents (MBCAs)

Microbial biological control agents (MBCAs) are applied to crops for the biological control of plant pathogens. They act through a series of modalities with indirect or direct action on pathogens.

Some MBCAs interact with plants by inducing resistance of plants without any direct interaction with the target pathogen. Other MBCAs act indirectly through nutrient competition or other mechanisms that modulate the growth conditions of the pathogen.

Direct actions with the pathogens include hyperparasitism and antibiosis. These interactions are regulated by metabolic events that may be signalling compounds, enzymes and metabolites produced *in situ* to interfere with the pathogen.

Plants are able to defend themselves against pathogens through the activation of biochemical mechanisms. These mechanisms can be induced by stimuli that are recognized by specific receptors. Typically recognized stimuli are pathogen-associated molecular patterns (PAMPs) which induce an increase in the defence pathways in the host plant against the recognized pathogen. Resistance can be induced locally in the attacked tissue or spread through the activation of systemic resistance mechanisms.

Induced resistance is a physiological state of increased defensive capacity triggered by specific environmental stimuli, so the plant's innate defences are enhanced against subsequent biotic

stress. Induced resistance can be distinguished in systemic acquired resistance (SAR) and systemic resistance induced (SRI) according to the nature of the elicitor and regulatory pathways involved. SAR can be triggered by exposing the plant to virulence, avirulent and non-pathogenic microbes. SRI is enhanced by microorganisms present in the rhizosphere called plant growth promoters (PGP), in this case, the stimuli produced by the microorganisms inducing resistance in the plant are called microbe-associated molecular patterns (MAMPs).

The induced defence mechanisms involve the production of reactive oxygen species, phytoalexins, phenolic compounds, proteins linked to pathogenesis or the formation of physical proteins barriers, such as changes to cell walls and cuticles in the plant (Freeman and Beattie, 2008).

The germination and growth of plant pathogens depend on nutrient uptake. Biotrophic pathogens are obliged to use only nutrients from infected living host cells and do not depend on any source of exogenous nutrients in the environment outside the host plant (Agrios, 2005). Most plant pathogens are necrotrophic, i.e. the source of nutrients is dead organic plant matter. Necrotrophic pathogens kill and then invade the tissues of the host plant using the available nutrients. The main competitive advantage of necrotrophic pathogens is that they are the first colonisers immediately after they have caused the death of the host. Successful host infection of most necrotrophic pathogens also depends on exogenous nutrients during spore germination and the formation of infectious structures on host tissues (Chou and Preece, 1968; Fokkema *et al*., 1983). This dependence on exogenous nutrients during significant parts of their life cycle makes necrotrophic pathogens vulnerable to nutrient competition (Köhl and Fokkema, 1998).

Potentially competitive MBCA must be able to occupy niches, survive and rapidly consume sources of nutrients essential for pathogen infection such as sugars, pollen and plant exudates on residual surfaces and plants, so that competing pathogens are unable to infect the host. The population of pathogens will decrease, but will not be killed by the antagonist.

Parasitism is the direct competitive interaction between two organisms in which one is acquiring nutrients from the other. If the host is also a parasite, e.g. a plant pathogen, the interaction is defined as hyperparasitism. This type of interaction is often observed between one fungus and another.

Hyperparasites invade the spores of the host after killing these cells. The main mechanisms of parasitism are the excretion of lytic enzymes, combined in some cases with the excretion of secondary metabolites, in close contact with the host cell which leads to openings in the cell wall and subsequent cell lysis. Cell wall degradation is typically caused by a series of chitinases, β-1,3-glucanase and proteases or, in the case of oomycetes hyperparasites, cellulase.

The researchers have studied the phenomenon of hyperparasitism and found many antagonistic fungal species for some groups of pathogens. The most studied mycoparasites belong to the genus *Trichoderma*. They produce structures for attachment and infection and kill their hosts through the production of lytic enzymes, often in combination with secondary antimicrobial metabolites (Harman *et al*., 2004; Harman, 2006; Mukherjee *et al*., 2012; Nygren *et al*., 2018). These lytic enzymes are not constitutive, but are produced after host recognition. Surface compounds such as lectins in the host cell wall, surface properties and secondary diffusible metabolites released by the host play an important role in recognition pathways. The recognition of the fungal host then leads to the transcription of the genes for the production of the enzymes involved in the attack of the host and its lysis. In practice, oligosaccharides and oligopeptides released by the host are recognised by MBCA receptors and therefore act as inductors (Karlsson *et al*., 2017).

Antibiosis is a direct mechanism of MBCA against pathogens, it consists of the production and release of antimicrobial substances into the surrounding environment.

Antimicrobial metabolites are secondary metabolites belonging to heterogeneous organic groups. Antimicrobial metabolites are often regarded as the most powerful mode of action that microorganisms have against competitors in environments with limited resources (Raaijmakers

and Mazzola, 2012). They are produced and released into the environment in small quantities by many microorganisms. Among the bacteria producing antimicrobial metabolites used in biocontrol are species of *Agrobacterium, Bacillus, Pseudomonas, Streptomyces*, and many others.

Many antibiotics are only produced when a microbial population reaches a certain threshold, such as *Pseudomonas* in the production of phenazine.

Fungal antagonists can also produce antimicrobials, the best known being *Trichoderma* spp. for the production of 6-pethyl-alpha-pyrone, glioviridine, gliotoxin, viridine and many other as yet unknown compounds (Ghorbanpour *et al*., 2018).

1.7. The *Trichoderma* case

The genus *Trichoderma* includes anamorphic filamentous fungi. They are normally considered saprophyte; however, many *Trichoderma* species are opportunistic and avirulent symbionts. They are fast-growing soil organisms with high adaptability to different environmental conditions and are able to grow in various types of substrates; for these characteristics, they have been widely studied and used in agriculture as biocontrol agents for their effective antagonism against soil and foliar pathogens.

The genus *Trichoderma* has been known since the beginning of the 19th century, the first association with the *Hypocrea* Fr. teleomorphs were recognised by the Tulasne brothers in 1865, however, in 1939 Bisby discovered that *T. viride* was morphologically distinct from the teleomorph (Bisby, 1939).

The first morphological distinction of "aggregated species" was made by Rifai in 1969, who during his research recognised nine distinct species not related to individual teleomorphic species, based on macroscopic and microscopic analogies (Kubicek and Harman, 2002).

In 1991, Bissett, based on the aggregated species described by Rifai, divided the genus into five sections:

- *Pachybasium*

- *Longibrachiatum*

- *Trichoderma*

- *Saturnisporum*

- *Hypocreanum*

The introduction of molecular analysis, through the use of molecular markers, has opened a new era for the systematics of fungi.

The first DNA-based phylogenetic studies of the genus *Hypocrea* were carried out by Spatafora and Blackwell, Rehner and Samuels, establishing the phylogenetic relationships of the *Hypocrea/Trichoderma* species.

Today 287 different species belonging to the genus *Trichoderma* are known and confirmed (Zhu, 2017).

Morphologically the colonies appear as an aerial hyaline mycelium, which can also take on a floccose appearance depending on the strain and medium, with a whitish-brown colour that can become greenish-brown depending on the stage of development. The conidiophores are arranged in a central axis that branches off to form irregular agglomerates with a final pyramid structure. Usually, the ramifications of conidiophores end in one or more phialides which can be cylindrical, subglobe or ampolliform. In some cases, the phialides can be grouped on the main axis (e.g. *Trichoderma polysporum*, *Trichoderma hamatum*) or they can be solitary and irregularly arranged (e.g. *Trichoderma longibrachiatum*).

The conidia are unicellular, typically green or hyaline, with a smooth or wrinkled wall. Their shape can vary in ellipsoidal, elongated, cylindrical or globular, with dimensions between 3-5 µm in diameter, generally appearing dry; however, in some species they can appear in gelatinous heads or heads.

The optimal temperature of growth is around 25-30 °C while it stops at temperatures above 35 °C.

These fungi have often been found to be secondary colonizers of decaying organic substances and rotting plant roots, especially if damaged by other fungi (Davet, 1979). The ability to use a wide range of carbon and nitrogen sources, together with the abundant production of conidia and adaptability to different environmental conditions give these species a high level of competitiveness with respect to the remaining soil microflora. The ability to compete is often combined with the production of toxic metabolites and mycoparasitic action, making these microorganisms excellent antagonists of numerous pathogenic fungi of economic importance. The competitive ability of some *Trichoderma* is due, among other things, to the ability to influence the availability of certain nutrients in the rhizosphere, such as iron, by subtracting it from other fungi (Sivan and Chet, 1989).

The fungi of the genus *Trichoderma* express their antibiotic activity through the production of a large number of metabolites secondary to antibiotic activity (Ghisalberti and Sivasithamparam, 1991; Harman *et al*., 2004a). The secondary metabolites produced by *Trichoderma* spp. vary according to the strain considered.

The best known antifungal metabolites, which are synthesised from the genus *Trichoderma*, are gliovirin, gliotoxin, peptaibols, trichodermin, trichozianin and 6-pentyl-α-pyrone (6-PP). For example, gliotoxin seems to play an important role in the biocontrol implemented by *Trichoderma virens* against *Rhizoctonia solani* and *Pythium ultimum* (Bisset, 1991; Wilhite *et al*.,1994).

Furthermore, it seems to exert beneficial effects on plants by inducing them to produce pesticide substances against other pathogens (Harman, 2006; Vinale *et al*., 2008, Vinale *et al*., 2014).

Trichoderma also produces another class of antibiotic compounds, peptaibols, which act synergistically with cell wall degradation enzymes during mycoparasitism (Sivasithamparam and Ghisalberti, 1998; Reino *et al*., 2007).

Mycoparasitism seems to have a primary role in the antagonism of *Trichoderma*. This complex phenomenon first involves the chemotrophic growth of *Trichoderma*'s hyphae towards the

pathogenic fungus hyphae, probably due to a gradient of exudates produced by the latter (Barak *et al*., 1985), then the antagonist fungus forms specialised structures similar to appressors and hooks, which allow it to anchor itself to the host fungus. The last phase, eventually, involves the complete degradation of the structures of the target fungus. Chitinase, glucanase and protease are used to degrade the fungal cell wall and are considered key compounds in the mechanism of mycoparasitism and biocontrol in *T. harzianum* (Lorito *et al*., 1993 a; Lorito *et al*., 1996).

Trichoderma preparations are very effective in controlling several phytopathogenic fungi, such as *R. solani, P.ultimum* or *Sclerotium rolfsii* through competition processes (fungistasis), or against *Fusarium oxysporum* and *Botrytis cinerea* through nutrient competition (Benìtez *et al*., 2004).

In addition to the ability to directly attack or inhibit the growth of phytopathogenic agents, some *Trichoderma* strains actively interact with the plant by promoting root and stem growth (Harman *et al*., 2004b).

The ability of some *Trichoderma* strains to biodegrade or otherwise tolerate a wide range of environmental pollutants has also been demonstrated in recent years. The literature reports the use of *Trichoderma* spp. in the bioremediation of hydrocarbon polluted soils (Harman *et al*., 2004c).

Trichoderma can bioremediate soils and waters polluted by various toxic substances, especially Trichoderma mycelium seems to be able to cleanse matrices contaminated by various heavy metals (Krantz-Rülcker *et al*., 1993).

Trichoderma spp. strains, by virtue of their peculiarities, are also used in soil recovery programmes; as rhizosphere-competent microorganisms, they contribute to the stability of microbial communities in the rhizosphere, control pathogenic and competitive microflora, improve plant health and increase root development and density. The T22 strain of *T. harzianum* has found commercial use in this field because it significantly increases the effectiveness of the

plants used for phytoremediation. Recent studies have shown that the treatment of ferns roots with *T. harzianum* strain T22 results in a drastic reduction of arsenic levels in contaminated soils and a significant increase in root biomass compared to control plants (Harman *et al*., 2004b).

1.8. Objectives of the thesis

Environmental pollution is a very topical subject on which most contemporary scientific research is focused. Particularly, in agriculture, it is evident how contamination, caused mainly by the excessive use of agrochemicals, can lead to long-term alterations in the surrounding ecosystem.

A valid alternative is the use of microorganisms, which have the innate ability to grow in the presence of contaminants, decompose harmful substances into other compounds or possibly remove them from the matrix through uptake mechanisms. In addition, some microorganisms possess the ability to produce antimicrobial substances that are useful in counteracting the onset of disease in plants; these substances, being of natural origin, are easily degradable and do not cause pollution.

The main aim of this study is to find possible eco-sustainable alternatives to the use of agrochemicals and to exploit the innate ability of some microorganisms to grow in the presence of contaminants, such as heavy metals, which are one of the main causes of contamination crops and consequently of human health through the food chain.

In this thesis, two selected strains of *T. atroviride* (TS) and *T. asperellum* (IMI 393899) were investigated, with the following objectives:

(i) to evaluate their antagonistic property towards the pathogenic oomycete *Phytophthora nicotianae* in the presence of a heavy metal (cadmium) and thus their ability to protect the plant from both stresses (biotic and abiotic);

(ii) to ascertain their ability to produce metabolites with antimicrobial activity in liquid culture and to test the inhibitory activity of these metabolites against different fungi and oomycetes pathogenic to agricultural and forestry plants;

(iii) to determine the effective dose of these metabolites on different plant matrices inoculated by mycotoxigenic fungi and the possible reduction of mycotoxin production by these metabolites.

The unequivocal objective is to limit pollution, through alternatives in reducing contaminants and combating plant diseases through more environmentally sustainable methods.

2. STUDY ON THE TOLERANCE OF *TRICHODERMA ASPERELLUM* TO HEAVY METALS AND ITS SIMULTANEOUS ANTAGONISTIC CAPACITY

2.1. Abstract

Heavy metal contamination is an important environmental issue due to the toxic effects on various organisms. Filamentous fungi of the genus *Trichoderma* have an important role in the bioremediation of heavy metals from contaminated wastewater and soils.

This study aimed at studying the behaviour of *Trichoderma asperellum* strain IMI393899 towards different heavy metals at various concentrations and the possible antagonistic capacity towards *Phytophthora nicotianane* in the presence of Cd. For this purpose *T. asperellum* was checked, at different time intervals, for its tolerance to mercury (Hg), cadmium (Cd), lead (Pb) and zinc (Zn) at various concentrations (0, 1, 2.5, 5, 7.5 and 10 mg/L) on Czapek-Dox-Agar (CDA) medium. At the same time, *T. asperellum* was grown in dual cultures with *P. nicotianae* in the presence of 10 mg/L Cd in PDA to verify its antagonist ability in the presence of heavy metals. The results showed that the fungus was able to grow at the maximum concentration of 10 mg/L of the heavy metals, compete with the pathogen on potato-dextrose-agar (PDA) and protect the plant from cadmium stress (in MS solution). Besides, the analysis of four genes (TrhHg5, TrhHg6, Clone001Hg and Clone031Hg) that appeared to be expressed by *Trichoderma* in the presence of heavy metals was conducted. Particularly, the genes Clone001Hg, TrhHg5 and Clone031Hg are significantly overexpressed in the presence of Cd at 10 mg/L in CDA, with $2^{-\Delta\Delta Ct}$ values of 57, 24 and 7, respectively, but none of the studied genes was significantly expressed in PDA or MS solution.

Keywords: *Trichoderma asperellum*; biological control; bioremediation; heavy metals.

2.2. Introduction

Trichoderma spp. are fungi normally present in the soil and in the plant rhizosphere. They are known as biocontrol agents of many plant diseases and are normally used in agriculture. Their main interest lies in their ability to defend the plant against pathogens. The mechanisms on pathogens can be direct-acting, such as antibiosis and hyperparasitism, and indirect, such as competition for nutrients and space (Köhl *et al*., 2019). In addition, some *Trichoderma* species can induce hormone production and plant growth promote (PGP) (Martinez-Medina *et al*., 2014). An important aspect is their ability to grow under extreme conditions. Many studies demonstrate the ability of some *Trichoderma* species to grow in the presence of pollutants and to be able to decontaminate soil and water (Harman *et al*., 2004).

Contamination of soil and water by toxic heavy metals and organic pollutants is a consequence of industrial activities due to increased globalisation. These pollutants are not biodegradable and can cause environmental and public health problems. In particular, heavy metals can be absorbed by plants and cause metabolic deterioration due to their similarity to certain ions essential for plant growth, such as calcium (Perfus-Barbeoch *et al*., 2002), which in some cases can lead to plant death. Besides, they can be a health risk when polluted and can re-enter the food chain and reach high concentrations through biomagnification. The main polluting metals are Cd, Hg, Pb, As, Ag and Cr; these can be present in soil and water at high concentrations, due to their presence also in fertilisers and pesticides (Gimeno-García *et al*., 1996).

Cd is a metal that is easily absorbed by the roots of the plant, and subsequently, a small part is moved through the xylem to the leaves and fruits (Wagner, 1993). It is one of the heavy metals most commonly found in plant foods and implies a high health risk.

Conventional techniques used for heavy metal decontamination are mainly physico-chemical and include precipitation, oxidation or reduction, ion extraction, reverse osmosis, filtration, electrochemical treatments, sludge separation and evaporative recovery (Volesky, 1994; Doménech, 1998). These techniques can be expensive, unspecific and cause secondary

contamination due to the use of other chemicals (e.g. EDTA). For these reasons, research is focusing on alternative methods, in which the use of microorganisms is a viable alternative.

The objective of this study was to evaluate the ability of the strain *Trichoderma asperellum* (IMI 393899) to protect tomato plants (*Solanum lycopersicum*) from the abiotic stress due to Cd, and from the biotic stress caused by the pathogen *Phytophthora nicotianae*. In addition, the possible expression of genes in *Trichoderma* that seem to be involved in the presence of heavy metals was studied.

2.3. Materials and methods

2.3.1. Fungal strains and maintenance

The oomycete *Phytophthora nicotianae* (Ph_nic) and the fungi *Trichoderma asperellum* (IMI 393899) were obtained from the collection of the Molecular Plant Pathology laboratory of the Di3A, University of Catania (Italy). The identification of *P. nicotianae* and *T. asperellum* was reported by La Spada *et al*. (2020).

Phytophthora nicotianae and *Trichoderma asperellum* were maintained on potato dextrose agar (PDA) at room temperature and subcultures were made every 20 days.

2.3.2. *Trichoderma* growth conditions in the presence of heavy metals

Evaluation of the growth capacity of *T. asperellum* IMI 393899 in the presence of heavy metals was conducted using Czapek Dox Agar (CDA) (Difco) with addition of $(C_2H_3O_2)_2Hg$, $(C_2H_3O_2)_2Cd$, $(C_2H_3O_2)_2Pb$ or $(C_2H_3O_2)_2Zn$. Initially, 100 mg/L stock solutions of Hg^{2+}, Cd^{2+}, Pb^{2+} and Zn^{2+} were prepared by dissolving the metal salt in sterile water, a known volume of the metal stock solution was added to the CDA under stirring and the final concentrations for each metal were of 1, 2.5, 5, 7.5 and 10 mg/L. A known volume of the metal stock solution was added to the CDA under stirring. The pH of the substrate was measured after autoclaving and after the addition of the metal salt, and maintained at 7.3 ± 0.2.

A plug (5 mm) of *Trichoderma* from a 5-7 day-old cultures grown on CDA was placed in the centre of the metal-CDA plate. At the same time, the control (plates with only CDA) was prepared. In addition, the possible interference of the acetate counter ion was assessed, working equally for the metal, by preparing plates of acetate-CDA at 1, 2.5, 5, 7.5 and 10 mg/L; the acetate counter ion gave negligible interference on *Trichoderma* growth (< 2%). Five repetitions for each thesis were made. All plates were incubated at 25 ±1 °C and the two orthogonal diameters of the grown mycelium were measured daily. The percentage inhibitory radial growth (PIRG) was calculated using the following formula (Sarkar *et al*., 2010):

$$\text{PIRG\%} = \frac{(Dc-Dt)}{(Dc)} * 100$$

where:

PIRG, percent of growth inhibition;

Dc, growth rate of the *Trichoderma* (control without Cd);

Dt, growth rate of the *Trichoderma* in presence of Cd**.**

At the end of the experiment, the mycelia growth with 5 and 10 mg/L of each metal were collected and stored in Eppendorf at -80°C for further analysis.

2.3.3. *In vitro* antagonistic properties in presence of cadmium

The growth capacity of *Trichoderma asperellum* in the presence of cadmium was evaluated on Potato Dextrose Agar (PDA) (Oxoid) to which $(CH_3COO)_2Cd$ solution, taken from a stock solution (100 mg/L of Cd), was added, obtaining plates with a final concentration of 10 mg/L of Cd. The decision to replace CDA with PDA was due to the fact that *Phytophthora* species do not grow on CDA. The pH after the addition of cadmium acetate was maintained at 6 ± 0.2. In this case, the mycelium plugs (5 mm) taken from 5-7 day-old cultures on PDA of *P. nicotianae* was placed at 1 cm from the edge of the plate. Only after growing *P. nicotianae* for 3 days, a plug (5 mm) of *T. asperellum* was placed at the opposite side of the *P. nicotianae*.

This choice was made to allow the pathogen growth as the two microorganisms have a different growth rate. The plan of the experiment is summarised in Tab. 2.1.

Tab. 2.1. Treatments assessed for *in vitro* and *in planta* assays for the evaluation of the antagonistic capacity of *Trichoderma* in the presence of the pathogen and/or cadmium.

Corresponding number of treatments *in vitro* and *in planta*	**Treatments**
1	*Phytophthora nicotianae*
2	*Trichoderma asperellum*
3	*P. nicotianae* and *T. asperellum*
4	*P. nicotianae* in presence of Cd
5	*T. asperellum* in presence of Cd
6	*P. nicotianae* and *T. asperellum* in presence of Cd
7	Tomato plant (only *in planta*)
8	Tomato plant in presence of Cd (only *in planta*)

The possible interference of the counter ion acetate was also assessed, preparing acetate-PDA media with final concentration of 10 mg/L acetate. According to the final report there was no interference of mycelium growth compared to the control (PDA only). The plates were incubated at 25 ± 1 °C and the radial growth of both microorganisms was measured every day. Ten replicates were assessed and the percentage inhibitory radial growth (PIRG) was calculated. At the end of the experiment, all mycelium was collected and stored in Eppendorf at -80°C for further analysis.

2.3.4. *In planta*, host-pathogen interaction in presence of cadmium

Evaluation of the ability of *T. asperellum* to defend the plant against pathogen and cadmium accumulation. For the *in planta* test, a host-pathogen-antagonist system was developed in the presence of metal. The tomato plant *Solanum lycopersicum* cv. cuore di bue (Vilmorin Italia S.R.L.) was used as the host. The plants were grown in hydroponics using the nutrient solution described by Murashige and Skoog, 1962. The choice of using a hydroponic system was taken in order to avoid soil interference on subsequent gene analyses. Tomato seeds were sterilised

for 20 min under agitation in 2% sodium hypochlorite; subsequently, they were rinsed with sterile water and seeded on bibulous paper in the dark. After 3 days, the grown seedlings were transferred to trays containing Murashige and Skoog (MS) nutrient solution and placed in the growth chamber at 25°C, 80% of relative humidity and a photoperiod of 16 hours of light and 8 hours of dark. Every 5-7 days the MS solution was renewed.

After four weeks, a conidial suspension of *Trichoderma* (10^6 spores/mL) was added, using sterile water for the samples not treated with *Trichoderma*, and 20 mL of the stock Cd solution (20 mg/L) was added to treatments 4, 5 and 6. The seedlings were subsequently inoculated with four plugs of *Phytophthora nicotianae* grown on PDA. All seedlings were harvested after one week when plants inoculated with *P. nicotianae* alone showed clear symptoms of wilting. Roots were separated from the aerial parts, weighed and stored at -80 °C for further analysis. Six repetitions were made for each treatment.

2.3.5. RNA extraction and quantification

Total RNA from samples collected from the *in vitro* and *in planta* study was isolated using the RNeasy Plant Mini kit (Qiagen) and treated with TURBO DNA-free™ Kit (Promega) to remove genomic DNA contamination, according to the manufacturer's instructions. The extracted RNA from each sample was first checked for quantity and quality using Nanodrop 2000 (Thermo Fisher Scientific), which shows concentrations between 600 and 100 ng/µL and appropriate 260/280 and 260/230 ratios both ≥ 2, and by denaturing agarose gel electrophoresis (Masek *et al*., 2005). RNA samples were diluted to 50 ng/µL with pure RNAse-free H2O and stored at -80 °C until their complementary DNA (cDNA) reverse transcription.

2.3.6. Reverse transcription and qPCR reaction

The cDNA synthesis was performed using 10 µL of total RNA (500 ng) according to the protocol instructions of the High Capacity cDNA Reverse Transcription™ Kit (Applied

Biosystems). The cDNA was subsequently stored at -80 °C until use for qPCR reactions. The cDNA samples were analysed by StepOnePlus Real time PCR (Applied Biosystems) for the assessment of gene overexpression by *Trichoderma* in the presence of heavy metals. For this purpose, four genes were selected based on a study carried out previously (Cacciola *et al*. 2015; Puglisi *et al*. 2012). The sequences of the primer pair used are shown in Tab. 2.2.

Tab. 2.2. Primer sequences used for qPCR analysis.

GenBank accession number	Primers	Sequences	Proteins	Genes
FG068297	Forward	5'-CAGTCACAAGAGCAGCCTGA-3'	Hydrophobin	TrhHg6
	Reverse	5'-CCCCTTGAAGACCATTTGTG-3'		
FG342068	Forward	5'-TTCCTGATCCCCGAGACCAA-3'	Glucose transporter	Clone001Hg
	Reverse	5'-CCTCAACGGTAGCCTTCTCC-3'		
FG342103	Forward	5'-AGATCCCCTGGTTGAACGAC-3'	ATPase	Clone031Hg
	Reverse	5'-GGCCTTGACATCCACGACAT-3'		
FG068296	Forward	5'-CAACCAAAATTGTCGAGGAC-3'	Putative DENN domain-containing protein	TrhHg5
	Reverse	5'-CAGAAGATCGGTGGAGAAGA-3'		
FG342114	Forward	5'-AGCGAAGGTGACGACCATAC-3'	Elongation factor	EF-1α
	Reverse	5'-AAGGAGACCAAGGCTGACAA-3'		

The different expression levels of the four genes encoding for the four putative proteins (Hydrophobin, DENN domain-containing protein, ATPase, glucose-transport proteins) isolated from *T. asperellum* mycelium grown in the presence of mercury, cadmium, lead, zinc at 5 or 10 mg/L, and in the absence of heavy metals (control) were studied. EF-1alpha, which encodes for the Elongation Factor, was chosen as the endogenous control gene (housekeeping).

Real-time amplification reactions were performed in 96 well plates using the PowerUp SYBR Green Master Mix kit (Applied Biosystems). Reactions were prepared in a total volume of 10 µL containing: 1µL of template, 1.4 µL of amplification primer mix (forward/reverse of each gene; 10 µM) and 5 µL of SYBR Green master mix. Non-template controls (NTCs) for each primer pair were also included, replacing the template with DNAse and RNA-free water. Before proceeding with the determination, it was ruled out that these primers could amplify regions in *Phytophthora* and tomato in order to avoid false positives by performing the analysis on samples of mycelium or roots only.

Determinations were performed in four independent experiments with two biological and three technical replicates each. Cycling conditions were set as default: initial denaturation step at 95 °C for 5 minutes to activate Taq DNA polymerase, followed by 40 cycles of denaturation at 95 °C for 15 minutes. Followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing temperatures at 55 °C for 15 sec and elongation at 72 °C for 1 min. The melting curve was generated by heating the amplicons from 60 to 90 °C. The baseline, threshold cycles (Ct) and primer parameter analysis were automatically determined using StepOne Plus version 2.3 software (Applied Biosystems). Quantification of gene expression was performed using Livak's method, which calculates the $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001):

$$\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{sample}} - (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{calibrator}}$$

Ct is the threshold cycle of each transcript, defined as the point at which the amount of amplified target reaches a fixed threshold above background fluorescence.

2.3.7. Statistical analysis

Statistical analysis of data was carried out using IBM SPSS Statistics version 23.0. Data were expressed as mean ± SE of different experiments. The statistical analysis of the results was performed by Student's t-test for paired samples. Differences between groups were statistically

analysed with one-way ANOVA followed by the Turkey HDS post-hoc test for multiple comparisons. The difference level of $p < 0.05$ was considered statistically significant.

2.4. Results and Discussion

2.4.1. Tolerance of *Trichoderma* to heavy metals

The results on the effect of the heavy metals mercury (Hg), cadmium (Cd), lead (Pb) and zinc (Zn) on the diametric growth of *T. asperellum* revealed several aspects. Fig. 2.1 shows the average diametric growth values of the subcultures grown over a period of eight days at different concentrations of Hg, Cd, Pb and Zn.

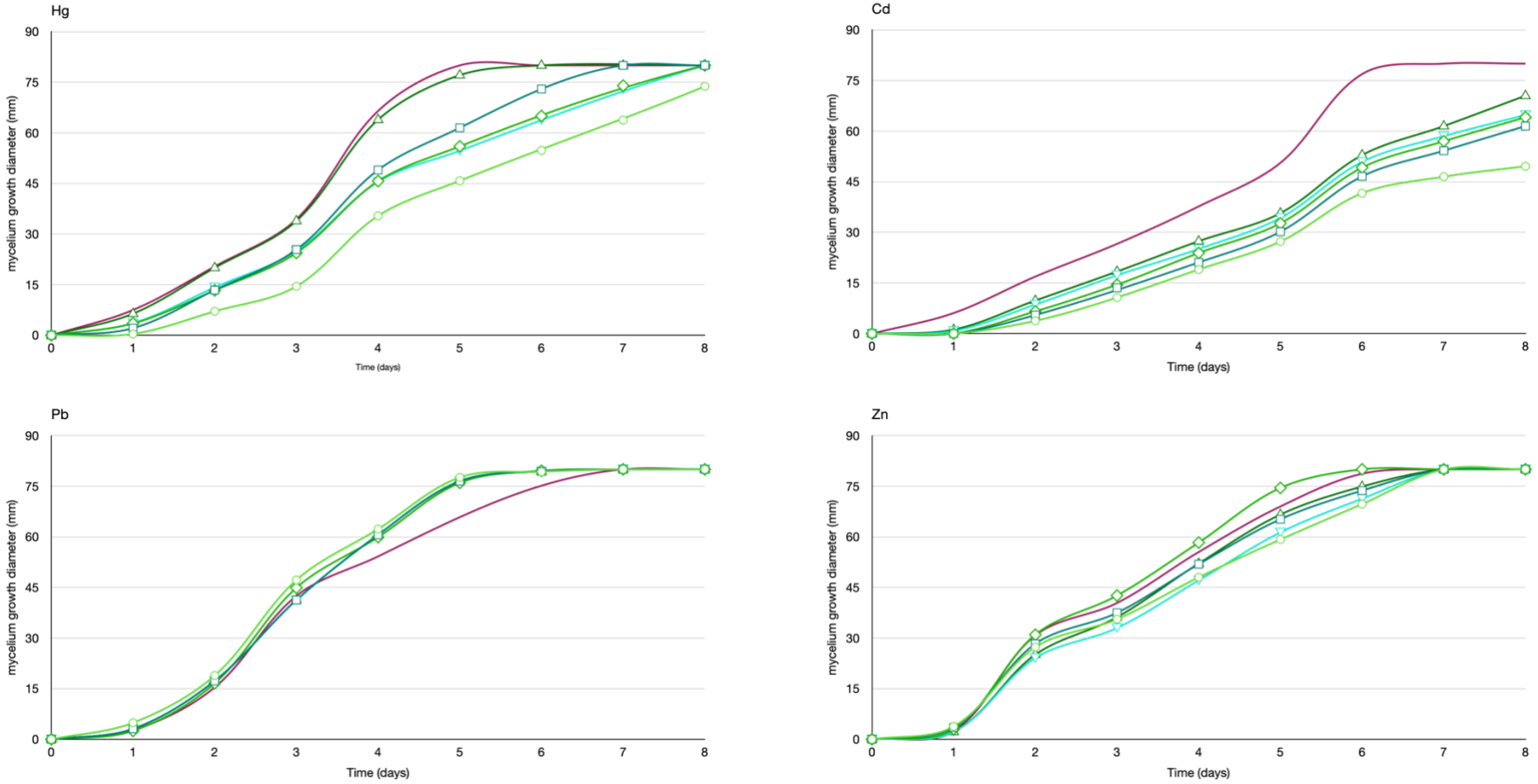

Fig. 2.1. Growth of *Trichoderma asperellum* IMI393899 during 8 days in CDA alone (—), or in the presence of mercury (Hg), cadmium (Cd), lead (Pb) or zinc (Zn) at 1 mg/L (◇), 2.5 mg/L (△), 5 mg/L (▽), 7.5 mg/L (□) and 10 mg/L (○).

In addition, Tab. 2.3 shows the percentages of inhibition (PIRG) of *T. asperellum*, grown on CDA substrate supplemented with heavy metals, compared to the control. It is evident that the growth of *Trichoderma* in the presence of metals depends on dose and time, and the growth changes according to the metal tested.

Tab. 2.3. The percentage inhibition of growth (PIRG) of *Trichoderma asperellum* IMI393899 with different concentrations of heavy metals on Czapek-Dox-Agar (CDA). Results are expressed as the ± SE of PIRG after 1- and 7-day incubation; [a] Not Determined

Heavy metal	Concentration (mg/L)	First Day of growth	Seventh Day of growth
Hg^{2+}	1	14.7 ± 3.3	ND[a]
	2.5	52 ± 4.4	20.3 ±
	5	53.3 ± 6	18.8 ± 5.2
	7.5	72 ± 4.9	8.8 ± 4.6
	10	94.7 ± 3.3	31.5 ± 4.9
Cd^{2+}	1	80.3 ± 5.6	31.2 ± 0.9
	2.5	86.9 ± 6.1	33.8 ± 2
	5	100 ± 0	36 ± 0.8
	7.5	100 ± 0	39.4 ± 1.1
	10	100 ± 0	45.9 ± 0.8
Pb^{2+}	1	ND	ND
	2.5	ND	ND
	5	ND	ND
	7.5	ND	ND
	10	ND	ND
Zn^{2+}	1	8 ± 4.9	4.6 ± 5
	2.5	8 ± 4.9	9.4 ± 4.5
	5	ND	ND
	7.5	ND	ND
	10	ND	11.4 ± 6.4

Hg and Cd had a negative effect on growth, and this effect increased with the concentration, especially Hg showed PIRG of 14 and 94% at day one for 1 and 10 mg/L, respectively. These values decreased significantly at 0 and 31%, at day seven of incubation; Cd significantly inhibited *Trichoderma* growth by showing PIRG higher than 80% at day one, and remaining higher than 30% at day seven. Whereas, Pb and Zn did not show negative growth inhibition, but seemed to facilitate *Trichoderma* growth.

The expression levels of the four genes, studied by RT-qPCR, are shown in Fig. 2.2. The results are expressed as average values of the target genes normalised to the corresponding EF-1alpha value and expressed as fold change compared to the control (without metal).

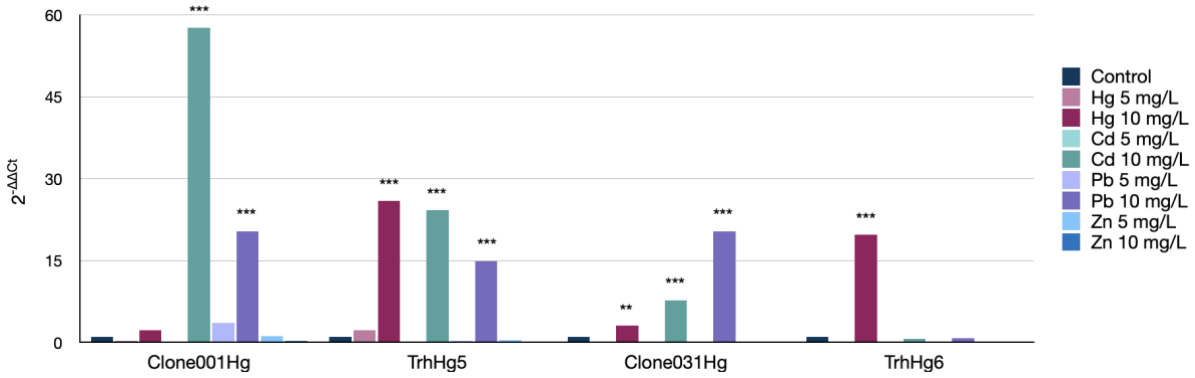

Fig. 2.2. Analysis of the relative mRNA expression levels of Clone001Hg, TrhHg5, Clone031Hg and TrhHg6 quantified in *Trichoderma asperellum* IMI393899 grown at 5 and 10 mg/L of Hg, Cd, Pb and Zn. The average of the target gene values was normalised to the corresponding EF-1alpha value and expressed as fold change compared to the control (without metal). (*) $p \leq 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ indicates a significant difference to the control.

In the presence of Hg at 10 mg/L concentration, the genes TrhHg6 and TrhHg5, encoding for hydrophobin protein and a putative DENN domain-containing protein, respectively, were significantly expressed compared to the control; the genes clone031Hg and clone001Hg, encoding for the putative plasma membrane protein ATPase and glucose transporter proteins, respectively, were low in expression and only the gene clone031hg was significantly expressed compared to the control. The genes Clone001Hg, TrhHg5 and Clone031Hg are significantly over-expressed in the presence of Cd at 10 mg/L, with $2^{-\Delta\Delta Ct}$ values of 57, 24 and 7, respectively. The same genes are also significantly overexpressed for Pb at 10 mg/L compared with the control. The presence of Zn did not lead to any significant expression of these tested genes. In addition, all metals at a concentration of 5 mg/L did not show significant expression in the tests.

2.4.2. Antagonistic properties of *Trichoderma* in presence of heavy metals

Trichoderma spp. are ubiquitous fungi in the soil and present in the rhizosphere of the plants where they can exert protection of plants against pathogens through direct and indirect antagonism mechanisms (Köhl *et al.*, 2019).

The results, expressed as PIRG%, of the antagonistic capacity of *T. asperellum* IMI393899 against *Phytophthora nicotianae* Ph_nic as well of tolerance to cadmium, are shown in Tab. 2.4.

Tab. 2.4. The percentage inhibition of growth (PIRG) of *Trichoderma asperellum* IMI393899 in dual cultures with *Phytophthora nicotianae* and in the presence of Cd (10 mg/L) on PDA. Results are expressed as the ± SE of PIRG after 4-days of incubation.

***T. asperellum* growth in the presence of**	**Fourth day of growth**
Cd (10 mg/mL)	8.35 ± 0.87
Phytophthora nicotianae	30.52 ± 0.8
P. nicotianae and Cd (10 mg/L)	36.58 ± 1.04

After 4 days of growth on PDA supplemented with Cd, the strain IMI393899 showed only 8% inhibition compared to the control (*Trichoderma* alone); the inhibition increased to 30% in the presence of the pathogen only, while it was 36% if Cd was also supplemented to the substrate. Therefore, the growth of *T. asperellum* IMI393899 is only partially inhibited, suggesting a tolerance to cadmium.

The mortality percentage of plants inoculated with *Phytophthora nicotianae* was 100%, and with Cd it was 83%. In the host-pathogen-antagonist system, *Trichoderma* showed no protection against the pathogen with a mortality rate of 83%. However, it was effective in protecting the plant from Cd stress, with a mortality rate of 17%. If both Cd and the pathogen were present, the mortality of tomato plants was 83%. This might suggest that *Trichoderma* is not able to compete in the aquatic environment against *Phytophthora*, which is favoured by the release of zoospores. Zoospores are biflagellate structures that can move and are released from sporangia in the presence of water (Judelson and Blanco, 2005).

Analysis of the genes involved in Cd tolerance showed no expression of the TrhHg6, TrhHg5, Clone001Hg and Clone031Hg genes in both systems, *in vitro* and *in planta*. The results of this experiments made it possible to hypothesise that this strain of *Trichoderma* has a tolerance towards Cd that depends not only on the concentration of the metal and the time of exposure to it, but also on the nature of the medium in which it is present. Indeed, in CDA, it would appear that Cd has a greater influence on the growth of *T. asperellum* IMI393899, whereas if Cd is supplemented to PDA, *Trichoderma* growth is less affected. In their study, Nongmaithem *et al*. (2016) demonstrated that several *Trichoderma* isolates showed high tolerance to Cd, even at concentrations of 200 mg/L. Many studies highlight that several factors may influence the tolerance and bioabsorption capacity of heavy metals by *Trichoderma* spp. For example, Hoseinzadeh *et al*. (2017) studied the uptake capacity of cadmium (Cd), lead (Pb) and nickel (Ni) by *T. asperellum* TS141 and *T. harzianum* TS103 strains, at different metal concentrations, temperatures and pH; thus showing that both strains exhibited excellent Cd uptake capacity at 200 mg/L at a temperature of 35 °C, while the optimal pH for strain TS141 was 9 and for TS103 was 4.

2.5. Conclusions

Pollutants such as pesticides, hydrocarbons and metals can accumulate in soils, water and plants. Significant reduction of hazardous waste, contaminated soil and water, can be achieved by using microbes that promote the degradation and uptake of xenobiotic compounds, such as heavy metals. *Trichoderma* spp. have remarkable tolerance to heavy metals and these can colonise the soil and help promote plant growth. This genus has the potential to control plant pathogens at polluted sites, making it a preferred choice for both biocontrol and plant growth promotion.

The *Trichoderma asperellum* strain IMI393899 showed good tolerance to heavy metals and was able to increase plant resistance to Cd by with a significant reduction in mortality. In the

host-pathogen-antagonist aquatic system, this strain did not show the ability to compete with the pathogen *Phytophthora nicotianae* Ph_nic in the presence of Cd, but further studies in the soil are needed to establish the ability of *T. asperellum* IMI393899 to absorb the metal and thus avoiding the uptake of metals or reduction of metal toxicity in plants, and the simultaneous protection against pathogens.

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3. ANTIFUNGAL ACTIVITY OF BIOACTIVE METABOLITES PRODUCED BY *TRICHODERMA ASPERELLUM* AND *TRICHODERMA ATROVIRIDE* IN LIQUID MEDIUM

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3.1. Abstract

Trichoderma spp. are known as biocontrol agents of fungal plant pathogens and have been recognized as a potential source of bioactive metabolites. The production of antimicrobial substances from strains *T. atroviride* (TS) and *T. asperellum* (IMI 393899) was investigated. The bioactivity of 10- and 30-day culture filtrate extracted with ethyl acetate was assessed against a set of pathogenic fungi and oomycetes. The 30-day extracts of both strains had significant cytotoxic effects against the tested pathogens, with values of minimum fungicidal concentration (MFC) ranging between 0.19 and 6.25 mg/mL. Dual culture assay (direct contact and non-direct contact) and the percentage inhibition of radial growth (PIRG) was calculated. The highest PIRG values were 76% and 81% (direct contact) with IMI 393899 and TS, respectively. Non direct contact does not show inhibition on any of pathogens tested, indicating that the inhibition is not due to the secretion of volatile substances. Culture filtrates were analyzed by GC-MS and HPLC-Q-TOF-MS for the identification of volatile organic compounds (VOCs) and nonvolatile organic compounds (nVOCs), respectively. Seven classes of VOCs and 12 molecules of nVOCs were identified. These results indicate that these strains of *Trichoderma* had antimicrobial activities and they are potential natural sources of compounds with biological activity.

Keywords: *Trichoderma asperellum*; *Trichoderma atroviride*; bioactive metabolites; biological control; plant pathogens

3.2. Introduction

Synthetic chemicals, such as fertilizers, herbicides and pesticides, used in horticultural production systems and during postharvest processes are environmental pollutants and potentially harmful to humans and animals (Wightwick *et al*., 2010). The excessive use of broad-spectrum synthetic fungicides can affect the microbiome of the rhizosphere, including symbiotic fungi and beneficial bacteria. These microorganisms interact with the plants, modifying and promoting the availability of nutrients in the soil, such as nitrogen fixation (Tinker, 1984). In addition, numerous studies have shown that there is the risk of selection of resistant strains of pathogens to synthetic fungicides (Brent and Hollomon, 1998). The excessive use of synthetic chemicals in farming systems has led governmental and international institutions to limit their use and shift interest towards sustainable food and agriculture (FAO, 2020). A valid alternative to synthetic fungicides is the use of biocontrol agents and/or their metabolites. Currently, several biocontrol agents are recognized including bacterial, such as *Agrobacterium*, *Bacillus*, and *Pseudomonas*, and fungal, such as *Ampelomyces*, *Aspergillus*, *Candida*, *Coniothyrium*, *Gliocadium*, *Pseudozyma*, *Streptomyces*, and *Trichoderma*, agents (Fravel, 2005). In particular, the Trichoderma genus have ability to adapt and thrive in different environmental conditions. This genus proved effective in the sustainable management of crop diseases caused by fungi. *Trichoderma* spp. are among the most frequently isolated soil inhabiting fungi and are common in the plant rhizosphere. These fungi are opportunistic, avirulent plant symbionts, and act as parasites and antagonists of many phytopathogenic fungi, thus protecting plants from disease (Harman *et al*., 2004). The antagonistic properties of *Trichoderma* spp. are based on the activation of indirect and direct mechanisms. The indirect mechanisms are competition for space and nutrients, promotion of growth, and induction of plant defenses, whereas the direct mechanisms are mycoparasitism and production of active metabolites and lytic enzymes. These indirect and direct mechanisms can act synergistically and depending on species and strain (Köhl *et al*., 2019). An interesting feature of Trichoderma

spp. is the production of volatile and nonvolatile secondary metabolites capable of inhibiting the growth of pathogens. However, little is known about their production which can vary from strain to strain.

Based on their structure, known metabolites with antibiotic activity can be classified into two main types: (i.) low-molecular-weight and volatile metabolites and (ii.) high-molecular-weight and polar metabolites. Low molecular weight and volatile metabolites include simple aromatic compounds, volatile terpenes, and isocyanide, and relatively nonpolar substances with significant vapor pressure (Cardoza *et al*., 2005).

These "volatile organic compounds" in the soil environment would be expected to diffuse over a distance through systems thus enhancing the performance of the organism by affecting the physiology of competitor organisms (Cardoza *et al*., 2005; Peñuelas *et al*., 2014). High-molecular-weight and polar metabolites, like peptaibols, may exert their activity through direct interactions between *Trichoderma* species and their antagonists (Cardoza *et al*., 2005). In this study, we tested the antagonistic ability and the production of secondary metabolites with inhibitory activity of strains of *Trichoderma asperellum* and *Trichoderma atroviride* on different pathogens of the genera *Fusarium*, *Aspergillus*, *Penicillium*, *Colletotrichum*, *Neofusicoccum*, and *Phytophthora*, that cause loss to agricultural crops.

3.3. Materials and methods

3.3.1. Chemical materials

HPLC-grade methanol and acetonitrile and analytical reagent grade ethyl acetate, formic acid (99%), and dimethyl sulfoxide (99.9% DMSO) were obtained from Thermo Fisher Scientific (Loughborough, UK). Magnesium sulfate (MgSO4) was obtained from Thermo Fisher Scientific (Kandel, Germany). Potato dextrose agar (PDA) and Potato dextrose broth (PDB) was obtained from Thermo Fisher Scientific (Basingstoke, UK). Ultrapure water (<18 MW/cm) was obtained from a Milli-Q purification system (Millipore Corp., Bedford, MA, USA).

3.3.2. Fungal strains, culture conditions and spore production

Six *Penicillium* strains (*P. digitatum* CECT 2954, *P. commune* CECT 20767, *P. expansum* CECT 2278, *P. roqueforti* CECT 2905, *P. camemberti* CECT 2267, *P. brevicopactum* CECT 2316) and four *Aspergillus* strains (*A. parasiticus* CECT 2681, *A. niger* CECT 2088, *A. steynii* CECT 20510, *A. lacticoffeatus* CECT 20581) were sourced from the Spanish Type Culture Collection (Spain).

Six *Fusarium* strains (*F. proliferatum* ITEM 12072, *F. verticillioides* ITEM 12052, *F. sporotrichoides* ITEM 12168, *F. langsethiae* ITEM 11031, *F. poae* ITEM 9151, *F. graminearum* ITEM 126) and two *Aspergillus* strains (*A. flavus* ITEM 8111, *A. carbonarius* ITEM 5010*)* were obtained from the Agro-Food Microbial Culture Collection (Italy).

Penicillium verrucosum VTT D-01847 was obtained from the VTT Culture Collection (Finland).

Two *Neofusicoccum* species (*N. batangarum*, *N. parvum*), two *Colletotrichum* species (*C. acutatum*, *C. gloeosporioides*), two species of the oomycete *Phytophthora* (*Ph. parvispora*, *Ph. nicotianae*) and two species of *Trichoderma* (*T. asperellum* IMI 393899, *T. atroviride* TS) were obtained from the collection of the Molecular Plant Pathology laboratory of the Di3A, University of Catania (Italy).

The mycotoxigenic fungi were cryopreserved in sterile 30% glycerol at –80°C, but before antifungal studies they were defrosted and cultured in PDB at 25 °C for 48 h and inoculated on PDA plates to obtain spores.

All other fungal strains were maintained on potato dextrose agar (PDA) at room temperature and subcultures were made every 20 days.

The strains of *Neofusicoccum* and *Colletotrichum* were grown on oatmeal agar (OMA) to increase the production of conidia (Amponsah *et al.*, 2008).

Phytophthora nicotianae and *Ph. parvispora* were grown on OMA for the production of sporangia. From 10-day-old cultures, a zoospore suspension was obtained by flooding each

culture plate with 10 mL sterile distilled water. The plates with the sterile distilled water were then refrigerated (5 °C) for 25 min and incubated in the dark at 25 °C for 30 min to induce zoospore release (Nyadanu *et al*., 2009).

The OMA was prepared with the protocol (Sinclair and Dhingra, 1995) slightly modified. In particular, 60 g of rolled oats were added to 600 mL of water and then heated for 40 min. The suspension was sieved and 12 g of agar were added, subsequently reaching the final volume of 1 L and autoclaved for 20 min at 121 °C. The OMA medium for *Phytophthora* was prepared as previously described, but with 75 g of rolled oats and 20 g of agar (Sinclair and Dhingra, 1995).

3.3.3. Isolation and identification *Trichoderma* stains

The strain of *Trichoderma asperellum*, IMI 393899, (Cacciola *et al*., 2015; Puglisi *et al*., 2012) had previously been identified morphologically as *Trichoderma harzianum*. However, recent DNA analysis has identified it as *T. asperellum*. *Trichoderma atroviride,* TS strain, was isolated from the basidiocarp of *Ganoderma lucidum* a wood parasite. The tissue fragments of the basidiocarp (5 mm), taken between the healthy tissue and the infected tissue, were washed with 1% NaClO for 2 min, rinsed with sterile distilled water and transferred onto plates with potato dextrose agar (PDA) with the addition of streptomycin sulfate (0.25 g/L). The plates were incubated at 25 °C for 24 hours. Pure subcultures were obtained from growing colonies on PDA.

For the identification of *Trichoderma* spp. the growing mycelium on PDA plates was taken and DNA was extracted and purified using the DNA PowerPlant® Pro kit. The identification of *Trichoderma* isolates was carried out by amplification and analysis of the regions of the Internal Transcribed Spacer (ITS) region of ribosomal DNA (rDNA). The amplification was made using Taq DNA polymerase, recombinant (Invitrogen ™) with the universal primer pairs ITS-5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990).

The reaction mixture for PCR amplification was PCR buffer (1X), dNTP mixture (0.2 mM), MgCl2 (1.5 mM), forward and reverse primers (0.5 mM), Taq DNA Polymerase (1 U) and 100 ng of DNA.

The conditions of the thermocycler were: 94 ° C for 3 minutes; followed by 35 cycles of 94 ° C for 30 seconds, 55 ° C for 30 seconds and 72 ° C for 30 seconds and finally 72 ° C for 10 min. The amplicons obtained were confirmed in 1% agarose gel and sequenced in both directions by an external service (Macrogen). Obtained sequences were analyzed by using FinchTV v.1.4.0 (**https://finchtv.software.informer.com/1.4/**). For species identification, blast searches in GenBank (**https://blast.ncbi.nlm.nih.gov/Blast.cgi**) were performed.

3.3.4. Liquid culture and metabolite production

Two plugs of each *Trichoderma* strains, obtained from actively growing margins of PDA cultures, were used to inoculate 750 mL flasks containing 500 mL of sterile potato dextrose broth (PDB). The liquid cultures were incubated for 10 and 30 days at 30 °C under stirring (100 rpm). The cultures were filtered under vacuum through filter paper, and the filtrates stored at 2 °C for 24 h before the biphasic extraction. Then 10- and 30-day culture filtrates (100 mL) and the relative PDB (controls) were stored at -80 °C and subsequently freeze-dried for the analysis of volatile organic compounds (VOCs).

3.3.5. Extraction of metabolites from liquid culture

The 10- and 30- day culture filtrates of *T. asperellum* IMI 393899 and *T. atroviride* TS were extracted with ethyl acetate (EtOAc) for 3 times with a final 1:1 ratio.

The combined organic fraction was dried ($MgSO_4$) and evaporated under reduced pressure at 35 °C. The red-brown residues recovered were dissolved with 10% DMSO or MeOH and stored at -20 °C until the subsequent analysis.

3.3.6. Agar diffusion test

The activity of EtOAc extracts of 10 and 30 days of the two *Trichoderma* strains was tested against different pathogens with the agar diffusion test. The spore suspension of pathogen was stratified on the surface of PDA plate using sterile cotton swabs and 10 µL of extract at different concentrations (0, 1, 10, 25, 50, 100 mg/mL) were distributed on the surface. The plates were incubated at 25 °C. The control 10% DMSO was used in the test. After 72 h of incubation, the diameter of the inhibition halos was measured. *Phytophthora* species are oomycetes which form zoospores as infectious propagules and have a different behaviour from spores. For this reason, the assay for two species of *Phytophthora* was performed in a different way. A plug (5 mm) of mycelium from an active growing culture was placed in the center of the PDA plate and 10 µL of extract were placed at a distance of 25 mm using the concentrations previously reported. The plates were incubated at 25 °C for three days. The inhibition of the growth was assessed by measuring the diameter of the inhibition halos in comparison with the control (10% DMSO).

3.3.7. Antimicrobic dilution assay

The minimum inhibitory concentration (MIC), defined as the lowest concentration of the treatment that inhibits the visible fungal growth, was determined with a microdilution method. A 100 µL aliquot of 30-day EtOAc extract (stock solution: 50 mg/mL), diluted with sterile PDB to obtain final concentrations of 0.09-25 mg/mL, was added to 96-well sterile microplates. Then, the wells were inoculated with 100 µL of a 5 x 10^4 spores (or zoospores)/mL suspension and incubated at 25 °C for 72 hours. The suspension of the pathogen strains was prepared by suspending the spores or zoospores in buffered peptone water with 1% Tween 20. Spore or zoospores were counted using the Neubauer chamber and the suspension was adjusted to the final concentration (Petrikkou *et al*., 2001). Wells with either PDB and the spores (or zoospores) of the pathogens or PDB and 10% DMSO were used as controls.

After determining the MIC, aliquots of the wells with concentrations corresponding to the MIC

as well as with higher concentrations were used to inoculate PDA plates for the determination of the minimum fungicidal concentration (MFC). After incubation of the plates at 25 °C for 72 h, the MFC, defined as the lowest treatment concentration required to kill a pathogen and corresponding to a non-visible growth of the subculture, was determined. Three replicates of each assay were assessed.

3.3.8. Dual culture assay

The competition ability for direct contact and production of non-volatile or volatile substances by *Trichoderma* strains were evaluated with two methods of dual culture assay. In both methods, a 5 mm plug, taken from 5-7 days cultures on PDA of the pathogen as well as of the antagonist, was placed on the opposite side of a PDA plate; however, in the second method, the plate was separated into two compartments. The first method evaluates the inhibition growth for direct contact and production of non-volatile substances while the second method determines the possibility of inhibition due to volatile substances, because they are not in direct contact.

All plates were incubated at 25 °C and radial growth of pathogens was measured daily. The controls were plates with only the pathogen and three repetitions were made per thesis. The percentage inhibitory of radial growth (PIRG) was calculated using the following formula:

$$\text{PIRG\%} = \frac{(\boldsymbol{Dc-Dt})}{(\boldsymbol{Dc})} * \mathbf{100}$$

where:

PIRG, percent of growth inhibition;

Dc, growth rate of the pathogen (control);

Dt, growth rate of the pathogen in presence of *Trichoderma.*

3.3.9. Analysis of VOCs

Lyophilized culture filtrate (200 mg) was mixed with 2 mL of water and placed in a 10-mL glass vial. VOCs were identified by gas chromatography with a single quadrupole mass spectrometer detector (GC/MS) analysis. Prior to analysis, samples were incubated in a water bath at 55 °C for 45 min, while being gently stirred with a rod. VOCs were extracted from the vial headspace by solid-phase micro-extraction (SPME). An SPME holder (Supelco, Bellafonte, PA, USA) containing a fused-silica fiber coated with a 50/30 μm layer of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) was used to trap VOCs in the vial headspace. The fiber was introduced into the split-less inlet of an Agilent 6890N GC system (Agilent Technologies, Palo Alto), and thermal desorption of the analytes was performed at 250 °C for 5 min. The GC system was equipped with an HP-5MS (30m × 0.25 mm, 0.25 μm 5% diphenyl/95% dimethylpolysiloxane) capillary column (J&W Scientific, Folsom, CA, USA). The oven was programmed to start at 40 °C (held for 2 min) and to ramp up to 160 °C at 6 °C/min, then increase to 260 °C at 10 °C/min (held for 4 min). Helium (99.999%) was used as the carrier gas, and the flow rate was 1 mL/min. The flow was transferred from the column into an Agilent 5973 MS detector (Agilent Technologies, Palo Alto). The ion source temperature was set at 230°C, the ionizing electron energy was 70 eV, and the mass range was 40–450 Da in full scan acquisition mode. Compounds were identified using the NIST Atomic Spectra Database version 1.6 (Gaithersburg, MD, USA), considering spectra with 95% similarity. Results were expressed as a percentage of the VOC by dividing the area of each peak by the total area of the chromatogram peaks (Guarrasi *et al.*, 2017). The analysis was carried out in triplicate.

3.3.10. Analysis of nVOCs

For the identification of non-volatile organic compounds (nVOCs), 50 mg of the crude extracts were re-suspended with 2 mL of methanol. All samples were filtered through 0.22 μm nylon

membrane prior injection. The HPLC system used for the chromatographic determination was an Agilent 1200 (Agilent Technologies, Santa Clara) equipped with a vacuum degasser, autosampler and binary pump. The column was a Gemini C18 (50 mm × 2 mm, 100 Å, 3-µm particle size; Phenomenex).

The binary mobile phases consisted of water (A) and acetonitrile (B) with 0.1% v/v formic acid. The initial gradient of the mobile phase was 5% B for 5 min and was increased to 95% B over 10 min. It was maintained at 95% B for 20 min, and then reduced to 5% B for 5 min and maintained for 5 min. The flow rate was maintained at 0.3 mL/min and 20 µl of each sample was injected.

Mass spectrometry (MS) analysis was performed using a Q-TOF-MS (6540 Agilent Ultra High Definition Accurate Mass), equipped with an Agilent Dual Jet Stream electrospray ionisation (Dual AJS ESI) interface in positive and negative ion mode over the range of m/z 50-1500. The parameters were as follows: drying gas flow (N_2), 8.0 L/min; nebuliser pressure, 30 psig; gas drying temperature, 350°C; capillary voltage, 3.5 kV; fragmentor voltage, 175 V. Targeted MS/MS experiments were carried out using collision energy values of 10, 20 and 40 eV. Integration and data elaboration were managed using MassHunter Qualitative Analysis software B.08.00 (Denardi-Souza *et al.*, 2018). The analysis was carried out in triplicate.

3.4. Results and Discussion

3.4.1. Antifungal activity on solid medium, MIC and MFC

The results of the antifungal activity of non-volatile or diffusible compounds obtained with the two *Trichoderma* strains tested, *T. asperellum* IMI 393899 and *T. atroviride* TS, showed that the extracted antifungal compounds inhibited the growth of all pathogens in the agar diffusion assay, as shown in Tab. 3.1.

Tab. 3.1. Agar diffusion test of 30-days EtOAc extracts. Several concentrations of the EtOAc extracts of *Trichoderma asperellum* IMI393899 and *T. atroviride* TS were tested on different pathogens; results were measured as the diameter of the inhibition halos. *range 5-10 mm (+), range 11-15 mm (++), range 16-20 mm (+++), range 21-25 mm (++++) and more than 26 mm (+++++).

Strains	***EtOAc extracts of**											
	T. asperellum						***T. atroviride***					
Concentrations mg/mL	**0**	**1**	**10**	**25**	**50**	**100**	**0**	**1**	**10**	**25**	**50**	**100**
Penicillium digitatum	-	-	+	++	+++	+++	-	-	+	++	++	++
Penicillium commune	-	-	+	+++	+++	+++	-	-	+	+	++	++
Penicillium expansum	-	+	++	++	+++	+++	-	-	+	++	++	++
Penicillium roqueforti	-	-	+	++	++	++++	-	-	+	+	+	++
Penicillium camemberti	-	++	++	+++	++++	+++++	-	-	+	+	++	++
Penicillium brevicopactum	-	-	+	++	+++	++++	-	-	+	+	+	++
Penicillium verrucosum	-	-	++	++	++	+++	-	-	+	+	+	++
Aspergillus parasiticus	-	-	+	+	+++	++++	-	-	-	-	+	++
Aspergillus flavus	-	-	++	++	++	++++	-	-	+	+	++	++
Aspergillus niger	-	+	+	++++	++++	+++++	-	-	+	+	++	++
Aspergillus steynii	-	++	+++	+++++	+++++	+++++	-	-	+	++	++	++
Aspergillus carbonarius	-	+	+	+++	+++++	++++	-	-	+	+	+	++
Aspergillus lacticoffeatus	-	-	++	+++	++++	+++++	-	-	+	+	++	++
Fusarium proliferatum	-	-	++	++	+++	+++	-	-	+	+	++	++
Fusarium verticillioides	-	-	+	++	+++	++++	-	-	-	+	++	++
Fusarium sporotrichoides	-	-	++	++	+++	++++	-	-	+	+	+	++
Fusarium langsethiae	-	-	+	+	++	+++	-	-	-	+	++	++
Fusarium poae	-	-	++	++	++	+++	-	-	+	+	++	++
Fusarium graminearum	-	-	+	++	+++	++++	-	-	-	+	++	++
Neofusicoccum batangarum	-	-	+	+++	+++++	+++++	-	-	+	+++	++++	+++++
Neofusicoccum parvum	-	-	+	+++	+++++	+++++	-	-	+	+++	++++	+++++
Colletotrichum acutatum	-	-	+	++	+++	+++	-	-	+	+	++	+++
Colletotrichum gloeosporioides	-	-	+	+	+++	+++++	-	-	-	+	++	+++
Phytophthora parvispora	-	-	++	+++	++++	+++++	-	-	+	++	++	++++
Phytophthora nicotianae	-	-	++	+++	++++	+++++	-	-	++	++++	+++	+++++

The agar diffusion test of the extract of the 10 days culture filtrate at maximum concentrations incited a change in the colony morphology of the pathogen, but not a significant reduction in growth. For this reason, all subsequent inhibition tests were carried out using 30-day extracts. Both 30-day EtOAc extracts were effective on all tested pathogens, showing inhibition halos that varied with the concentration of the extract.

In particular, the extract of *T. asperellum* showed an inhibition halo at a concentration of 1 mg/mL on *P. expansum, P. camemberti, A. niger, A. steynii* and *A. carbonarius*, with an average halo of 8 mm. The inhibition was visible on all pathogens at a concentration of 10 mg/mL with an average halo of 9.7 mm. At this concentration, the most susceptible was *A. steynii* with an area of inhibition of 17 mm and the least susceptible was *C. gloesporioides* with an area of inhibition of 5 mm. At the maximum concentration of 100 mg/mL extract, the average inhibition values were 24 mm and exceeded 30 mm for of *P. camemberti, A. niger, A. steynii, Neofusicoccum* spp. and *Ph. parvispora* and *Ph. nicotianae*. At this concentration, the lowest value was 18 mm of inhibition area for *P. verrucosum* and *F. poae*, respectively.

The extract of *T. atroviride* was effective at the minimum concentration of 10 mg/mL on all pathogens with the exception of *A. parasiticus, F. verticillioides, F. langsethiae* and *F. graminearum*; moreover, *A. parasiticus* was not inhibited at the concentration of 25 mg/mL and the inhibition halo was observed at concentrations of 50 and 100 mg/mL with values of 7 and 11 mm, respectively.

At a concentration of 10 mg/mL, the average inhibition area of all pathogens was 7 mm, the lowest value was 4 mm for *F. proliferatum* and the highest value was 13 mm for *Ph. nicotianae*. For the maximum tested concentration of 100 mg/mL, the mean inhibition area was 14 mm, species of *Neofusicoccum* were the most susceptible to the extracts with an inhibition halo of 27 mm while the minimum value of 10 mm was obtained on *P. brevicopactum, F. langsethiae* and *F. poae*.

Both extracts were shown to have inhibitory activity on all tested pathogens. In particular the

extract of *T. asperellum* was more effective; as it was able to inhibit at the minimum concentration (10 mg/mL) *A. parasiticus*, the major fungus responsible for aflatoxin formation in crop seeds (Klich, 2007).

Interestingly, a week after test initiation, the inhibition halo persisted (Fig. 3.1-2), thus showing that these extracts have a great inhibition potential *in vitro*.

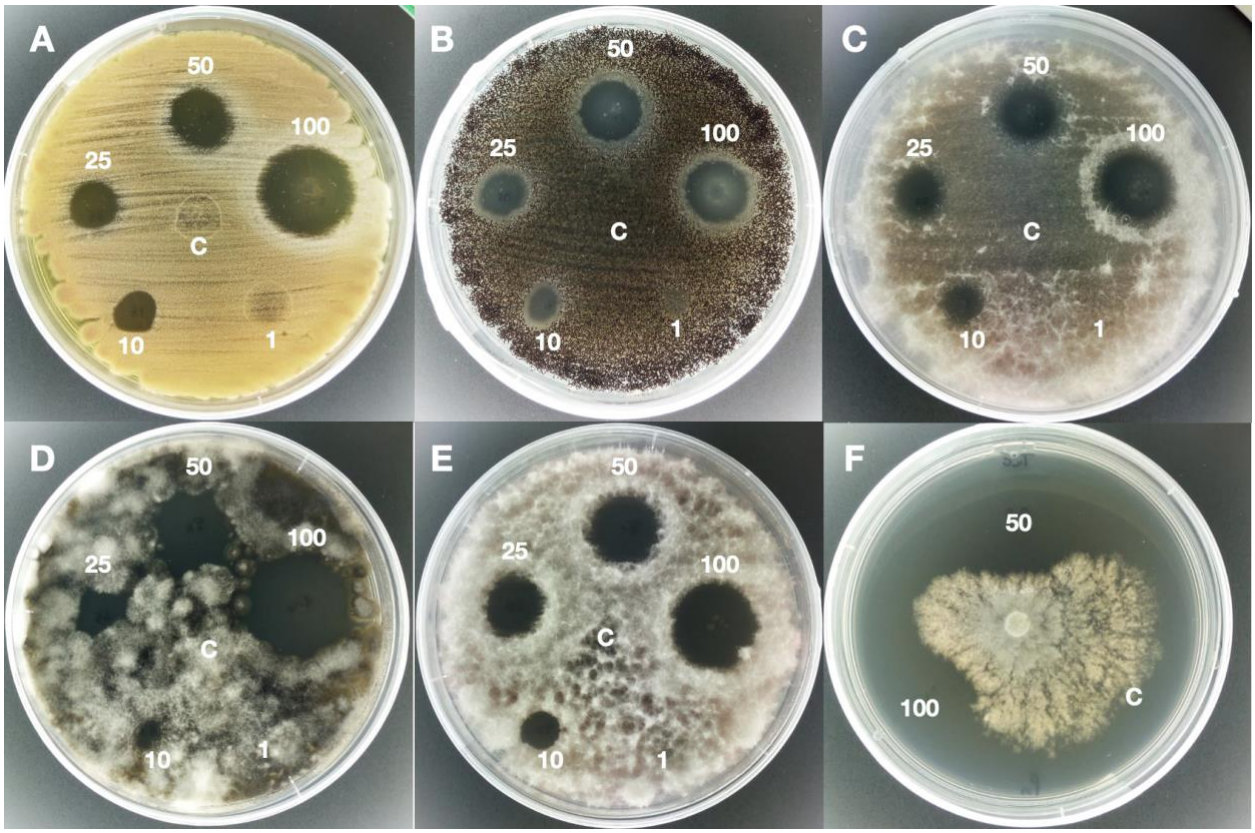

Fig. 3.1. Agar diffusion test of *Trichoderma asperellum* IMI393899 extract. The picture shows the halos of antifungal activity at different concentrations (1, 10, 25, 50 and 100 mg/mL) of 30-days EtOAc extract and in comparison with the "C" control (10% DMSO) on *Penicillium camemberti* (A), *Aspergillus carbonarius* (B), *Fusarium verticillioides* (C), *Neofusicoccum parvum* (D), *Colletotrichum acutatum* (E) and *Phytophthora nicotianae* (F).

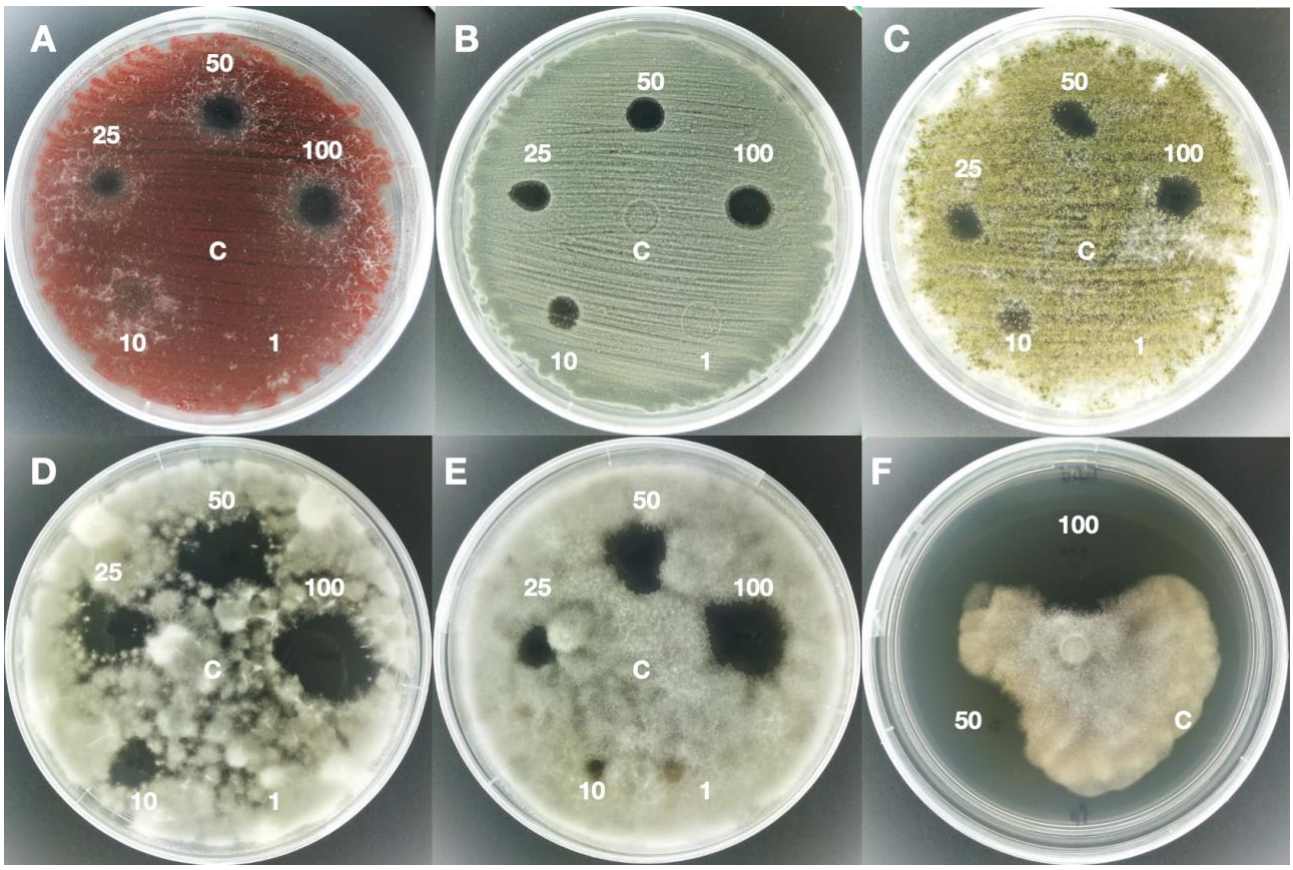

Fig. 3.2. Agar diffusion test of *Trichoderma atroviride* TS extract. The picture shows the halos of antifungal activity at different concentrations (1, 10, 25, 50 and 100 mg/mL) of 30-days EtOAc extract and in comparison with the "C" control (10% DMSO) on *Fusarium proliferatum* (A), *Penicillium brevicopactum* (B), *Aspergillus Flavus* (C), *Neofusicoccum batangarum* (D), *Colletotrichum gloeosporioides* (E) and *Phytophthora parvispora* (F).

The results of the quantitative analysis of the EtOAc extracts obtained with the MIC and MFC are shown in Tab. 3.2.

The MIC of *T. asperellum* IMI 393899 extract ranged between 0.09 and 0.78 mg/mL and values of MFC were in the range of 0.19-1.56 mg/mL.

The extract of *T. atroviride* TS, despite higher concentrations, proved effective with values in the range of 0.19-3.13 and 0.39-6.25 mg/mL of MIC and MFC, respectively.

Penicillium spp. were the most susceptible to the antifungal activity of both extracts; these pathogens are among the most common in post-harvest rot, which cause significant economic damage due to loss of production (Ibatsam and Rukhsana, 2014).

Tab. 3.2. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC). Values in mg/mL of the EtOAc extracts of *Trichoderma asperellum* IMI393899 and *T. atroviride* TS on tested fungus and oomycete pathogens.

Strains	EtOAc extract of *T. asperellum*		EtOAc extract of *T. atroviride*	
	MIC	**MFC**	**MIC**	**MFC**
Neofusicoccum batangarum	0.39	0.78	3.13	3.13
Neofusicoccum parvum	0.78	0.78	3.13	6.25
Colletotrichum acutatum	0.39	1.56	1.56	3.13
Colletotrichum gloeosporioides	0.39	1.56	1.56	3.13
Phytophthora parvispora	0.39	0.78	1.56	3.13
Phytophthora nicotianae	0.19	0.78	1.56	3.13
Penicillium digitatum	0.09	0.39	0.19	0.39
Penicillium commune	0.09	0.39	0.19	0.39
Penicillium expansum	0.09	0.39	0.19	0.78
Penicillium roqueforti	0.09	0.39	0.19	0.39
Penicillium camemberti	0.19	0.78	0.39	0.78
Penicillium brevicopactum	0.09	0.39	0.19	0.78
Penicillium verrucosum	0.39	0.78	1.56	1.56
Aspergillus parasiticus	0.39	0.78	3.13	6.25
Aspergillus flavus	0.39	0.78	3,13	6.25
Aspergillus niger	0.39	0.78	1.56	3.13
Aspergillus steynii	0.09	0.19	0.78	1.56
Aspergillus carbonarius	0.39	0.78	1.56	3.13
Aspergillus lacticoffeatus	0.39	0.78	3.13	3.13
Fusarium proliferatum	0.39	0.78	0.78	0.78
Fusarium verticillioides	0.78	0.78	0.78	1.56
Fusarium sporotrichoides	0.19	0.78	0.19	0.78
Fusarium langsethiae	0.39	1.56	0.78	1.56
Fusarium poae	0.39	0.78	0.78	0.78
Fusarium graminearum	0.39	0.78	0.78	1.56

3.4.2. Antagonistic properties

In the first method, inhibition by direct contact and/or by secretion of metabolites, both *Trichoderma* species showed an inhibition of the mycelial growth of all the tested pathogens. Table 3 shows the PIRG observed on the 1st, 3rd and 7th day of the test. In some cases, the inhibition is observed from the first day and, as matter of fact, it appears that the growth rate of the pathogen could influence the efficiency of *Trichoderma* in inhibiting the growth of the pathogen. This observation is supported by Vinale *et al*. (2009) who demonstrated the production and the class of metabolites produced by *Trichoderma* are influenced by the presence and type of host pathogen.

In the case of *Penicillium* strains with a slower growth rate, the inhibition is observed only on the third day when *Trichoderma* has filled half of the plate and is close to the pathogen.

On the seventh day, the highest PIRG was observed when *Trichoderma* came into direct contact with the pathogen. In particular, both *Trichoderma* strains showed PIRG values higher than 40% and the maximum values of PIRG were obtained for *Ph. nicotianae* with 76% and 81% for *T. asperellum* and *T. atroviride*, respectively. Only *P. expansum* showed less than 20% radial growth inhibition.

In Fig. 3.3., a 7-day test of *F. graminearum*, *P. commune* and *A. parasiticus* grown with *T. atroviride* and *T. asperellum* clearly demonstrates the inhibition of the growth in comparison with the control (only the pathogen). Control cultures of the tested pathogens showed a faster growth with respect to dual culture. The dual culture plates showed rapid initial growth of the fungus that stopped at the point of contact with the antagonist. In some cases, the biological antagonist was not only able to inhibit the mycelium growth of the pathogen, but it grew on it, causing degradation of the mycelium (Tab. 3.3), as in the case of *P. commune* (Fig. 3.3Ba).

Tab. 3.3. The Percentage Inhibition of Radial Growth (PIRG) in dual culture assay. Results are expressed as the ± Standard Error of PIRG after 1, 3 and 7-day incubation; * represents pathogens that showed signs of mycelial lysis due to mycoparasitism by *Trichoderma asperellum* IMI393899 and *T. atroviride* TS.

Strains	***Trichoderma asperellum***			***Trichoderma atroviride***		
	1st day	**3rd day**	**7th day**	**1st day**	**3rd day**	**7th day**
Neofusicoccum batangarum	3.64 ± 1.82	37.78 ± 2.00	63.75 ± 2.6 *	14.55 ± 3.64	36.67 ± 1.67	67.50 ± 1.44 *
Neofusicoccum parvum	6.45 ± 3.23	43.75 ± 0.83	54.17 ± 0.42 *	8.06 ± 2.79	41.35 ± 0.48	63.33 ± 2.32 *
Colletotrichum acutatum	ND	3.17 ± 1.59	46.28 ± 2.19	ND	ND	47.93 ± 1.43
Colletotrichum gloeosporioides	ND	10.26 ± 1.28	59.01 ± 0 *	13.33 ± 3.33	14.10 ± 1.28	58.39 ± 2.24
Phytophthora parvispora	ND	9.09 ± 0	48.62 ± 0.92 *	ND	9.09 ± 0	44.95 ± 0
Phytophthora nicotianae	5.77 ± 1.92	22.39 ± 1.49	76.24 ± 2.97 *	7.69 ± 0	25.37 ± 1.49	81.19 ± 0.99 *
Penicillium digitatum	ND	3.03 ± 1.52	56.52 ± 1.26 *	ND	ND	71.01 ± 2.61 *
Penicillium commune	ND	20.00 ± 4.00	51.85 ± 1.85	ND	12.00 ± 4.00	68.52 ± 1.85 *
Penicillium expansum	ND	ND	18.18 ± 3.15	ND	ND	30.91 ± 7.93
Penicillium roqueforti	ND	ND	51.67 ± 0.83 *	ND	7.02 ± 1.75	75.83 ± 6.01 *
Penicillium camemberti	ND	ND	25.71 ± 12.45	ND	9.09 ± 4.55	28.57 ± 10.30
Penicillium brevicopactum	ND	11.11 ± 5.56	43.90 ± 2.44	ND	22.22 ± 14.70	68.29 ± 4.88
Penicillium verrucosum	ND	15.79 ± 5.26	42.42 ± 6.06 *	ND	21.05 ± 9.12	63.64 ± 5.25 *
Aspergillus parasiticus	2.70 ± 0	4.82 ± 1.20	46.05 ± 0.66	13.51 ± 2.70	16.87 ± 7.23	50.00 ± 7.59
Aspergillus flavus	ND	4.11 ± 1.37	38.52 ± 0	ND	4.11 ± 2.74	22.95 ± 3.57
Aspergillus niger	ND	8.99 ± 1.95	45.86 ± 1.69	ND	ND	41.40 ± 3.87
Aspergillus steynii	ND	ND	42.22 ± 1.11	ND	ND	25.56 ± 1.11 *
Aspergillus carbonarius	ND	6.82 ± 1.14	40.58 ± 0.72	ND	ND	23.91 ± 2.17
Aspergillus lacticoffeatus	ND	4.11 ± 2.74	34.55 ± 1.57	8.11 ± 2.70	9.59 ± 4.11	21.82 ± 0.91
Fusarium proliferatum	ND	10.94 ± 2.71	50 ± 0.96 *	ND	6.25 ± 0	42.31 ± 1.67 *
Fusarium verticillioides	7.69 ± 0	9.59 ± 0	45.83 ± 3.00	7.69 ± 0	8.22 ± 1.37	35.83 ± 1.67 *
Fusarium sporotrichoides	ND	5.88 ± 3.11	50 ± 2.47 *	ND	3.53 ± 1.18	39.73 ± 1.37 *
Fusarium langsethiae	ND	1.67 ± 1.67	42.57 ± 0.99 *	ND	3.33 ± 1.67	30.69 ± 5.51
Fusarium poae	ND	12.82 ± 5.13	67.15 ± 2.53 *	ND	2.56 ± 1.28	48.91 ± 1.93 *
Fusarium graminearum	ND	3.03 ± 3.03	61.19 ± 5.22 *	ND	6.06 ± 1.52	51.49 ± 1.97

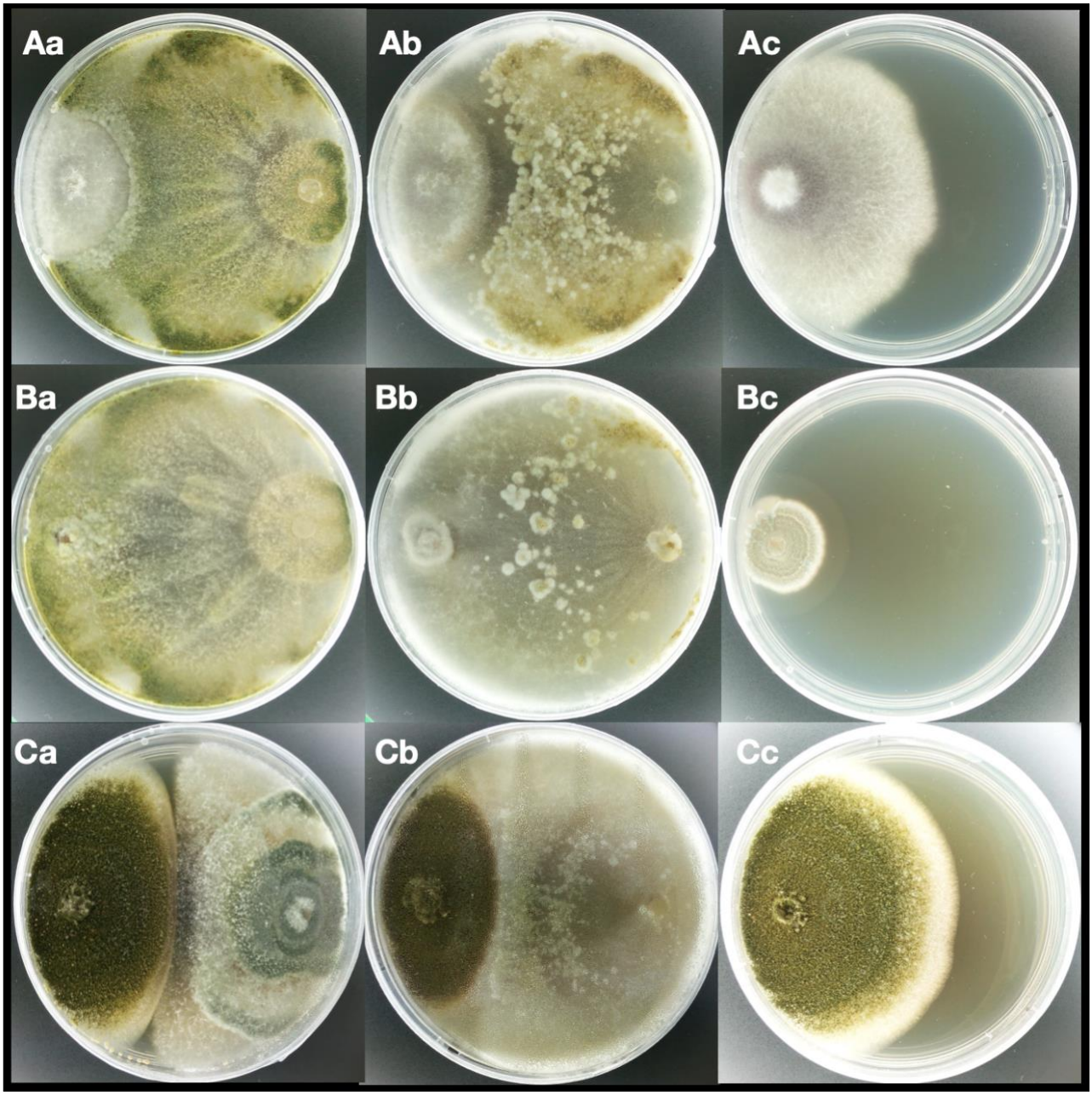

Fig. 3.3. Dual culture assay after seven-days of incubation. *Fusarium graminearum* (A), *Penicillium commune* (B) and *Aspergillus parasiticus* (C) in co-culture (left side of each plate) with *Trichoderma atroviride* TS (a), *Trichoderma asperellum* IMI393899 (b) and the control (c).

According to the generally accepted definition, antibiosis is the mechanism mediated by specific metabolites such as enzymes, volatile compounds and non-volatile antibiotics (Fravel, 1988). The involvement of enzymes in biological control complicates the distinction between mycoparasitism and antibiosis.

Mycoparasitism is one of the main strategies of *Trichoderma* spp. to antagonize pathogens as supported by previous studies. John *et al.* (2010) reported mycoparasitic activity from

Trichoderma viride on *Pythium arrhenomanes* and *Fusarium oxysporum* f. sp. *adzuki*; *Trichoderma* showed a mycoparasitic activity on both pathogens, but mainly on *Pythium*. Krauss *et al*. (1998), the MP11 variety of *Trichoderma* spp. was able to mycoparasitize various pathogens (*Fusarium* spp., *Colletotrichum* spp., *Botryodiplodia theobromae* and *Nigrospora sphaerica*). In another study, Hibar *et al*. (2005) showed the invasion of the mycelium of *Trichoderma harzianum* on *Fusarium oxysporum* f. sp. *radicis-lycopersici* cultures after six days in direct contact. Microscopic observations, made at the level of the contact area between *T. harzianum* and *F. oxysporum* f. sp. *radicis-lycopersici*, showed a profound change in the mycelium of the pathogen characterized by significant lysis, in which the hyphae of *Trichoderma* were enveloping those of the pathogen. In the study of Tondje *et al*. (2007) in which the four strains of *Trichoderma* were used to control the black pod disease of cocoa caused by a *Phytophthora megakarya*, the biological principle of their actions was discussed. According to these authors, the mycoparasitic activity of the *T. asperellum* strains involves both direct penetration and enveloping the hyphae of the pathogen; in the latter case a high level of hydrolytic enzymes is produced before the destruction of the hyphae.

In the second method of the double culture test, which makes it possible to evaluate the inhibition by secretion of volatile substances, no inhibition was observed for the 3 days in which the test was conducted (data not shown). This may suppose either that the inhibition of the two strains of *Trichoderma, T. asperellum* IMI 393899 and *T. atroviride* TS, is not mainly due to the secretion of volatile substances or that antibiotic volatile substances are not normally produced if *Trichoderma* is not approaching the pathogen.

Calistru *et al*. (1997) showed the inhibition growth of *A. flavus* and *F. moniliforme* was due to volatile substances produced by two strains of *T. harzianum* (T1, T2) and two strains of *T. viride* (T5, T6). The growth of *A. flavus* and *F. moniliforme* was inhibited when the colonies were exposed to the trapped atmosphere from cultures of *Trichoderma* spp., with the exception of *T. harzianum* T1. *Trichoderma harzianum* T2 which only inhibited the growth of *A. flavus*, while

T. harzianum T1 did not reduce the growth of *F. moniliforme* and *A. flavus*. This shows that not all *Trichoderma* strains are able to inhibit the *in vitro* growth by producing volatile substances.

3.4.3. Identification of VOCs and nVOCs

A total of 62 VOCs were identified in the lyophilized samples of *Trichoderma* strains and only seven were also present in the control medium (PDB). The VOCs identified in the lyophilized culture filtrates of the two *Trichoderma* strains and the control medium with a percentage > 0.01% are shown in Table 3.4. The identified compounds are classified according to the chemical class into alcohols, aldehydes, acids, ketones, pyrazines, esters and others.

Both culture filtrates presented high levels of alcohols and pyrazines compared to the control. Among the compounds most identified in *T. asperellum* IMI 393899 culture filtrate were 3-methyl-1-butanol (4.47%), phenylethyl alcohol (19.06%), tetramethyl pyrazine (4.81%), 6-pentyl-alpha-pyrone (42.66%), α-Zingiberene (5.86%) and α-Sesquiphellandrene (6.60%), while in the culture filtrate *T. atroviride* TS were 3-methylbutanol (5.29%) tetramethylpyrazine (56.79%) and 1-Hydroxy-2.4-di-tert-butylbenzene (6.17%).

Tab. 3.4. Volatile organic compounds (VOCs) produced in the culture filtrate of *Trichoderma asperellum* IMI393899 and *T. atroviride* TS identified by GC-MS. Results are expressed as mean relative abundance percentages (as obtained by dividing the area of each peak by the total area of the chromatogram peaks) ± Standard Deviation.

Compound (VOCs)	**Strains**	
	Trichoderma asperellum	***Trichoderma atroviride***
Alcohols		
Ethanol	0.58 ± 0.01	1.02 ± 0.07
2-methyl-1-propanol	0.70 ± 0.01	0.68 ± 0.05
3-methyl-1-butanol	4.47 ± 0.13	5.29 ± 0.23
3-methylacetate-1-butanol	0.35 ± 0.01	ND
2-methylacetate-1-butanol	0.18 ± 0.01	ND
Benzyl alcohol	0.35 ± 0.05	ND
Phenylethyl Alcohol	19.06 ± 0.08	ND
1-phenyl-2-propanol	ND	0.15 ± 0
2-(4-methoxyphenyl)ethanol	0.71 ± 0.03	ND
Aldheydes		
Benzaldehyde	0.13 ± 0	0.20 ± 0
Benzeneacetaldehyde	0.06 ± 0	0.91 ± 0.04
3-methyl-benzaldehyde	0.30 ± 0.01	ND
Nonanal	ND	0.11 ± 0
Acids		
1-methyl-6-oxopyridine-3-carboxylic acid	ND	0.20 ± 0.01
Ketones		
2,3-butandione	ND	1.25 ± 0.04

Compound (VOCs)	Strains	
	Trichoderma asperellum	***Trichoderma atroviride***
3-ethyl-2-cyclopenten-1-one	0.41 ± 0.03	0.87 ± 0.08
Phenylacetone	ND	0.23 ± 0.02
5,6,6-trimethyl-3,4-undecadiene-2,10-dione	0.27 ± 0.02	1.55 ± 0.02
Pyrazines		
Trimethyl pyrazine	0.09 ± 0.01	0.43 ± 0.03
Tetramethyl pyrazine	4.81 ± 0.04	56.79 ± 0.29
2,3,5-trimethyl-6-ethylpyrazine	0.45 ± 0.01	1 ± 0.06
2,3,5-trimethyl-6-propylpyrazine	0.14 ± 0	0.14 ± 0.01
2-methyl-3,5-diethylpyrazine	ND	0.74 ± 0.02
trimethylisobutylpyrazine	ND	0.86 ± 0.07
2,3-dimethyl-5-(1-propenyl)pyrazine	0.04 ± 0	ND
Esters		
Ethyl Acetate	0.62 ± 0.01	0.46 ± 0
Ethyl isobutyrate	0.15 ± 0.02	ND
Isobutyl butanoate	0.22 ± 0.01	ND
Ethyl β-hydroxybutyrate	0.11 ± 0.01	0.22 ± 0
Isoamyl 2-methylpropanoate	0.18 ± 0.01	ND
Butyl isobutyrate	0.10 ± 0	ND
Ethyl 3-acetoxybutanoate	ND	0.34 ± 0.01
β-phenylethyl formate	0.14 ± 0.01	ND
Ethyl benzeneacetate	ND	1.16 ± 0.09
2-phenethyl acetate	1.15 ± 0.05	ND
Pentanoic acid, oct-4-yl ester	ND	0.66 ± 0.07
Isobutyl phenylacetate	ND	0.08 ± 0.01
α-phenylethyl butyrate	0.41 ± 0.01	ND
Butyl adipate	ND	0.26 ± 0.02
Others		
2,2-diphenyl-2H-1-benzopyran	0.22 ± 0.02	ND
Trimethyloxazole	ND	0.83 ± 0.03
2-pentyl Furan	0.19 ± 0.01	ND
Methyl hydroxytriazaindolizine	0.08 ± 0	ND
5,6,7,8-tetrahydro-2-methyl-4H-chromen-4-one	0.23 ± 0.01	1.38 ± 0.08
Methyl 3,4-di-O-acetyl-2-O-methylfucofuranoside	0.16 ± 0.01	ND
1,3-di-tert-butylbenzene	0.20 ± 0.02	0.48 ± 0.18
2,4-dimethyl-3-acetylpyrrole	ND	0.29 ± 0.04
6,7-dimethyl-1,2,3,5,8,8a-hexahydronaphthalene	0.22 ± 0.03	ND
3-methylhexyl isothiocyanate	ND	1.39 ± 0.10
6-pentyl-alpha-pyrone	42.66 ± 0.15	0.26 ± 0
α-Himachalene	2.14 ± 0.13	0.61 ± 0.01
α-Zingiberene	5.86 ± 0.01	2.67 ± 0.14
1-hydroxy-2,4-di-tert-butylbenzene	2.50 ± 0.04	6.17 ± 0.04
Butylated Hydroxytoluene	0.58 ± 0.01	1.66 ± 0.11
α-Sesquiphellandrene	6.60 ± 0.07	2.43 ± 0.05
Nerolidol	ND	0.86 ± 0.01
Cedrene	0.34 ± 0.08	ND
6,7-dihydro-2-trans-farnesol	0.63 ± 0.04	ND
1,2,4,4,6-pentamethyl-1,4-dihydropyridine-3,5-dicarbonitrile	ND	1.19 ± 0.01
Ledol	0.95 ± 0.02	3.04 ± 0.36
Phenol, 2-(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl	0.26 ± 0	1.15 ± 0.76

The compound 6-pentyl-alpha-pyrone (6-PP) is an unsaturated lactone with a strong coconut-like aroma, which was first characterized by Collins & Halim (1972), and identified to be one of the bioactive compounds of several species of *Trichoderma*, such as *T. viride* (Collins and

Halim, 1972), *T. harzianum* (Claydon *et al*., 1987; Bonnarme *et al*., 1997) and *T. atroviride* (Reithner *et al*., 2005; Reithner *et al*., 2007). The biological effects of 6-PP are the reduction of the production of the mycotoxin deoxynivalenol by *F. graminearum* (Cooney *et al*., 2001) and the fusaric acid by *F. moniliform* (El-Hasan *et al*., 2007); moreover, it exerts antifungal properties, reducing the mycelial growth rate of *Rhizoctonia solani* and *F. oxysporum* f. sp. *lycopersici* (Scarselletti and Faull, 1994). Finally, Vinale *et al*. (2008) reported that 6-PP has a growth promoting effect on tomato seedlings which compared to control plants had a more extensive and vigorous root system.

Tetramethylpyrazine, also known as Lanzorite, is a compound belonging to the alkylpyrazines with the characteristic nutty and roasted flavor (Rosales *et al*., 2018); it has been found in fermented cocoa beans and soybeans fermented by *Bacillus* spp. (Hashim *et al*., 1998; Besson *et al*., 1997). There is no report in the literature on this substance produced by *Trichoderma* spp.

Pyrazines are known as substances with antifungal and nematicidal activity (Gu *et al*., 2007; Chen *et al*., 2008), previous studies have shown that tetramethylpyrazine inhibits the growth of *Moniliophthora perniciosa* and *F. oxysporum* f. sp. *lactucae* (Chaves-López *et al*., 2015). The analysis of the non-volatile organic compounds (nVOCs) of the culture filtrates of *Trichoderma* strains tested, *T. asperellum* IMI 393899 and *T. atroviride* TS, led to the identification of 12 molecules present in the literature, namely diterpene lactone trichodermaerin, peptabolite asperilins A, H and E, dichetopyrazine methylcordysinin A, steroids 3,5,9-trihydroxyergosta-7,22-dien-6-one and ergosta-7,22-dien-3-ol, sterol beta-sitosterol, adenine nucleoside, cyclopentenone atrichodermone B, sesquiterpene atrichodermone C and the pyrone derivative 6-penta-1-enyl-pyrane-2-one. The molecules identified in the extract after 10 and 30 days and their molecular structure are shown in Tab. 3.5. and in Fig. 3.4. All the molecules known for their antimicrobial activity were recovered in the extract of the culture filtrate after 30 days with the exception of beta-sitosterol

and ergosta-7,22-dien-3-ol found in the 10-day extracts. This could explain why the 30-day extracts were more active than the 10-day ones. In particular, 3,5,9-trihydroxyergosta-7,22-dien-6-one was isolated from *Trichoderma* spp. by Xuan *et al.* (2014) (Xuan *et al.*, 2014) and showed antimicrobial properties against *E. coli*, *B. subtilis*, *P. oryzae*, *C. albicans*, *A. niger* and *A. alternata*. Beta-sitosterol isolated from *T. asperellum* (Li *et al.*, 2019) and *T. harzianum* (Ahluwalia *et al.*, 2015) showed inhibitory activity against *R. solani*, *S. rolfsii*, *M. phaseolina* and *F. oxysporum* (Ahluwalia *et al.*, 2015).

Tab. 3.5. Non-volatile compounds (nVOCs) in the EtOAc extracts after 10 and 30 days incubation identified by HPLC-Q-TOF-MS.

Compound (nVOCs)	MW	Molecular formula	m/z	Ion	RT	Days of fermentation	Ref.
trichodermaerin	316.20	$C_{20}H_{28}O_3$	317.2104	$[M+H]^+$	15.9	30	(Chantrapromma *et al.*, 2014)
aspereline H	978.62	$C_{47}H_{82}N_{10}O_{12}$	977.6099	$[M-H]^-$	16.7	10	(Chen *et al.*, 2013)
aspereline A	936.60	$C_{45}H_{80}N_{10}O_{11}$	935.5956	$[M-H]^-$	19.3	10	(Chen *et al.*, 2013)
aspereline E	952.60	$C_{45}H_{80}N_{10}O_{12}$	951.5935	$[M-H]^-$	16.6	10	(Chen *et al.*, 2013)
methylcordysinin A	240.15	$C_{12}H_{20}N_2O_3$	299.1628	$[M+CH3COO]^-$	20.7	10	(Song *et al.*, 2018)
3,5,9-trihydroxyergosta-7,22-dien- 6-one	444.32	$C_{28}H_{44}O_4$	489.3224	$[M-HCOO]^-$	9.4	30	(Xuan *et al.*, 2014)
ergosta-7,22-dien-3-ol	398.36	$C_{28}H_{46}O$	397.3486	$[M-H]^-$	30.3	10 - 30	(Li *et al.*, 2019)
beta-sitosterol	414.39	$C_{29}H_{50}O$	413.3784	$[M-H]^-$	33.3	10	(Chantrapromma *et al.*, 2014)
adenine nucleoside	267.10	$C_{10}H_{13}N_5O_4$	312.0953	$[M-HCOO]^-$	5.5	30	(Li *et al.*, 2019)
atrichodermone B	156.08	$C_8H_{12}O_3$	215.0914	$[M+CH3COO]^-$	9.3	30	(Zhou *et al.*, 2017)
atrichodermone C	236.18	$C_{15}H_{24}O_2$	295.1906	$[M+CH3COO]^-$	23.5	30	(Zhou *et al.*, 2017)
6-pent-1-enyl-pyrane-2-one	164.08	$C_{10}H_{12}O_2$	165.0915	$[M+H]^+$	9.9	30	(McMullin *et al.*, 2017)

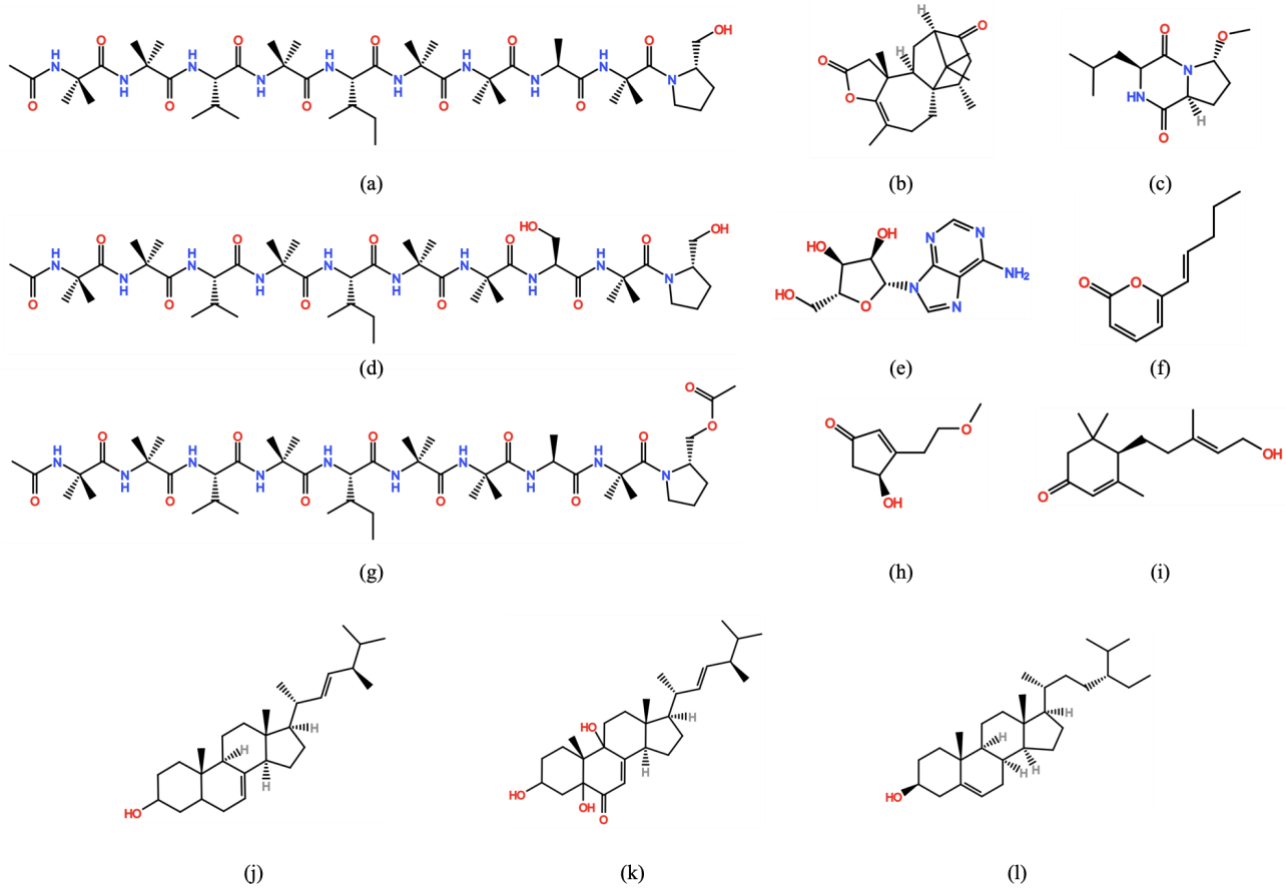

Fig. 3.4. Chemical structure of the non-volotile compounds (nVOCs) identified in the *Trichoderma asperellum* (IMI 393899) and *Trichoderma atroviride* (TS) EtOAc extracts. (a) aspereline A; (b) trichodermaerin; (c) methylcordysinin a; (d) aspereline E; (e) adenine nucleoside; (f) 6-pent-1-enyl-pyrane-2-one; (g) aspereline H; (h) atrichodermone B; (i) atrichodermone C; (j) ergosta-7,22-dien-3-ol; (k) 3,5,9-trihydroxyergosta-7,22-dien-6-one; (l) beta-sitosterol.

However, few studies on the identification of nVOCs produced by *Trichoderma* spp. are present in the literature and many more studies should be performed to understand the antimicrobial activity of the secondary metabolites produced.

3.5. Conclusions

The two *Trichoderma* strains tested in this study, *T. asperellum* IMI 393899 and *T. atroviride* TS, inhibited the growth of tested pathogens when they came in contact with them. In particular, the extract of *T. asperellum* showed the highest inhibition activity and was active even at a low concentration. Moreover, it was demonstrated that both *Trichoderma* strains produced compounds with antifungal activity against the pathogenic fungi and oomycetes tested.

These results highlight the potential use of these two *Trichoderma* strains as antagonists in biological control of plant pathogens and their ability to produce secondary bioactive metabolites that might be used for the management of crop and post-harvest diseases as an alternative to synthetic fungicides.

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4. INHIBITION OF MYCOTOXIGENIC FUNGI IN DIFFERENT VEGETABLE MATRICES BY EXTRACTS OF *TRICHODERMA* SPECIES

4.1. Abstract

Post-harvest diseases of plant products are a serious concern leading to economic losses and health risks. Moreover, the use of chemical fungicides to prevent post-harvest fungal diseases is limited due to toxic residues.

This study aimed at determining the effective dose of extracts of *Trichoderma asperellum* IMI393899 (TE1) and *Trichoderma atroviride* TS (TE2) in inhibiting the contamination by mycotoxigenic fungi on different plant matrices. Extracts were previously tested *in vitro* obtaining the MIC and MFC. Starting with MFC, three concentrations were tested *in vivo* on tomatoes contaminated by *Fusarium verticillioides* and Fusarium *graminearum*, wheat contaminated by *Penicillium verrucosum* and maize contaminated by *Aspergillus flavus*. The efficacy of extracts was evaluated at two time intervals after treatment, 4 and 11 days for tomato, 10 and 20 days for wheat and maize. Both extracts showed a significant inhibitory activity on mycotoxigenic pathogens and reduced significantly the Log CFU/g compared to the control. Moreover, the extracts reduced mycotoxins production in a dose dependent manner and with a long-lasting effect. The ochratoxin A was reduced by both extracts but only the extract TE2 was effective in reducing aflatoxins, whereas TE1 treatment increased in their synthesis.

Keywords: *Trichoderma asperellum*; *Trichoderma atroviride*; bioactive metabolites; biological control; mycotoxins

4.2. Introduction

Diseases caused by fungi are the main causes of production losses in agriculture. Fungi are responsible for numerous plant diseases and their infections can occur in the field and affect the fruit. Often the disease in the fruit does not appear immediately in the field but during the storage and distribution processes. From contaminated fruits, the disease can spread to other fruits leading to huge losses (Dutot *et al.*, 2013). In addition, some fungi are able to produce toxic compounds called mycotoxins. Mycotoxins are secondary metabolites produced by some species of fungi, which are consequently referred to as mycotoxigenic fungi, such as *Fusarium*, *Penicillium*, *Aspergillus* and *Alternaria* (Stankovic *et al*., 2007; Somma *et al*., 2014; Aloi *et al*., 2021). Mycotoxins are not involved in fungal development or growth. However, they do play a role in plant diseases as virulence, and pathogenicity factors (Masi *et al*., 2020). The proliferation of fungi and the production of mycotoxins on food and feed is always favoured by some environmental factors, such as humidity and temperature, as well as by the vegetable matrix.

Although more than 400 mycotoxins have been identified, the most studied are aflatoxins (AFs), ochratoxin A (OTA), Fusarium toxins, fumonisins (FBs), zearalenone (ZEA), trichothecenes (TCT) and deoxynivalenol (DON), which cause greater health risks and economic losses (Alshannaq and Yu, 2017).

Mycotoxins are very commonly found in cereals and cereal products (Bentivenga *et al*., 2021). They can also be found in dairy products, spices, nuts, coffee, vegetable oils, wine and fruit juices (Malir *et al*., 2014; Drusch *et al*., 2003). These compounds are dangerous to animal and human health as they can be lethal, carcinogenic, mutagenic, teratogenic, immunosuppressive, or interfere with hormonal processes (Chu, 1991; Fung and Clark, 2004). Their activity depends on the type of toxin and their concentration in food. Mycotoxins can be produced before and after harvest, and their levels may increase during post-harvest, handling and storage. They can reach consumers either through direct contamination of plant materials or derived products or

through the accumulation of mycotoxins and their metabolites in animal tissues, milk and eggs after intake of contaminated feed (Bryden, 2007).

In addition, this hazard remains in processed foods because these metabolites are not removed by normal industrial processing, and the risk may increase if mouldy fruits or other part of the plant are used in by-product processing (Bullerman and Bianchini, 2007).

Inhibition of fungal growth in crops, fresh fruit and vegetables is therefore necessary to reduce the risk of mycotoxin contamination. Fungal growth inhibition is often controlled by the use of chemical fungicides in both pre-harvest and post-harvest. The first step in controlling fungal contamination is the application of fungicides in the field. Fungicides can be also applied in post-harvest, provided that they do not adversely affect the appearance or quality of the treated produce (Amiri *et al*., 2008).

The indiscriminate and excessive use of fungicides in crops has been indicated as one of the main cause of the development of resistant populations of pathogens, resulting in the use of higher concentrations of these antifungals and the consequent increase in toxic residues in food products (Merrington *et al*., 2002). Moreover, some of these compounds are non-biodegradable, so they can accumulate in the soil, water and plants, causing environmental contamination and, through the food chain, they can be hazardous to human health. Because of these undesirable effects, the need has arisen for the development of new, safe, biodegradable alternatives that are effective and economically feasible. A viable alternative appears to be the use of microorganisms and their metabolites. In particular, the genus *Trichoderma* is known and widely used as a biological control in agriculture. Some species of this genus are able to produce antimicrobial metabolites that inhibit the growth of fungal pathogens (Ghisalberti and Sivasithamparam, 1991; Harman *et al*., 2004; Stracquadanio *et al*., 2020).

The objective of this study was to evaluate the capacity of metabolites extracted from two *Trichoderma* species, *T. asperellum* (IMI 393899) and *T. atroviride* (TS), to inhibit the growth of mycotoxigenic fungi, *Fusarium verticillioides*, *F. graminearum*, *Penicillium verrucosum* and

Aspergillus flavus, and the production of their mycotoxins in different plant matrices, including tomato, wheat and maize.

4.3. Materials and methods

4.3.1. Chemical materials

HPLC-grade methanol and acetonitrile, analytical reagent grade methanol, ethyl acetate, formic acid (99%), and dimethyl sulfoxide (99.9% DMSO) were obtained from Thermo Fisher Scientific (Loughborough, UK). Magnesium sulfate (MgSO4) was obtained from Thermo Fisher Scientific (Kandel, Germany). Potato dextrose agar (PDA) and Potato dextrose broth (PDB) were obtained from Thermo Fisher Scientific (Basingstoke, UK). Ultrapure water (<18 MW/cm) was obtained from a Milli-Q purification system (Millipore Corp., Bedford, MA, USA).

Aflatoxins (AFB1 and AFB2), Ochratoxin A (OTA), Fumonisins (FB1 and FB2), (Zearalenone (ZEA) and Deoxynivalenol (DON) standards were obtained from Sigma-Aldrich (St. Louis, MO, USA).

4.3.2. Fungal strains and culture conditions

Two *Fusarium* strains (*F. verticillioides* ITEM 12052 and *F. graminearum* ITEM 126) and *Aspergillus flavus* ITEM 8111 were obtained from the Agro-Food Microbial Culture Collection (Bari, Italy). *Penicillium verrucosum* VTT D-01847 was obtained from the VTT Culture Collection (Finland). Two species of *Trichoderma* (*T. asperellum* IMI 393899 and *T. atroviride* TS), characterized in previous studies (Stracquadanio *et al*., 2020; La Spada *et al*., 2020), were obtained from the collection of the Molecular Plant Pathology laboratory of the Di3A, University of Catania (Catania, Italy).

The mycotoxigenic fungi were cryopreserved in sterile 30% glycerol at –80°C, but before antifungal studies, they were defrosted and cultured in PDB at 25 °C for 48 h and inoculated on

PDA plates to obtain spores. All fungal strains were maintained on potato dextrose agar (PDA) at room temperature and subcultures were made every 20 days.

4.3.3. Liquid culture, production and extraction of metabolites

Two plugs of each *Trichoderma* strains, obtained from actively growing margins of PDA cultures, were used to inoculate 750 mL flasks containing 500 mL of sterile potato dextrose broth (PDB). The liquid cultures were incubated for 30 days at 30 °C under stirring (100 rpm) (Stracquadanio *et al*., 2020). The cultures were filtered under vacuum through filter paper, and the filtrates stored at 2 °C for 24 h before the biphasic extraction. The culture filtrates of *T. asperellum* IMI 393899 and *T. atroviride* TS were extracted with ethyl acetate (EtOAc) for 3 times with a final 1:1 ratio.

The combined organic fraction was dried ($MgSO_4$), filtered and evaporated under reduced pressure at 35 °C. The two red-brown residues recovered were dissolved with 10% DMSO and stored at -20 °C until the subsequent analysis.

4.3.4. Inoculum preparation

The inoculum of the pathogens consisted of a spore suspension and it was prepared by suspending the spores in buffered peptone water with 1% Tween 20. Spores were counted using the Neubauer chamber and the suspension was adjusted to the final concentration used in the assay (Petrikkou *et al*., 2001).

4.3.5. Study of the extracted metabolites in different matrices

In the search for alternative antifungals to be applied in vegetable matrices, one issue to consider is their efficacy *in vivo*. Although *in vitro* screening has revealed good results, *in vivo* vegetable matrices can interact with bioactive compounds, decreasing their efficacy.

The evaluation of the antimicrobial activity of *Trichoderma* extracts was carried out in three different vegetable matrices inoculated with different mycotoxigenic pathogens. The matrices used were tomato fruits inoculated with *F. verticillioides* and *F. graminearum*, wheat inoculated with *P. verrucosum* and maize inoculated with *A. flavus*.

Based on the previous study in which the extracts of *T. asperellum* IMI393899 and *T. atroviride* TS were effective *in vitro* on the pathogens examined; the results were expressed as minimum fungicidal concentration (MFC) (Stacquadanio *et al*., 2020). Three different concentrations were tested: the MFC, the double and the quadruple of MFC (MFCx2 and MFCx4), respectively. The concentrations are reported in Tab. 4.1.

	***Extract (mg/ml or mg/g)**					
	TE1			**TE2**		
Strain	MFC	MFCx2	MFCx4	MFC	MFCx2	MFCx4
Fusarium verticillioides	0.78	1.56	3.12	1.56	3.12	6.25
Fusarium graminearum	0.78	1.56	3.12	1.56	3.12	6.25
Penicillium verrucosum	0.78	1.56	3.12	1.56	3.12	6.25
Aspergillus flavus	0.78	1.56	3.12	6.25	12.5	25

Tab. 4.1. Concentrations of extracts TE1 and TE2. Three different concentrations were tested: the MFC as determined in a previously study (Stracquadanio *et al*., 2020), the double (MFCx2) and the quadruple (MFCx4) of MFC; *the concentration of extracts as mg/mL for tomatoes and mg/g for maize and wheat.

4.3.5.1. Tomato fruits

The evaluation of the antifungal activity of *T. asperellum* (TE1) and *T. atroviride* (TE2) extracts against the two *Fusarium* species was carried out on tomato fruits. The cherry tomatoes were obtained from the supermarket chain Mercadona (Valencia, Spain). The fruits were initially washed under running water, sterilised for 2 min in 2% sodium hypochlorite and then rinsed with sterile water. Wet fruits were placed in plastic trays (sterilised with 70% ethanol and under UV) and dried for 2 h under a laminar flow cabinet (Telstar MH 100, Terrassa, Spain). The fruits were wounded with a sterile needle and 20 µL of a suspension of spores of the pathogen

at a concentration of 10^4 spores/mL was inoculated onto the wound; subsequently, the drop was dried for 1 h. Finally, tomato fruits were treated with 20 µL of TE1 or TE2 at three different concentrations (MFC, MFCx2, MFCx4). The MFC concentrations for both *Fusarium* species were 0.78 and 1.56 mg/mL of TE1 and TE2, respectively. The treated tomato fruits were dried under a laminar flow cabinet until the droplet was completely dry and the closed trays were incubated at room temperature. For each test, 40 tomato fruits were used. The untreated positive control was inoculated with the spore suspension of the pathogen and the negative control was the wounded fruit only (untreated and not inoculated). Fruits were monitored daily and results were evaluated at two time intervals, at 4^{th} and 11^{th} days of incubation. The percentage of infected fruit (%IF) was calculated based on the number of fruits with visible symptoms of infection out of the total treated fruits. The diameter of the lesions was also measured.

Subsequently, 14 tomato fruits from each test were taken ramdomly and eight were freeze-dried for mycotoxin analysis and six were examined for the viable microbial count.

4.3.5.2. Wheat and maize

The wheat (Biocesta, Valencia, Spain) was obtained from an organic supermarket and the maize was purchased from the supermarket chain Mercadona (Valencia, Spain).

The wheat and maize kernels were sterilised into autoclave at 120 ºC for 20 min. Afterwards, 5 g of wheat or maize were placed in sterile small plates (55 mm) and inoculated with 500 µL of the pathogen spore suspension (10^3 spores/g). Finally, the kernels were treated with 500 µL of TE1 or TE2 at three different concentrations (MFC, MFCx2 and MFCx4, determined as mg/g of kernels). The MFCs of TE1 were 0.78 mg/mL for both *P. verrucosum* in wheat and *A. flavus* in maize; while for TE2 the MFCs were 1.56 and 6.25 mg/mL for *P. verrucosum* and *A. flavus*, respectively. After both inoculation and treatment, the kernels were dried for 2 h under a laminar flow cabinet. Plates were placed in glass flasks (sterile) and incubated at room temperature in the dark.

The control was wheat or maize inoculated with the pathogen without treatment with extracts (untreated control). For each test, 12 plates were made in order to obtain 6 repetitions per test, at 10 and 20 days after treatment. Some samples were used for viable microbial counting. Samples for mycotoxin analysis were stored at -80 ºC to stop fungal growth and proliferation.

4.3.6. Viable microbial counting

The viable spore count test was performed using six tomato fruits at 4 and 11 days after treatment and 15 g of wheat and maize at 10 or 20 days after treatment. All vegetable matrices were homogenised with sterile buffered peptone water at a ratio of 1:10 (w/v) in a Stomacher (IUL, Barcelona, Spain) for 30 s. From the homogenate, seven serial decimal dilutions were prepared in sterile falcon (15 mL) with 9 mL of peptone water (1:10 v/v ratio). Three repetitions were performed for each sample. Subsequently, 100 µL of each tube was plated in PDA plates. The plates were incubated at 25°C, and the number of viable colonies was counted at 72 hours of incubation.

Finally, from the number of colonies per plate the viability was calculated and expressed as Log CFU/g (WHO, 2019).

4.3.7. Mycotoxin analysis

Mycotoxins were extracted using the method described by Quiles *et al*. 2019. Maize and wheat kernels were ground using an Oester Classic grinder (Madrid, Spain) in order to reduce particle size. Tomato fruits were frozen at -80 °C and freeze-dried before grinding. The powdered samples were weighed into 2 g of tomato fruits and 3 g of maize or wheat kernels in a Falcon tube (50 mL); to each sample, 25 mL of MeOH was added and homogenised for 3 min with an Ultra Ika T18 Ultra-turrax (Staufen, Germany). Subsequently, the extract was centrifuged at 4000 rpm for 15 min at 4 °C, and the supernatant was evaporated to dryness with Büchi Rotavapor R-200 (Postfach, Switzerland). Finally, the dry extracts were resuspended in 2 mL

methanol (HPLC-grade) and filtered through 0.22 μm before analysis. The samples were extracted in triplicate.

The HPLC system used for the chromatographic determination was an Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA) equipped with a vacuum degasser, autosampler, and binary pump. The column was a Gemini NX-C18 (150 mm x 2 mm, 110 Å, and 3 m particle size; Phenomenex, Torrance, CA, USA).

The binary mobile phases consisted of water (A) and acetonitrile (B) with 0.1% v/v formic acid. The initial gradient of the mobile phase was 5% B and was increased to 95% B over 30 min. It was decreased to 5% B in 5 min, and then maintained for 3 min. The flow rate was maintained at 0.3 mL/min and 10 μL of each sample was injected.

Mass spectrometry (MS) analysis was performed using a Q-TOF-MS (6540 Agilent Ultra High Definition Accurate Mass, Santa Clara, CA, USA), equipped with an Agilent Dual Jet Stream electrospray ionisation (Dual AJS ESI, Santa Clara, CA, USA) interface in negative ion mode for the FB1, FB2, DON and ZEN, and in positive ion mode for OTA, AFB1 and AFB2. The MS range m/z was 100-1100 and the MS/MS range m/z was 50-800. The parameters were as follows: drying gas flow (N_2), 5.0 L/min; nebulizer pressure, 60 psig; gas drying temperature, 325 °C; capillary voltage, 3.5 kV; and fragmentor voltage, 175 V. Targeted MS/MS experiments were carried out using collision energy values of 10, 20, and 40 eV. Integration and data elaboration were managed using MassHunter Qualitative Analysis software B.08.00 (Agilent, Santa Clara, CA, USA). The analysis was carried out in triplicate.

4.3.8. Statistical analysis

Statistical analysis of data was carried out using IBM SPSS Statistics version 23.0. Data were expressed as mean ± SE of different experiments. Difference between groups were statistically analysed with one-way ANOVA followed by the Turkey HDS post-hoc test for multiple comparisons. The difference level of $p < 0.05$ was considered statistically significant.

4.4. Results and Discussion

4.4.1. Tomato fruits bio-preservation

The results of application of TE1 and TE2 extracts to tomato fruits inoculated with *F. verticillioides* and *F. graminearum* are shown in Fig. 4.1. and Fig. 4.2, respectively.

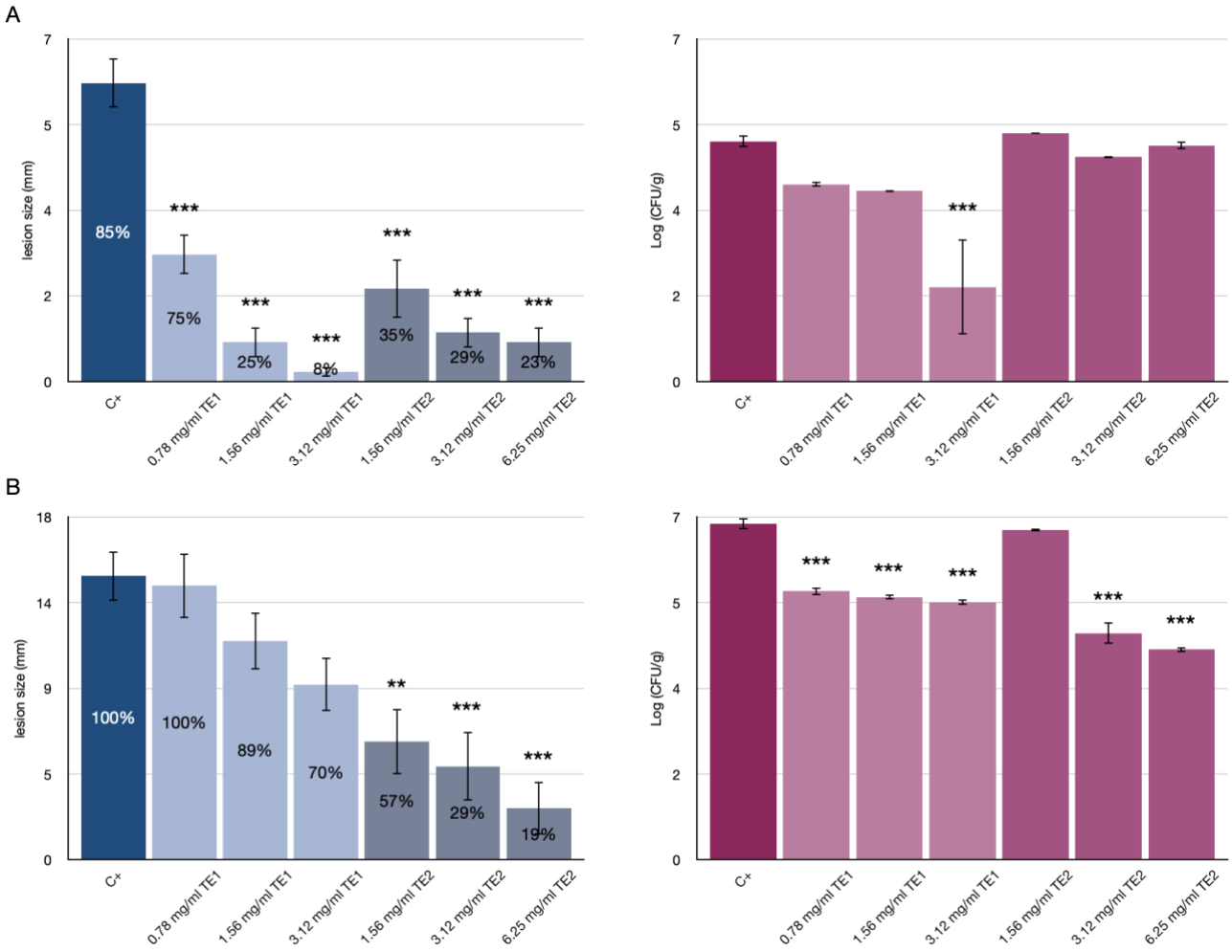

Fig. 4.1. Effects of TE1 and TE2 on the growth of *Fusarium verticillioides* in tomatoes. Results expressed as % infected fruit (IF), lesion size (mm) and microbiological count (log10 CFU/g) at 4 days (A) and 11 days (B). Statistically significant differences for each treatment are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Results are expressed as mean ± standard error.

At 4 days of storage, tomato fruits inoculated with *F. verticillioides* and treated with the extracts showed an increase in shelf-life compared with the untreated control. The untreated control had a % of IF (Infected Fruit) of 85% and 100% at 4 and 11 days of storage, respectively. Tomatoes

treated with the two extracts maintained a lower % IF than the untreated control. Notably, TE2 was effective at the lowest concentration (1.56 mg/mL) with 35% IF and showing treatment persistence even at 11 days of storage with 57% IF. At 4 days, TE1 was effective at the highest concentrations with 25% IF at 1.56 mg/mL and 8% IF at 3.12 mg/mL, whereas at 11 days it was unsatisfactory with 70% IF even at the highest concentration (3.12 mg/mL).

These results were consistent with the analysis of lesion diameter, showing a significant reduction compared to the untreated control at all concentrations tested, with the only exception of TE1 at 11 days which showed no significant reduction in lesion diameter compared to the control (Fig. 4.1B). The results of the viable count analysis expressed in Log CFU/g revealed that the treatments were effective over a long time, showing a significant inhibition, compared to the control with differences at 10 days and at the highest concentration of 1.61 and 2.58 Log CFU/g for TE1 and TE2, respectively. Although the TE1 extract did not shown a clear efficacy in reducing % IF in the long time (11 days), it was effective in reducing the Log CFU/g at the highest concentration (3.12 mg/mL) at 4 days of storage and at all concentrations tested at 11 days of storage. The TE2 extract showed a clear efficacy in reducing % IF at 4 and 11 days, moreover it maintained stable the Log CFU/g values at 4 and 11 days, being significantly ($p < 0.001$) effective in the long time compared to the control.

This could be explained by assuming these extracts have different mechanisms of action as indicated by the different growth pattern of the mycelium on treated fruits. The TE2 extract showed a greater persistence over time, as indicated by the reduction in IF % and lesion diameter. In this case, the extract might have acted by preventing the sporulation of the pathogen on the surface of the fruit (Fig. 4.3A). Conversely, the TE1 extract showed a lower persistence on the fruit, nevertheless it could have induced the biosynthesis and activation of substances or proteins with antimicrobial activity in the fruits. Yao *et al*. (2005) observed that post-harvest treatment of sweet cherry fruit with salicylic acid (SA) or methyl jasmonate (MeJA) induced β-1,3-glucanase and peroxidase (POD) activities in early storage as a defense

response induced, but it did not reduce the incidence of brown rot caused by *Monilinia fructicola*.

Tomatoes treated with the extracts and inoculated with *F. graminearum* showed a significant increase in shelf-life at 4 and 11 days, showing both lower IF % and lesion diameter respect to the control (Fig. 4.2).

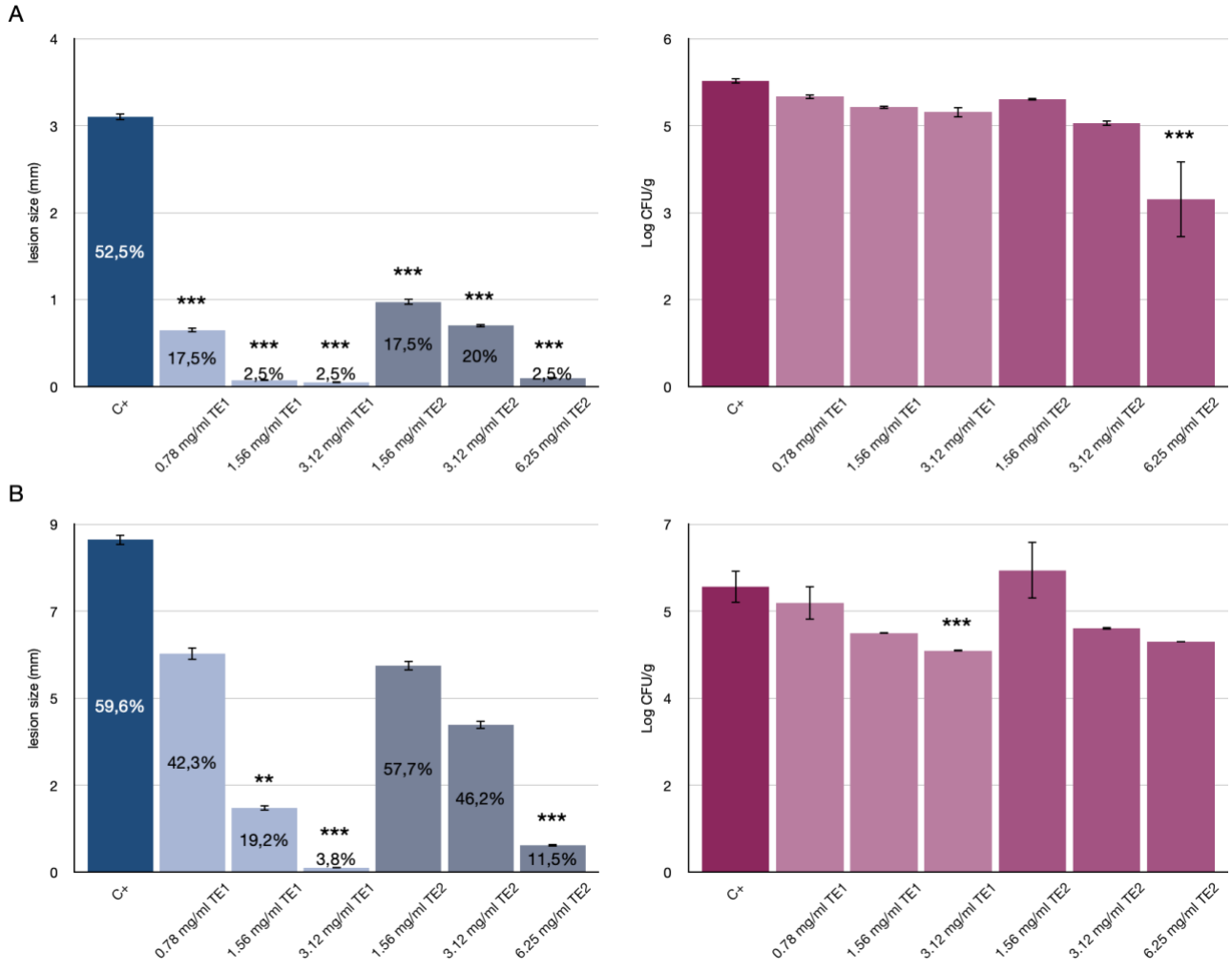

Fig. 4.2. Effects of TE1 and TE2 on the growth of *Fusarium graminearum* in tomatoes. Results expressed as % infected fruit (IF), lesion size (mm) and microbiological count (log10 CFU/g) at 4 days (A) and 11 days (B). Statistically significant differences for each treatment are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Results are expressed as mean ± standard error.

However, the efficacy could be attributed to the low incidence of infection of this pathogen on tomato fruits, with less than 60% IF in the untreated control. Treatment with the TE2 extract at the highest concentration tested (6.25 mg/mL) showed a reduction in microbial counts at 4 days

and no significantly different values at 11 days (Fig. 4.2), whereas the TE1 extract was effective only at the highest concentration (3.12 mg/mL) and 11 days of storage.

The mycotoxins normally produced by *Fusarium* species (FB1, FB2, ZEN and DON) were not detected by HPLC-MS-Q-TOF analysis, even in the untreated controls. Tomato fruit rot caused by *Fusarium* species is an obvious problem in postharvest production loss (Bakar *et al*., 2013), but to the best of our knowledge no study has reported the presence of mycotoxins in tomatoes produced by *Fusarium*. In the study of Haidukowski (2004), the same strain *F. graminearum* ITEM 126 showed mycotoxin production in contaminated wheat kernels. This could be possibly due to the fact that this strain does not produce mycotoxins when infecting tomatoes but only in small grain cereals and maize (Munkvold, 2017).

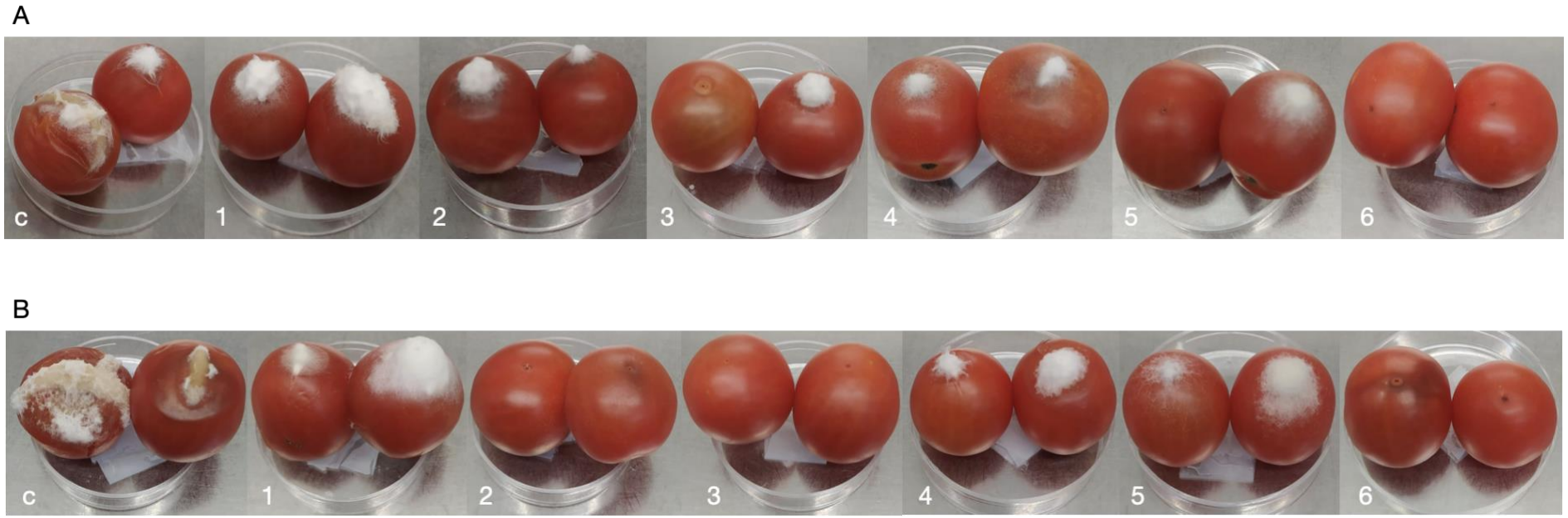

Fig. 4.3. Mycelium growth of *Fusarium verticillioides* (A) and *Fusarium graminearum* (B) on tomato treated with (1-3) TE1, (4-6) TE2 and (c) untreated at 11 days incubation. (1) 0.78 mg/mL TE1, (2) 1.56 mg/mL TE1, (3) 3.12 mg/mL TE1, (4) 1.56 mg/mL TE2, (5) 3.12 mg/mL TE2, (6) 6.25 mg/mL TE2.

4.4.2. Bio-preservation of wheat

The application of the two extracts on wheat inoculated with *P. verrucosum* showed significant differences compared to the untreated control. Visibly less fungal growth was observed on the wheat as the concentration increased, for both extracts at 10 and 20 days of storage (Fig. 4.4). Mycelium growth in the untreated control was observed after 3 days of storage, while at 7 days it was observed only in the treatments at the lowest concentrations, and only in the case of the highest concentration of TE1 no growth of *P. verrucosum* was observed until day 10 of storage (Fig. 4.4A). The same results were confirmed by the vital units count analysis, which showed a reduction in the number of colonies (Fig. 4.5A).

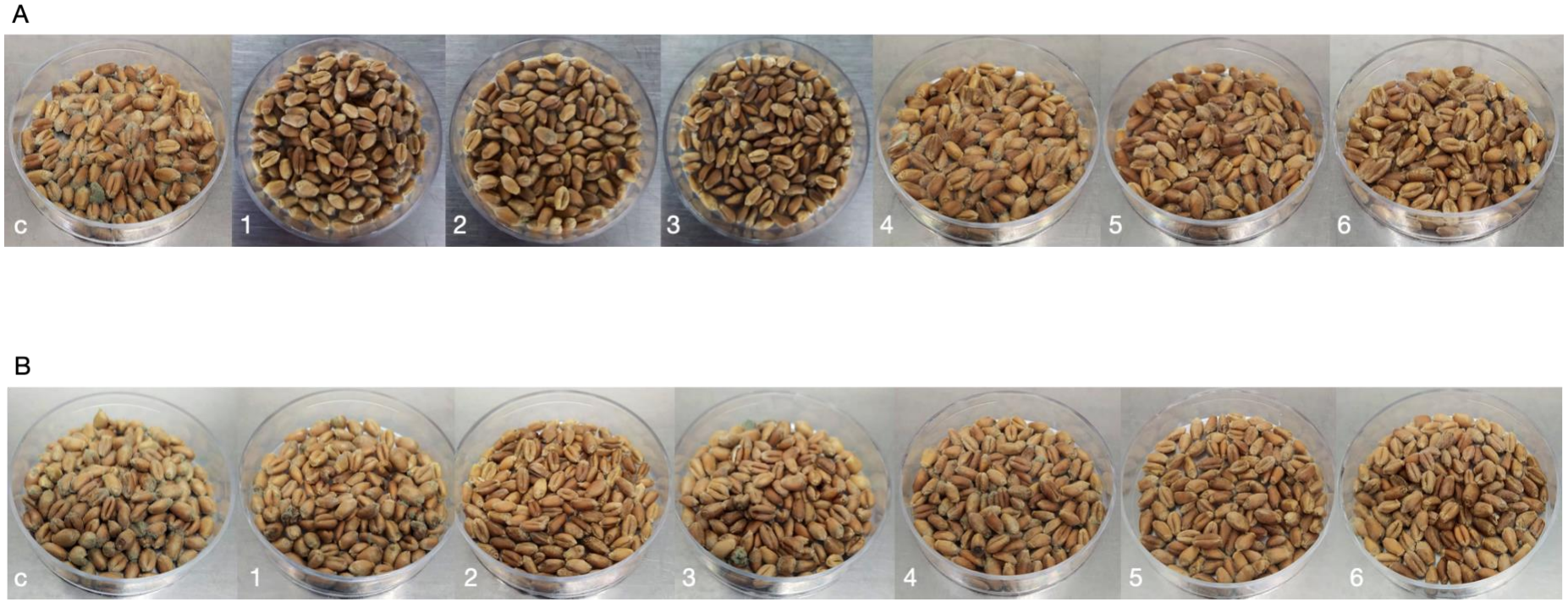

Fig. 4.4. Growth of *Penicillium verrucosum* on wheat treated with (1-3) TE1, (4-6) TE2 and (c) untreated, at (A) 10 days and (B) 20 days incubation. (1) 0.78 mg/mL TE1, (2) 1.56 mg/mL TE1, (3) 3.12 mg/mL TE1, (4) 1.56 mg/mL TE2, (5) 3.12 mg/mL TE2, (6) 6.25 mg/mL TE2.

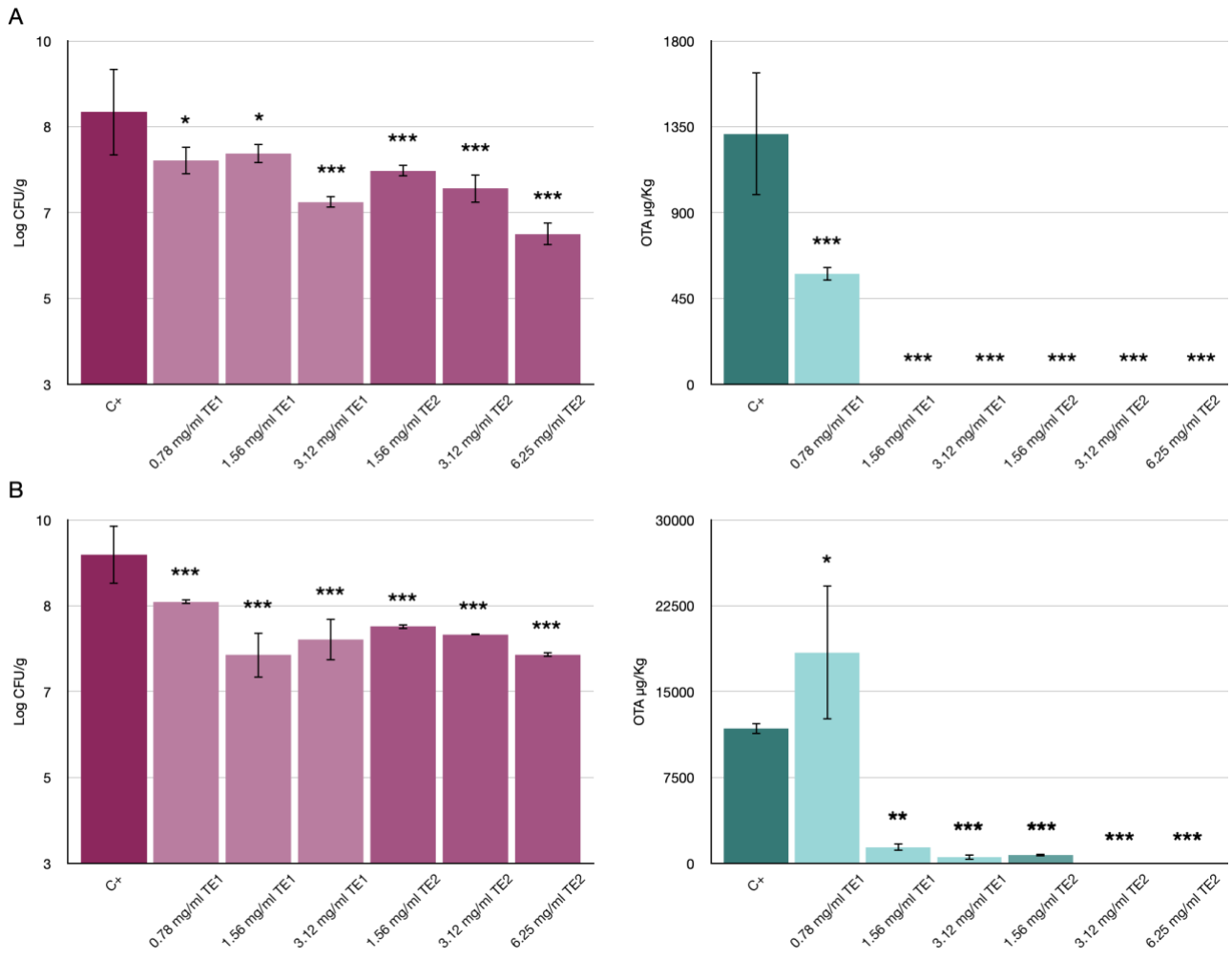

Fig. 4.5. Effects of TE1 and TE2 on the growth of *Penicillium verrucosum* in wheat. Results expressed as microbiological count (log10 CFU/g) and OTA concentration detected (µg/Kg) at 10 days (A) and 20 days (B). Statistically significant differences for each treatment are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Results are expressed as mean ± standard error.

The results of the analysis of the OTA produced showed a reduction at both 10 and 20 days of storage (Fig. 4.5). In particular, at 10 days, OTA was not detected in all treatments except for the treatment with TE1 at the lowest concentration (0.78 mg/mL), but in this case the OTA produced with value of 579 µg/Kg was lower compared to the untreated control (1312 µg/Kg). While at 20 days, only the treatments with TE1 at the highest concentrations (3.12 and 6.25 mg/mL) showed a significant reduction with values less than 1300 µg/Kg compared to the untreated control (11770 µg/Kg); while the lowest concentration of TE1 seems to have increased OTA production by *P. verrucosum*, probably due to the stress induced by the extract. Oddly, some fungicidal substances can stimulate the production of mycotoxins and pose a threat

because of the healthy visual appearance of crops contaminated by mycotoxins. The application of fungicide azoxystrobin e.g. in wheat contaminated by *Fusarium* spp. increased the production of the mycotoxin DON (Simpson *et al*. 2001).

TE2 extract at all concentrations tested induced a reduction of OTA. In particular OTA was detected only in the treatment at the lowest concentration (1.56 mg/mL) after 20 days of storage but at a significantly lower level compared to the untreated control.

4.4.3. Bio-preservation of maize

Treatment with TE1 and TE2 extracts in maize inoculated with *A. flavus* resulted in a drastic reduction of mycelium growth. , which could be clearly noticed by visual inspection. The untreated control showed an abundant aerial mycelium on the whole plate already after three days of storage. In maize treated with TE1 and TE2 at various concentrations, a stunted mycelium growth was observed at seven days of storage. After 10 days a green sporulation appeared that was not present in the untreated control (Fig. 4.6A1-4 and Fig. 4.6B1-6).

A reduction in the viability of *A. flavus* was observed, with significantly lower Log CFU/g values compared with the untreated control. In particular, treatment with TE1 at the highest concentration showed a good control that was maintained up to 20 days after treatment. At 10 days, the TE2 treatments showed similar values for all three concentrations (Fig. 4.7A); at 20 days, only the highest concentration of TE2 showed stable efficacy, while for the other two concentrations an increase in Log CFU/g was observed (Fig. 4.7B).

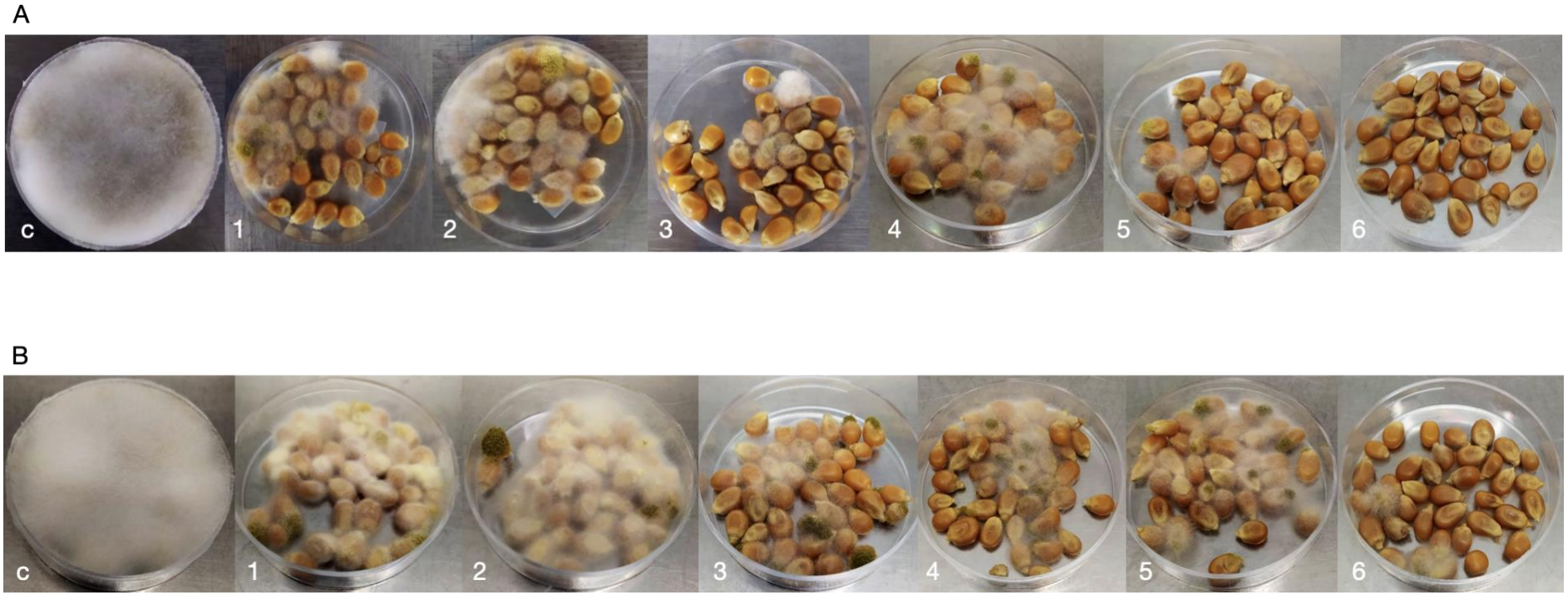

Fig. 4.6. Growth of *Aspergillus flavus* on maize treated with (1-3) TE1, (4-6) TE2 and (c) untreated, at (A) 10 days and (B) 20 days incubation. (1) 0.78 mg/mL TE1, (2) 1.56 mg/mL TE1, (3) 3.12 mg/mL TE1, (4) 6.25 mg/mL TE2, (5) 12.5 mg/mL TE2, (6) 25 mg/mL TE2.

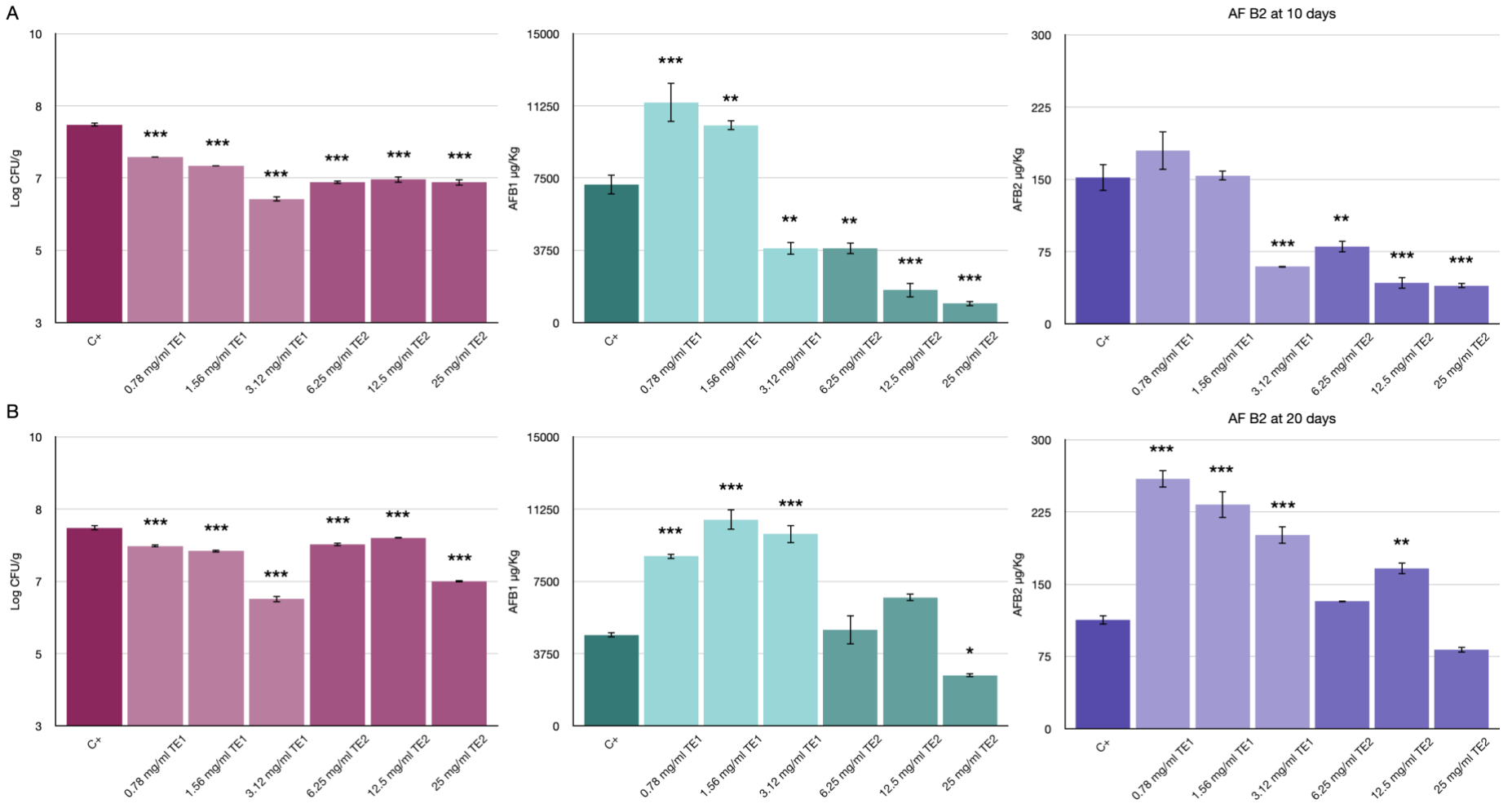

Fig. 4.7. Effects of TE1 and TE2 on the growth of *Aspergillus flavus* in maize. Results expressed as microbiological count (log10 CFU/g) and AFs concentration detected (µg/Kg) at 10 days (A) and 20 days (B). Statistically significant differences for each treatment are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Results are expressed as mean ± standard error.

The results of the analysis of aflatoxins produced by *A. flavus* (AFB1 and AFB2) are shown in Fig. 4.7. At 10 days, treatments with TE2 at all concentrations tested showed a significant reduction in AFB1 and AFB2, with values < 3,000 µg/Kg for AFB1 and < 80 µg/Kg for AFB2 , compared to the untreated control (7,174 and 152 µg/Kg of AFB1 and AFB2, respectively); only the highest concentration of TE1 (3.12 mg/mL) showed a reduction in AFB1 (3,857 µg/Kg) and AFB2 (59 µg/Kg), while at the other two concentrations a significant increase in AFs was observed compared to the control. At 20 days, only the treatment with TE2 at the highest concentration showed a reduction in the production of mycotoxins; for all other treatments, an increase in the production of AFs was observed that shows the stress induced by the treatments stimulates the fungus to produce mycotoxins as a defence mechanism. However, the use of higher concentrations may inhibit viable growth and consequently mycotoxin production.

Partial inhibition of fungal growth cannot be correlated with the inhibition of mycotoxin production because this fungistatic activity may trigger secondary metabolism as a stress response. Previous studies have shown that contact with plant extracts or essential oils can enhance aflatoxin production by fungi. dos Santos Oliveira and Furlong (2008) observed that aflatoxin B1 production by *A. flavus* was inhibited in the presence of methanolic extracts of banana pulp and orange peel, aubergine and potato pulp. However, these authors found that in the presence of banana pulp and potato pulp extracts, *A. flavus* produced aflatoxin B2, which was not detected in the control.

Prakash *et al*. (2010) reported that the production of AFB1 from an *A. flavus* strain treated with *Piper betle* var. *magahi* essential oil at low concentration (0.1 µL/mL) was higher than the control. At a higher concentration of essential oil, the inhibitory effect on aflatoxin was observed, and complete inhibition was observed at 0.6 µL/mL of essential oil. These Authors suggested that low doses of fungicides induced a certain stress condition that might have been responsible for increased production of mycotoxins as a defence mechanism.

4.5. Conclusions

The search for alternative antifungal substances is of great concern to the agricultural sector, mainly because of the substantial post-harvest losses occurring due to fungal contamination. Meanwhile, environmental protection agencies and organizations are expressing concern about the widespread use of synthetic fungicides that contaminate soil and water, and can reach and be harmful for the consumer through residues on food. The possibility of using naturally extracted compounds to control fungal and mycotoxin contamination is a promising alternative. However, when a new antifungal is to be tested, it is important to keep in mind the complexity of the food matrices and the behaviour of microorganisms when exposed to the new compound. The results obtained show that metabolites extracted from *T. asperellum* and *T. atroviride* can be used in the prophylaxis of post-harvest diseases and are also able to increase the shelf-life of the produces and inhibit the production of mycotoxins. In particular, the *T. atroviride* extract showed a long-lasting efficacy and reduced both fungal growth and mycotoxin production. Further studies, including the evaluation of toxicological implications, are needed to establish whether these extracts can be used for post-harvest treatments. Another aspect that deserves to be investigated is whether a synergistic use of both extracts is possible, which could allow a reduction in the application doses.

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5. GENERAL CONCLUSION AND FUTURE PERSPECTIVES

Environmental pollution is one of the most prominent problems of recent years. A significant part of the presence of pollutants in the environment is represented by invasive and incorrect agricultural practices, such as the excessive use of agrochemicals.

Synthetic agrochemicals can lead to increased pollution, the emergence of resistance in pathogens and a reduction in the beneficial microflora present in the soil. These consequences have alarmed the institutions, which have decreed a reduction in the use of chemical pesticides (2009/128/EC). In addition, public awareness has shifted towards a demand for less processed, more environmentally sustainable and natural food products.

For this reason, scientific research has shifted the focus to the search for natural methods that can limit or eliminate the presence of pollutants in the environment and possible alternatives to invasive and unsustainable agricultural practices. In this regard, the use of microorganisms is a valuable resource; in particular, fungal microorganisms are ubiquitous in the soil and have the ability to resist extremely unfavorable environmental conditions, this potential has found wide application in various sectors, including the environmental and agricultural sectors.

In this context, the present PhD study explored the potential of *Trichoderma* strains to protect plants from biotic and abiotic stresses. In particular, strain *Trichoderma asperellum* (IMI393899) was studied for its tolerance to heavy metals; through a host-pathogen-antagonist system in the presence of cadmium, its ability to protect the plant from cadmium stress and the pathogen *Phytophthora nicotianae* was assessed.

The antagonistic properties of the strains *Trichoderma asperellum* (IMI393899) and *Trichoderma atroviride* (TS), by means of mycoparasitism and antibiotic mechanisms, were evaluated against different fungal pathogens of the genera *Neofusicoccum*, *Collethotricum*, *Aspergillus*, *Penicillium* and *Fusarium* and oomycetes of the genus *Phytophthora*.

The positive results led to interest in the production of secondary metabolites in liquid culture, testing them against these pathogens and in three plant matrices contaminated by the

mycotoxigenic fungi *Penicillium verrucosum* in wheat, *Aspergillus flavus* in maize, and *Fusarium verticillioides* and *Fusarium graminearum* in tomato fruits.

The studies carried out showed the great potential of these two *Trichoderma* strains for their direct antagonistic capacities or for the possible production of their metabolite extracts to be useful in pre-harvest and post-harvest disease control. Moreover, the metabolites are natural products, easily degradable and can be a viable alternative to chemical fungicides.

This line of research lays the foundation for a possible field trial on the use of *Trichoderma* extracts. Further studies on the simultaneous protection of the plant against biotic and abiotic stresses should be carried out using a soil system and establish the correlation between tolerance and possible uptake of heavy metals.

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APPENDIX

List of abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
6-PP	6-pentyl-alpha-pyrone
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFs	Aflatoxins
BCA	Biological control antagonist
CDA	Czapek dox agar
cDNA	Complementary deoxyribonucleic acid
Ct	Threshold cycles
Cys	Cysteine
DDT	para-dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
EDTA	Ethylenediaminetetraacetic acid
EME	Haemoglobin
ESI	Electrospray ionization
EtOAc	Ethyl acetate
FB1	Fumonisin B1
FB2	Fumonisin B2
FBs	Fumonisins

GAP	Good agricultural practices
GC/MS	Gas chromatography/Mass spectrometry
Glu	Glutathione
Gly	Glycine
HPLC-QTOF-MS/MS	Hight performed liquid chromatography-quadrupole-time of flight
IF	Infect fruits
ITS	Internal transcribed spacer
MAMPs	Microbe-associated molecular patterns
MBCAs	Microbial biological control agents
MeOH	Methanol
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MS solution	Murashige and Skoog solution
ND	Not determined
NTCs	Non-template controls
nVOCs	Non-volatile organic compounds
OMA	Oatmeal agar
OTA	Ochratoxin A
PAMPs	Pathogen-associated molecular patterns
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PGP	Plant growth promoter
PIRG	Percentage inhibition of radial growth
ppm	Part per million
PSII	Photosystem II

qPCR	Quantitative polymerase chain reaction
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
RT	Reverse transcription
SAR	Systemic acquired resistance
SE	Standard error
SRI	Systemic resistance induced
TCT	Trichothecenes
TE1	*Trichoderm asperellum* extract
TE2	*Trichoderma atroviride* extract
v/v	Volume/volume
VOCs	Volatile organic compounds
w/v	Weight/volume
WHO	World Health Organisation
ZEA	Zearalenone

List of chemical symbols

$(C_2H_3O_2)_2Hg$	Mercury acetate
$(C_2H_3O_2)_2Pb$	Lead acetate
$(C_2H_3O_2)_2Zn$	Zinc acetate
$(C_2H_3O_2)_2Cd$	Cadmium acetate
Ag	Silver
Al	Aluminium
As	Arsenic
Cd	Cadmium

CdO	Cadmium oxide
Co	Cobalt
Cr	Chrome
Cu	Copper
Fe	Iron
FeS_2	Thiosulphate
Hg	Mercury
Mg	Magnesium
$MgSO_4$	Magnesium sulphate
Mn	Manganese
Mo	Molybdenum
Ni	Nickel
Pb	Lead
Sn	Tin
Zn	Zinc